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MORPHOLOGICAL VARIATION AND POLYSACCHARIDES BIOSYNTHESIS GENE EXPRESSION IN COLCHICINE-INDUCED AUTOPOLYPLOID AND ALLOPOLYPLOID IN *DENDROBIUM*

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ABSTRACT

Dendrobium officinale is a medicinal herb in many countries. It produces various phytochemicals, particularly polysaccharides, which have nutraceutical and pharmaceutical values. In this study, diploid stems of *in-vitro* plantlets derived from *Dendrobium officinale* 'Hu' × *Dendrobium officinale* 'Guanghua' and *Dendrobium officinale* 'Guanghua' × *Dendrobium nobile* 'Beijing' were used for autopolyploid and allopolyploid induction. Autopolyploid and allopolyploid were induced by colchicine treatment with 0.1 mg.L^{-1} for 4 and 5 days, respectively. The diploid and induced tetraploid were strongly confirmed by the consistency of results from flow cytometer analysis and counted chromosomes. The tetraploid plants had thicker and shorter stem and root than the diploid plants. Among nine studied genes, namely: *CSLA2-1, CSLA3-1, g1gc3-1, GMP1-3, GMPP, iPGM1-2, Man2-2, PGM1* and *PMM2-1*, six and seven of these were significantly up-regulated in autopolyploid and allopolyploid, respectively. In autopolyploid, the *CSLA2-1*, *g1gc3-1, GMP1-3*, *GMPP*, *iPGM1-2* and *Man2-2* genes might increase the polysaccharide content in tetraploid while these genes were *CSLA2-1, CSLA3-1, GMP1-3, GMPP, iPGM1-2, Man2-2* and *PGM1* in tetraploid of allopolyploid. The results from this study might provide valuable information for orchid breeding program to increase biomass production and polysaccharides content in *Dendrobium officinale*

Key words: Chromosome, *Dendrobium officinale*, polyploidy, polysaccharides genes

INTRODUCTION

The genus *Dendrobium*, one of the largest genera in the family *Orchidaceae*, are distributed mainly in tropical and subtropical Asia, eastern Australia (Wood 2006) and south, east and southeast Asia, including China, Japan, India, Philippines, Indonesia and Vietnam. The *Dendrobium* orchids are one of the most well-known orchids in global horticultural trade due to their beautiful flowers and ideal characteristics as houseplants (Teixeira da Silva et al. 2016). Moreover, many species in this genus have been extensively used as traditional herbal medicine in many Asian countries for hundreds of years (Bao et al. 2001) because of its bio-compounds, particularly polysaccharides that confer medicinal importance including antitumor activity (Lu et al. 2014), immunomodulating activity (Xie et al. 2016), antidiabetic activity (Hou et al. 2016), neuroprotective activity (Yang et al. 2015), hepatoprotective activity (Yang et al. 2007), anti-inflammatory activity (Hwang et al. 2010), antioxidant activity (Luo et al. 2016) and antifungal activity (Sattayasai et al. 2009). The *Dendrobium officinale* Kimura et Migo is a rare and endangered species in some countries (Li et al. 2008b). Because *D. officinale* has high medicinal and ornamental values, it has often been adulterated with other *Dendrobium* species in the market (Xu et al. 2012).

Polyploid plants, depending on their genomic origin, can be divided into autopolyploid (species that have genomes derived from a single ancestral species) and allopolyploids (species derived from the hybridization of two different genomes followed by genomic multiplication). Polyploid induction in *Dendrobium* was first reported by using colchicine treatment in 1961 (Nakasone and Hy 1961). Somatic doubling is associated with mitotic events such as endomitosis or endoreduplication, which may occur either in a zygote cell or in apical meristematic tissues, giving rise to mixoploids or even completely polyploid organisms. Despite being constantly used to attain artificial polyploids, somatic doubling is supposed to have a minor role in the origin of natural polyploid organism (Ramsey et al. 2014). Since then, autopolyploids of medicinal *Dendrobium* species, including *Dendrobium phalaenopsis* (Liu et al., 2023; Chaicharoen 1981), *Dendrobium cariniferum* (Zhang and Gao, 2021), *Dendrobium devonianum* (Han et al. 2005), *Dendrobium secundum* (Atichart and Bunnag, 2007), *Dendrobium scabrilingue* (Sarathum et al. 2010), *Dendrobium officinale* (Yang 2013), *Dendrobium chrysotosum* (Atichart 2013), *Dendrobium formosum* (Yenchon et al. 2014), *Dendrobium nobile* (Vichiato et al. 2014), *Dendrobium draconis* (Bunnag and Hongthongkham 2015) and *Dendrobium ochreatum* (Wang et al. 2017), have been successfully induced by the use of colchicine treatment. Polyploid of *Dendrobium phalaenopsis* × *Dendrobium loddigesii* were developed using colchicine and APM treatment (Grosso et al. 2018)

Genetic identification is necessary and helpful for further utilizing and controlling the quality of *Dendrobium* resources. In recent years, the development and application of molecular markers have increased rapidly and these showed potential value in identification of *Dendrobium* species in particular, as well as other herbs in general (Li et al. 2017). Moreover, the PCR and electrophoresis based molecular markers, such as AFLP, TRAP, RAPD, DAMD, ISSR and SCoT, had also been employed for identification of *Dendrobium* (Feng et al. 2015). More and more molecular markers closely linked to medicinal and ornamental traits have been studied and developed. These would be necessary for further commercial application of *Dendrobium*. However, to the best of our knowledge there are no reports on allopolyploid induction in *Dendrobium officinale*. Therefore, in this study, real-time PCR was used in the identification of the expressed genes related to polysaccharides in induced autopolyploid of *Dendrobium officinale* as well as in allopolyploid generated from *Dendrobium officinale* and *Dendrobium nobile*. Both *Dendrobium* are valuable in the traditional medicinal plant system in Asia. Moreover, their different phenotypes and uses may create the potential hybrid progeny.

