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Research Article

The Effect of *Thidiazuron* and *Naphtalene Acetic Acid* on *In Vitro* Development of *Eria hyacinthoides* (Blume) Lindl Orchid

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ABSTRACT
Eria hyacinthoides (Blume) Lindl. is an Indonesian orchid species found in Sumatra, Java, and Bali. This orchid is a sympodial orchid with flowers that has fragrant aroma, suspected containing phytochemicals for herbal medicines, so mass plant propagation is necessary. The aim of this research is to obtain the best *in vitro* conditions for this orchid through somatic embryos using growth regulators and analysing the structure of the *Dendrobium Orchid Homeobox 1* (*DOH1*) homologous gene in *E. hyacinthoides* to *Dendrobium* 'Madame Thong-In' which is known to induce bud formation. The method used in this study: (1) the leaves of the plant spread about 20 - 30 days from shoots measuring about 6.3-6.7cm on the mother plant aged ± 8 years, stored in an incubation room with picture of 16 hours of light and 8 hours of darkness in