This study sought to evaluate the effects of colchicine on autopolyploid and allopolyploid induction of *Dendrobium officinale*, examine the morphological changes after chromosomes doubling in autopolyploid and allopolyploid seedlings and assess the expression of polysaccharides genes related in autopolyploid and allopolyploid.

MATERIAL AND METHODS

Plant materials. Diploid stems of *in-vitro* plantlets derived from *Dendrobium*: *Dendrobium officinale* 'Hu' × *Dendrobium officinale* 'Guanghua' called *DO.201-3 D1* and *Dendrobium officinale* 'Guanghua' × *Dendrobium nobile* 'Beijing' called *DON 200-7 D1* were used for polyploidy induction.

Protocorm like bodies (PLBs) induction. The 4-5 cm stems of *in vitro* seedlings of hybrids *Dendrobium* were used for PLBs induction. The stems were decontaminated sequentially with 70% ethanol for 1 min, and thoroughly rinsed with sterilized distilled water for 30s, then cut into segments of about 0.4-0.5 cm in length and inoculated on ½ MS medium supplemented with 0.5 $mg \cdot L^{-1}$ 6-benzyladenine (6-BA), 20% coconut water (CW), 30 $g \cdot L^{-1}$ sucrose, 7 $g \cdot L^{-1}$ agar and 0.1 $g \cdot L^{-1}$ activated carbon (AC). The pH of the media was adjusted to 5.8 before autoclaving. The materials were cultured under dark conditions for one month.

PLB proliferation. PLBs were formed after a month culture. PLBs were cut off from stem segments and then inoculated on ½ MS medium supplemented with 0.5 $mg.L^{-1}$ 6-BA, 20% CW, 0.1 $g.L^{-1}$ AC, 30 $g.L^{-1}$ sucrose and 7.5 $g.L^{-1}$ agar and incubated at 25±2°C under dark conditions. The proliferation of PLBs were repeated 2-3 times on the same medium until enough PLBs applied to colchicine treatments.

Colchicine treatment. PLBs of 3-4 mm in size were used as explants for polyploid induction. One g of fresh PLBs (60 PLBs) was used for each colchicine treatment and transferred to 100 ml flasks containing ½ MS medium supplemented 1.0 $mg{\cdot}L^{-1}$ 6-BA, 0.1 $mg{\cdot}L^{-1}$ α-NAA, pH=5.8, without agar and incubated on a rotatory shaker at 120 rpm under dark conditions at 25±2°C for 3, 4, 5, 6 and 7 days (d). The different colchicine treatments were 0.00, 0.05, 0.1 and 0.15 $mg{\cdot}L^{-1}$ (v/v). Each treatment was repeated three times

The PLBs were then transferred to full strength MS medium (pH=5.8), supplemented with 0.5 $mg.L^{-1}$ 6-BA, 0.2 $mg.L^{-1}$ α-NAA, 0.2 $g.L^{-1}$ AC, and incubated under a 16/8 hours (light/dark) with light intensity of 35 µmol $m^{-2}s^{-1}$ provided by 36W cool-white fluorescent tubes (Ople, China) at 25±2°C. The survival of PLBs was recorded after two-month incubation. Polyploid induction was recorded after six months. The survival rate of PLBs and the polyploid induction were calculated using the formulas:

$$\text{Survival rate of PLBs} = \frac{\text{Number of PLBs that survived}}{\text{Total number of PLBs}} \times 100$$

$$\text{Polyploid induction rate} = \frac{\text{Number of polyploid inductions}}{\text{Total number of PLBs}} \times 100$$

Flow cytometer analysis. This was carried out using leaf tissues (about 0.5 cm^2) of six-month seedlings. The fresh leaves were cut finely with a sharp razor blade in a glass Petri dish where 0.4 mL of Partec HR-A solution and 1.6 mL Partec HR-B solution were added, then the suspension was filtered through a 30 µm Partec Celltrics® filter. After about 4-5 min under dark conditions, the ploidy was detected by Partec CyFlow® Ploidy Analyse (Partec, Münster, Germany).

Determination of chromosome number. Root tips of seedlings which was confirmed as diploid and tetraploid were cut about 1 cm in long. Root tips were treated with 2 mM 8-hydroxyquinoline for 6 hours (h) at 17°C in dark. After being washed with distilled water, the root tips were transferred to fresh Carnoy's solution (ethanol: acetic acid, 3:1(v/v)) and stored at 4°C for at least 24 h. After being washed with distilled water twice, the root tips were immersed in 1 M HCl for 8 min at 65°C, followed by staining with 1% (v/v) aceto-orcein. The dyed root tips were excised 1-2 mm long and squashed on a glass slide with a drop of 45 % (v/v) acetic acid. The slide was immediately observed and photographed under an Olympus light microscope (LX71, Olympus Corporation, Tokyo, Japan) at 100x magnification with immersion oil. Chromosome numbers were confirmed by counting three squashes per slide for each treatment.

Morphological examination. The morphological traits of a total of 90 six-month-old plantlets of each sample including three replications and 30 plantlets/each replicate were investigated. The height and stem diameter, length and width of leaf and length and diameter of root were measured by using a vernier caliper. The fresh weight of whole plantlet was measured by an analytical balance. The number of leaf and root per plantlet were recorded.

Stomata traits. The stomata dimension and density were measured according to the method of Stoddard (1965). A drop of clear nail polish was applied to the abaxial surface of the third leaf from the

meristem per plantlet. After drying, a piece of Scotch 3M Transparent Tape (Scotch 3M; St. Paul, MN) was set on the top of dried nail polish. The polish with imprint affixed to the tape was peeled off and mounted on the microscope slides for observation. Stomata was viewed using a model compound biological microscope, and displayed the images on a computer via the microscope's built-in camera (Moticam2306, 3.0 M Pixel USB 2.0) with Motic Images Advanced 3.2 software. Digital images of the leaf and stomata imprints were viewed at 100x magnification (for counting stomata) and 400x (for measuring the length and width of stomata), and saved as digital tagged image file (.tif) with 1024 x 768 pixel resolution for the later analysis. The images of the leaf imprint were analyzed and the stomatal lengths were measured by the Image J.2 software. Fifteen images per sample were observed.

Quantitative real-time RT-PCR (qRT-PCR) validation. Total RNA from different samples were extracted from 6-month-old seedlings that used for morphological analysis using an OmniPlant RNA Kit (Cwbio, China) and FastKing RT Kit (Tiangen, China) according to the manufacturer's instructions. The mRNAs were fragmented into small fragmented using divalent cautions under high temperature. The final cDNA library was produced using mRNA-seq sample preparation kit (FastKing RT Kit, Tiangen Biotech Beijing Co.,LTD, China). The primers sequences were designed by Primer 5.0. Basing on the results of transcriptome in *D. officinale* (P101SC17122636-01-B1-16 supported by Novogene Co., LTD, Beijing, China) and unigene database for polysaccharides, and synthesized by Sangon Biotech (Shanghai) Co., Ltd.

For qRT-PCR validation, nine keys genes and their isoforms related to expression of polysaccharide were selected. The expressions of studied genes *CSLA2-1*, *CSLA3-1*, *g1gc3-1*, *GMP1-3*, *GMPP*, *iPGM1-2*, *Man2-2*, *PGM1* and *PMM2-1* were normalized against *Actin* and calculated by the ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen 2001). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene for each sample (Pham *et al.* 2024). The procedure of qRT-PCR was described as follow: 2 µL of a 2/10 dilution of cDNA in dd H_2O was added to 7 µL Mix 10 (Sangon Biotech (Shanghai) Co., Ltd.) and primer 1 µL total is 10 µL for each sample. The PCR conditions were 95ºC for 10 min, 40 cycle of 95ºC for 15 s and 60ºC for 60s. A histogram was constructed using the average values to visualize the expression levels in different samples. Differences between values were calculated using one-way ANOVA with Student's t-test at $P<0.05$ in Excel 2016 with 'Analysis ToolPak' add-in program. All expression analysis was performed for three biological repeats and figures show average values of three repeats.

Statistical analysis. Data were evaluated by statistical program SPSS 22.0 (IBM Corporation, Somers, NY). Comparison among treatment means was examined by Duncan's multiple range test (DMRT) at 5 % significance level.

RESULTS AND DISCUSSION

Survival and polyploid induction rate of *D. officinale*. The significantly highest PLB survival of autopolyploid was 84.00% in MS medium without colchicine (Table 1). At 0.05 $mg.L^{-1}$ colchicine, the rates decreased from 83.33% to 39.17% at 3 days (d) to 7d, respectively. At 0.1 $mg.L^{-1}$ of colchicine treatment, the rates decreased from 76.67% to 30.67% at 3d to 7d, respectively. At the highest colchicine treatment (0.15 $mg.L^{-1}$), the PLB survival rate decreased from 68.33% to 17.33% at 3d to 7d, respectively. Lower survival rates of PLBs were observed at higher colchicine concentration and longer duration time.

A similar tendency was observed with allopolyploid. The significantly highest PLB survival of allopolyploid was 90.37% without colchicine treatment. At 0.05 $mg.L^{-1}$ colchicine, the rates decreased from 90.00% to 39.17 % for 3d to 7d, respectively. At 0.1 $mg.L^{-1}$ of colchicine, these decreased from 76.67% to 30.67% at 3d to 7d, respectively. At 0.15 $mg.L^{-1}$ of colchicine concentration, these decreased from 68.33% to 17.33% at 3d to 7d, respectively.

Autopolyploid was not induced by 0.05 mg.L^{-1} colchicine while allopolyploids were obtained with 7.50%, 10.00% and 10.06% at 5d, 6d and 7d, respectively (Table1). Both autopolyploid and allopolyploid were induced at 0.1 mg.L^{-1} and 0.15 mg.L^{-1} colchicine. The highest of autopolyploid induction efficiency was 46.67% after 5d at 0.1 mg.L^{-1} colchicine and 50.00% after 4d at 0.1 mg.L^{-1} colchicine for allopolyploid. The autopolyploid seedling - *DO.201-3.T1* and allopolyploid seedling *DON.200-7.D1* were induced from PLB diploid *DO.201-3.D1* and *DON.200-7.D1* at 0.1mg.L^{-1} colchicine after 5d and 4d, respectively.

Table 1. Effects of colchicine treatment on PLB survival and tetraploid induction of *D. officinale.*

Autopolyploid PLB			
Colchicine concentration (mg.L^{-1})	**Time of colchicine treatment (day)**	**Survival rate (%)**	**Polyploid induction rate (%)**
Control		84.00±3.39 a	0.00±0.00 f
0.05	3	83.33±5.27 a	0.00±0.00 f
	4	82.83±6.47 a	0.00±0.00 f
	5	68.33±4.08 abc	0.00±0.00 f
	6	60.00±6.89 bc	0.00±0.00 f
	7	39.17±7.17 de	0.00±0.00 f
0.1	3	76.67±4.08 ab	20.00±2.04 cde
	4	72.38±3.49 abc	33.33±2.61 b
	5	60.00±7.17 bc	46.67±3.33 a
	6	52.50±6.89 cd	18.00±4.64 cde
	7	30.67±10.87 ef	14.00±6.36 de
0.15	3	68.33±1.67 abc	10.00±4.08 ef
	4	53.33±9.35 cd	21.67±2.04 cd
	5	53.33±6.24 cd	26.67±4.08 bc
	6	36.00±4.85 de	10.00±6.12 ef
	7	17.33±5.31 f	3.33±3.33 f
Allopolyploid PLB			
Colchicine concentration (mg.L^{-1})	**Time of colchicine treatment (day)**	**Survival rate (%)**	**Polyploid induction rate (%)**
Control		90.37±1.84 a	0.00±0.00 f
0.05	3	90.00±6.12 a	0.00±0.00 f
	4	78.33±2.04 abc	0.00±0.00 f
	5	67.50±3.06 cd	7.50±3.06 ef
	6	57.50±5.00 de	10.00±6.12 def
	7	30.00±3.06 gh	10.06±6.13 def

Colchicine concentration (mg.L^{-1})	Time of colchicine treatment (day)	Survival rate (%)	Polyploid induction rate (%)
0.1	3	85.00±4.67 ab	30.00±3.06 b
	4	60.00±6.12 de	55.00±3.06 a
	5	60.00±4.67 de	27.50±2.50 b
	6	40.00±6.12 fg	15.00±2.50 cde
	7	17.50±3.06 hi	5.00±5.00 ef
0.15	3	76.67±4.08 bc	20.00±2.04 bcd
	4	56.67±4.08 de	28.33±5.00 b
	5	50.00±3.95 ef	25.00±0.00 bc
	6	30.00±3.06 gh	20.00±3.06 bcd
	7	7.50±5.00 i	0.00±0.00 f

Different letters in the same column indicate significant differences based on Duncan's Multiple Range Test (DMRT) at 5% level. Survival rate observed after two-month incubation and polyploid induction rate observed after six-month induction

The average PLB survival rate of autopolyploid and allopolyploid after 2-month culture were 56.95% and 53.78%, respectively. The average rate of polyploid induction after 6-month culture were 13,58% for autopolyploid and 16.59% for allopolyploid (Fig. 1). The result of our study showed that, survival rate of PLB depended on both colchicine concentration and time duration. At low colchicine concentration and longer time duration, the survival rate was lower than high colchicine concentration and shorter time duration (Table 1). This tendency was consistent with the results of a previous study where protocorm induction was observed in *Dendrobium officinale* (Liu et al. 2023)

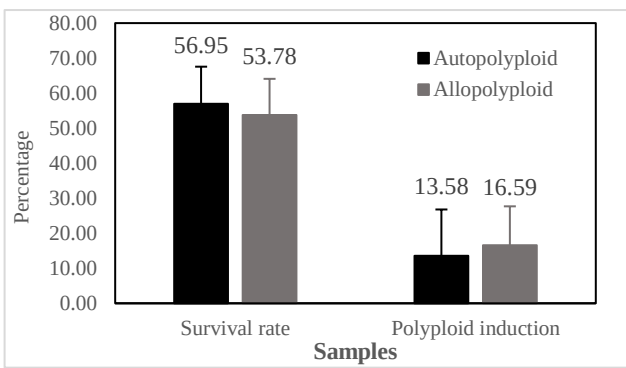

Figure 1. Survival rate of autopolyploid and allopolyploid in *D. officinale.*

Undifferentiated embryonic cells such as protocorms and PLBs are the best plant materials to use for polyploid induction in orchids (Griesbach 1981; Wimber and Van Cott 1966). One method to induce polyploid is use of chemical compounds to inhibit mitosis. The chemical compound must be in direct contact or close to meristematic tissue. Explants must be exposed to chemicals at levels high enough to saturate plant tissue and induce polyploidy without redoubling of epidermal tissue before reaching the meristem (Kermani et al. 2003). Other studies evaluated the effect of colchicine treatments on polyploid induction of plants, orchids and *Dendrobium* genus, such as *Dendrobium officinale* with 0.2% colchicine for 48 h (Liu et al. 2023), *Dendrobium cariniferum* Rchb. f. with 0.05% colchicine for 24 h (Zhang and Gao, 2021), *Dendrobium scabrilingue* with 0.075% colchicine for 336h (Sarathum et al. 2010), *Dendrobium devonianum* with 0.030% colchicine for 24 h (Li et al. 2008a), *Dendrobium*

officinale with 0.010% colchicine for 150-300 h (Chen et al. 2008) and *Phalaenopsis* 0.005% colchicine (Griesbach 1981). But allopolyploid induction from colchicine treatments in *Dendrobium* genus has not been reported up to now. In our present study, the highest allopolyploid induction was 55.00% using 0.1 $mg.L^{-1}$ colchicine for 4d, while the latest study on autopolyploid induction of *Dendrobium officinale* showed the tetraploid induction was 10% using 0.2% colchicine for 48 h (Liu et al. 2023).

Polyploid confirmation. Flow cytometer analysis indicated that diploid seedlings of *DO.201-3 D1* showed a large peak of diploid nuclei at 50, and a small peak of tetraploid nuclei at 100 as determined by analysis of standards which known ploidy level (Fig. 2A); tetraploid of *DO.201-3 T1* showed a large peak shift at 100 and small peak at 200 (Fig. 2C). These results were consistent with the results of determining the number of chromosomes in autopolyploid, chromosome counting in root-tips of diploid in all four samples as *DO.201-3 D1* was 38 (Fig. 2B) and it was 76 in tetraploid *DO.201-3 T1* (Fig. 2 D). Similar results were observed in diploid *DON.200-7 D1* and tetraploid *DON.200-7 T1*. The large and small peaks were at 60 and 120 for diploid *DON.200-7 D*, respectively (Fig. 2E); and at 120 and 240 for tetraploid *DON.200-7 T1* respectively (Fig. 2G). Once again, the results of flow cytometer analysis were consistent with the microscope counts in allopolyploid, these were 38 and 76 for *DON.200-7 D1* and *DON.200-7 T1*, respectively (Fig. 2F-H). These results confirm that the polyploids were generated by colchicine treatment.

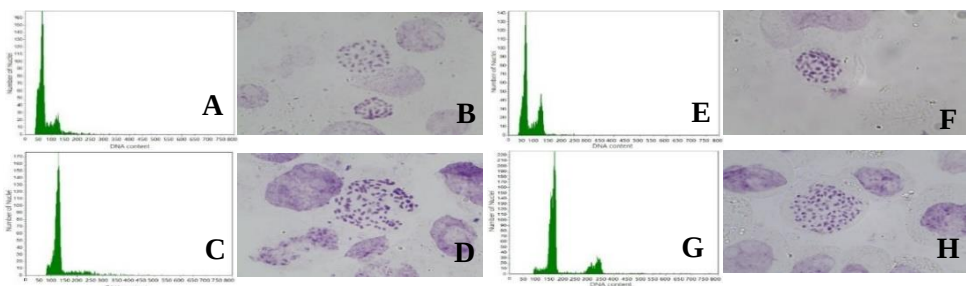

Figure 2. Relative DNA content and chromosome number of diploid and tetraploid in autopolyploid and allopolyploid of *D.officinale*.
A- DNA content of diploid *DO.201-3 D1*, B- chromosomes of diploid *DO.201-3 D1*, C- DNA content of tetraploid *DO.201-3 T1*, D- chromosomes of *DO.201-3 T1*, E- DNA content of diploid *DON.200-7 D1*, F- chromosomes of diploid *DON.200-7 D1*, G- DNA content of tetraploid *DON.200-7 T1* and H- chromosome of *DON.200-7 T1*.

Morphological changes. The morphology of both autopolyploid and allopolyploid PLBs was obviously changed after chromosome doubling, where significant differences were observed between diploid, autopolyploid and allopolyploid seedlings in stem height, stem diameter, number of root, root length and fresh weight (Table 2). Diploid seedlings were taller than autopolyploid and allopolyploid seedlings, while stem diameter in tetraploid seedlings of autopolyploid and allopolyploid were thicker than diploid seedlings. Root length of diploid seedlings were longer than tetraploid in autopolyploid and allopolyploid seedlings, while diameter of root in tetraploid seedlings were thicker than diploid seedlings.

For autopolyploid, diploid-*DO.201-3 D1* showed significantly higher values in stem height and root length (5.52 and 5.94, respectively) in comparison to tetraploid-*DO.201-3 T1* (3.12 and 2.16, respectively). In contrast, tetraploid-*DO.201-3 T1* showed significantly higher stem diameter and number of roots (0.43 and 0.20, respectively) in comparison to diploid-*DO.201-3 D1* (0.28 and 0.14, respectively) (Table 2).

For allopolyploid seedlings, diploid-*DON.200-7 D1* showed significantly higher values of stem height, number of roots and root length (6.42, 5.80 and 2.99, respectively) when compared to

tetraploid- *DON.200-7 T1*(3.60, 3.20 and 1.21, respectively) while the tetraploid- *DON.200-7 T1* showed significantly higher values in stem diameter and roots (0.62 and 0.18, respectively) in comparison with diploid- *DON.200-7 D1* (0.46 and 0.08, respectively). Seedlings of tetraploid of autopolyploid and allopolyploid had shorter and thicker stems and roots than diploid seedlings (Table 2).

Table 2. Characteristic changes in morphology and stomata of autopolyploid and allopolyploid in *D.officinale*

Traits	Autopolyploid		Allopolyploid	
	DO.201-3 D1	*DO.201-3 T1*	*DON.200-7 D1*	*DON.200-7 T1*
Stem height (cm)	5.52±0.67 a	3.12±0.44 b	6.42±0.12 a	3.60±0.22 b
Stem diameter (cm)	0.28±0.01 c	0.43±0.06 b	0.46±0.07 b	0.62±0.03 a
Number of leaves	6.20±0.80 a	4.80±0.66 a	5.20±0.37 a	5.80±0.73 a
Number of roots	5.20±0.58 a	4.80±0.58 ab	5.80±0.58 a	3.20±0.49 b
Root length (cm)	5.94±0.78 a	2.16±0.22 bc	2.99±0.25 b	1.21±0.22 c
Root diameter (cm)	0.14±0.01 b	0.20±0.01 a	0.08±0.01 c	0.18±0.01 a
Fresh weight (g)	0.88±0.24 bc	0.74±0.11 c	1.54±0.27 a	1.39±0.10 ab
Number of stomata*	46.00+2.30 a	23.00+1.30 c	33.40+0.75 b	23.20+1.07 c
Length of stomata (µm)	30.38+0.45 d	34.18+0.32 c	35.55+0.46 b	49.60+0.48 a
Width of stomata (µm)	15.56+0.53 b	15.90+0.25 b	15.36+0.89 b	24.57+1.45 a
Area of stomatal pore (μm^2)	1208.14+24.25 c	1400.37+35.99 b	1203.06+19.59 c	1795.09+57.53 a

*Total numbers of stomata counted at 100x magnification per one observation. Different letters within the same row indicate significant difference at 0.05 level according to Duncan's Multiple Range Test (DMRT).

Our results were very similar to some of the morphological features of some plants, such as *Mitracarpus hirtus* L. (Pansuksan et al. 2014) and *Centella asiatica* (Kaensaksiri et al. 2011). Chromosome duplication due to polyploidization generally caused the increase of organ size (Osborn et al. 2003), so tetraploid plants had a significantly higher biomass production (Corrêa et al. 2016; Hannweg et al. 2016).

Stomatal trait changes. The number of stomata cells were measured and presented as stomata density in each field microscope with (10× and 40× magnification). The diploid showed the significantly higher value on number of stomata (46.00 for autopolyploid and 33.40 for allopolyploid) than that in tetraploid (23.00.and 23.20, respectively). However, the tetraploid had bigger stomata area (1400.37 for autopolyploid and 1795.09 for allopolyploid) than that of tetraploid (1208.14 and 1203.06, respectively) (Table 2).

Moreover, stomata size increased significantly but stomata density decreased in induced tetraploid of autopolyploid and allopolyploid plants (Fig. 3) which was in agreement with previous researches (Jiang et al. 2014; Salma et al. 2018). It has been reported that the number of guard cells per unit area decreased in accordance with the increased level of polyploidy (De Oliveira et al. 2004). Some early studies considered the lower frequency of stomata in tetraploids as probably due to the larger epidermal and guard cells (Gantait et al. 2011) as well as reduced stomata differentiation (Tu et al. 2018).

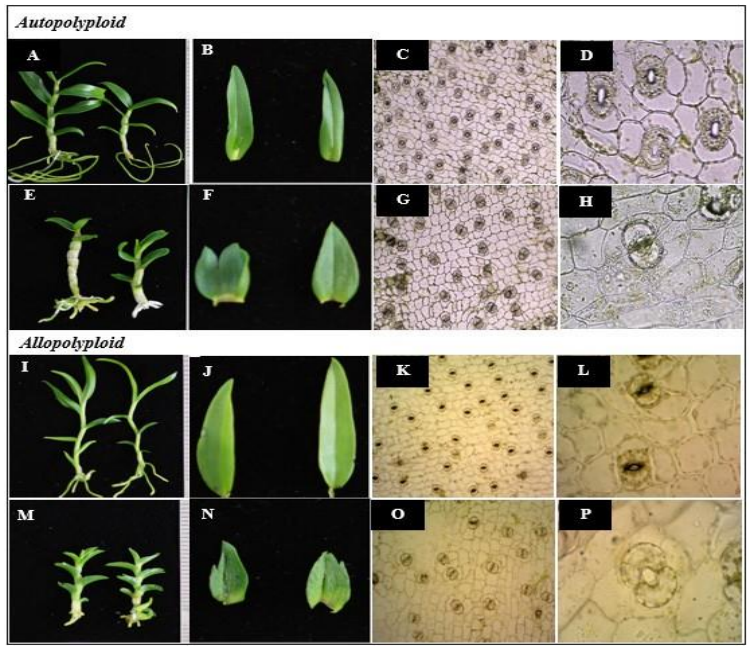

Figure 3. The six-month-old seedlings and leaves; stomata density (100×) and size (400×) of diploid and tetraploid *D.officinale*

Diploid of *DO.201-3 D1*, six-month-old seedling (A), leaf (B), stomata density-100× (C), stomata size-400× (D) Tetraploid of *DO.201-3 T1,* six-month-old seedling (E), leaf (F), stomata density-100× (G), stomata size-400× (H) Diploid of *DON.200-7 D1,* six-month-old seedling (I), leaf (J), stomata density-100× (K), stomata size-400× (L)

Quantitative real-time PCR (qRT-PCR) validation. The optical density (OD) and gel electrophoresis results of all samples revealed the quality of RNA was purified enough for further experiments as well as the amount of RNA was calculated (Data not shown). The expected size of cDNA of diploid and tetraploid of autopolyploid and allopolyploid in *D. officinale* was obtained at approximately 750bp (Fig. 4). This demonstrated that total RNA extracted from any samples of *D. officinale* could meet the requirements of related molecular biological researches.

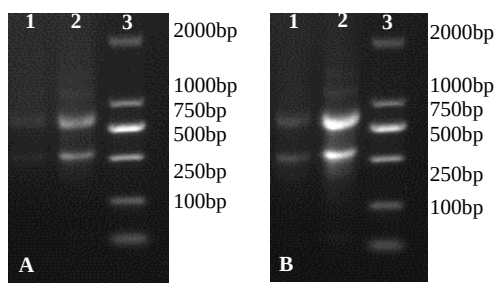

Figure 4. Agarose gel electrophoresis of the RT-PCR tubulin products

A, lane 1 diploid of *DO.201-3 D1*, lane 2 tetraploid *DO.201-3 T1,* lane 3 Marker

B, lane 1 diploid of *DON.200-7 D1*, lane 2 tetraploid *DON.200-7 T1*, lane 3 Marker

A total of 9 pairs of primers were selected by using Primer 5.0 soft-wave based on the results of transcriptome in *D. officinale* and unigene database. These 9 pairs of primers were subsequently tested for allele polymorphism, the number of nucleotides (Nu) ranging from 18 to 24 (Table 3).

Table 3. Selected primer pairs

Cluster	***Oligo Name***	**Sequence (5' to 3')**	**Number of Nucleotides**
Cluster-29035.194335(-2.8937) 1223-407）	*Man2-2F*	AACTCTGCGAACACCACCG	19
Cluster-29035.194335(-2.8937) 1223-407）	*Man2-2R*	GCAACAGCCCACATCTACCC	20
Cluster-29035.65822(-2.393)（596-524）	*g1gc3-1F*	TGCGTCGCCTACTGGTCTCTTA	22
Cluster-29035.65822(-2.393)（596-524）	*g1gc3-1R*	GATACTGTGATGATGGGTGCTGA	23
Cluster-29035.132552 (-3.9378)	*PGM1F*	ACGCTTGTTGTTTCTGGG	18
Cluster-29035.132552 (-3.9378)	*PGM1R*	AGCACCTGTAGCCTTGGA	18
Cluster-29035.113664 (4.1887)（854-324）	*iPGM1-2 F*	TGAAACGAGCACCTGGAC	18
Cluster-29035.113664 (4.1887)（854-324）	*iPGM1-2 R*	AAGGAATAAATCTGGGAAACC	21
Cluster-29035.194251 (191-946)	*PMM2-1 F*	GAGGATGCCAGGGCTTATTGT	21
Cluster-29035.194251 (191-946)	*PMM2-1 R*	CCCTCCAACGACACCAACTG	20
Cluster-29035.94132 (896-1868)	*GMP1-3 F*	CGATCCAGGCTTCCTTCACT	20
Cluster-29035.94132 (896-1868)	*GMP1-3 R*	TCCGACGAAACTGAGCAAGAT	21
Cluster-29035.190181 (362-1996)	*CSLA3-1F*	TTTCCCTCAAGTAGTGATTCCG	22
Cluster-29035.190181(362-1996)	*CSLA3-1 R*	CCTTCACTCTCCCTGCCTCTA	21
Cluster-29035.122404 (12-1168)	*CSLA2-1 F*	TCTCTTCCATTACCACAACACCAT	24
Cluster-29035.122404 (12-1168)	*CSLA2-1 R*	ATAAAGTTCCACAAATCACACGCT	24
Cluster-29035.108496	*GMPP F*	GCCGTAGGTTCGCACTTT	18
Cluster-29035.108496	*GMPP R*	CAATGATGCTGCTTGAGACA	20

In autopolyploid seedlings, the expression of *CSLA2-1, g1gc3-1, GMP1-3, GMPP, iPGM1-2 and Man2-2* were significantly up-regulated from diploid to tetraploid, while the expression of *PMM2-1, CSLA3-1* and *PGM1* were significantly down-regulated from diploid *DO.201-3 D1* to tetraploid *DO.201-3 T1*. These results revealed that *CSLA2-1, g1gc3-1, GMP1-3, GMPP, iPGM1-2* and *Man2-2 genes* might increase the polysaccharide content in tetraploid seedlings (Fig. 5).

In a similar trend with autopolyploid, the results of allopolyploid indicated that the expression of *CSLA2-1, CSLA3-1, GMP1-3, GMPP, iPGM1-2, Man2-2* and *PGM1* genes of *DON.200-7 T1* tetraploid were higher than that of *DON.200-7 D1* diploid. The expression of *g1gc3-1* gene had no significant difference between diploid and tetraploid seedlings while that of the PGM2-2 was lower in tetraploid in comparison to diploid seedlings (Fig. 6).

Research on *D. nobile* polysaccharides has been of great interest, especially studies on their content (Wang et al. 2011) composition (Luo et al. 2016; Wang 2010) and pharmacological effects (Zhang et al. 2013). Based on an understanding that *D. officinale* stems are the principal vessel for storage of polysaccharides (He et al. 2015), the roots, stems and leaves from six-months old *D.*

officinale seedlings were used to determine the relative expression level of some genes related with polysaccharides by qRT-PCR. In our study, nine genes related to the biosynthesis of polysaccharide as *CSLA 2-1, CSLA 3-1, g1gc3-1, GMP1-3, GMPP, iPGM1-2, Man2-2, PMM2-1* and *PGM1* were applied to determine the relative expression level of polysaccharide in stem of diploid and tetraploid of autopolyploid and allopolyploid seedlings. In autopolyploid, 6 per 9 genes (*CSLA 2-1, g1gc3-1, GMP1-3, GMPP, iPGM1-2* and *Man2-2)* exposed high significant of polysaccharide expression from diploid to tetraploid of *D. officinale*, while these were 7 per 9 genes (*CSLA 2-1, CSLA3-1, GMP1-3, GMPP, iPGM1-2, Man2-2* and *PGM1)* in allopolyploid. Previous studies also confirmed some group of genes regulate polysaccharides in *Dendrobium* such as *SCLA* (*DoCSLA1* to *DoCSLA9*) (He et al. 2015) or *PMM, GMPP* (Li et al. 2017) or *PMM* (He et al. 2017), but comparison of polysaccharide genes expression between diploid with tetraploid of autopolyploid and allopolyploid seedlings is not available in present literature. This is the most prominent study in this paper.

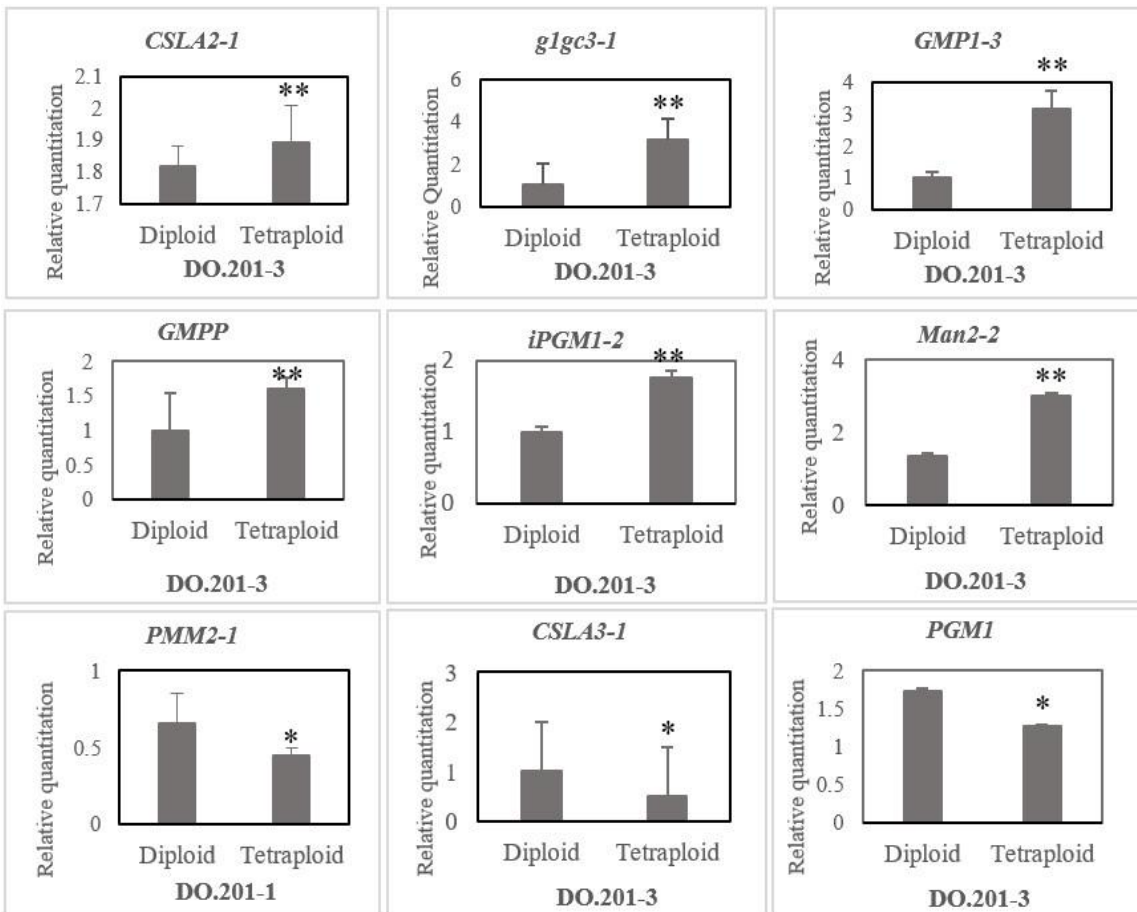

Figure 5. Expression levels of nine genes related to the polysaccharide process in autopolyploid. The expression of nine selected polysaccharide-related genes were validated with diploid and tetraploid of DO.201-3. Each bar shows the mean ±SD (n=3) of three biological repeats. The significant changes ($P<0.05$) in expression levels of studied gene between diploid and tetraploid were indicated by (*) significantly down-regulated from diploid to tetraploid and (**) significantly up-regulated from diploid to tetraploid.

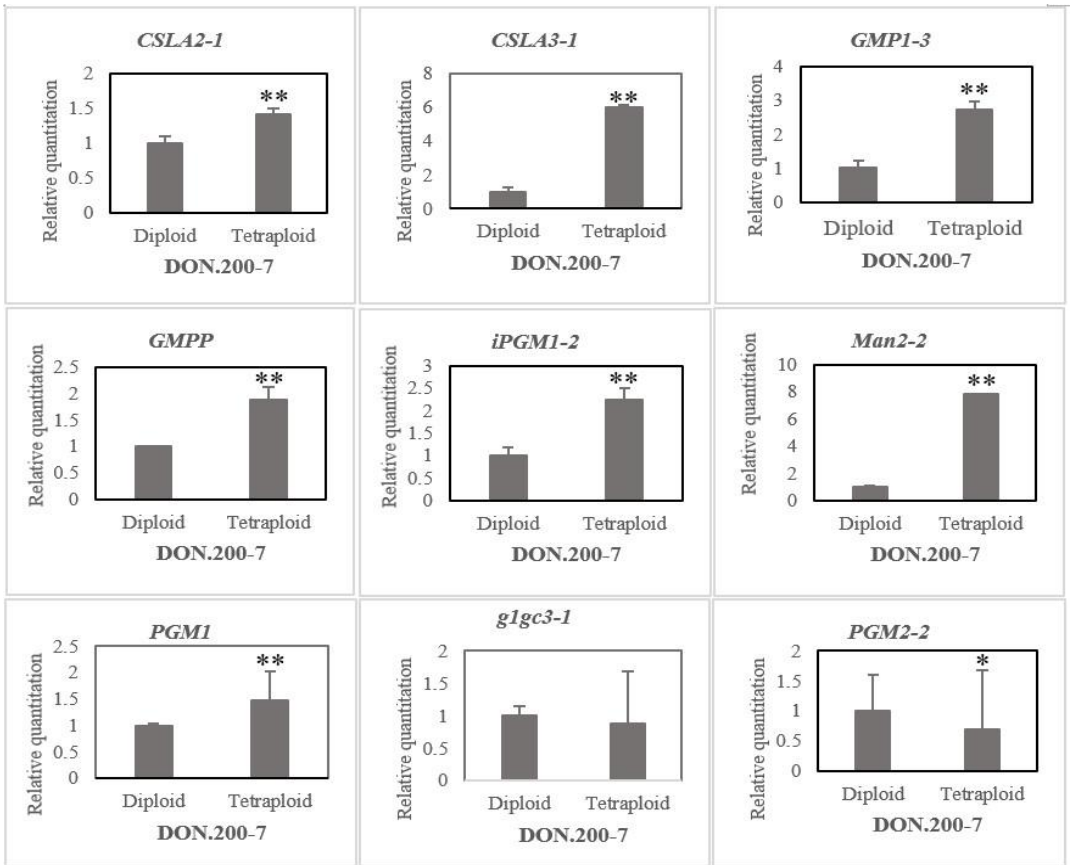

Figure 6. Expression levels of nine genes related to the polysaccharide process in allopolyploid. The expression of nine selected polysaccharide-related genes were validated at diploid and tetraploid of *DON.200-7*. Each bar shows the mean ±SD (n=3) of three biological repeats. The significant changes ($P<0.05$) in expression levels of these gene between diploid and tetraploid were indicated by (*) significantly down-regulated from diploid to tetraploid and (**) significantly up-regulated from diploid to tetraploid.

CONCLUSION

Colchicine affected the survival rate of PLB and polyploid induction. The higher colchicine concentration and longer treatment duration led to lower rate of PLB survival. The highest autopolyploid and allopolyploid induced by colchicine treatment used 0.1 mg.L^{-1} for 4 and 5 days, respectively. The seedlings of tetraploid of both autopolyploid and allopolyploid had shorter and thicker stem and root than diploid. This led to lighter fresh weight in diploid of both autopolyploid and allopolyploid. The diploid showed the significantly higher value on number of stomata than tetraploid. But the tetraploid had higher value of area of stomata pore than diploid. Among the studied genes, both the up-regulated and down-regulated from diploid to tetraploid were expressed significantly. In autopolyploid the *CSLA2-1*, *g1gc3-1*, *GMP1-3*, *GMPP*, *iPGM1-2* and *Man2-2* genes might increase the polysaccharide content in tetraploid while in allopolyploid these were *CSLA2-1*, *CSLA3-1*, *GMP1-3*, *GMPP*, *iPGM1-2*, *Man2-2* and *PGM1*. The results of this study might provide vital information for further studies related to breeding for high biomass production and polysaccharide content for medicinal as well as ornamental value of *Dendrobium officinale*.

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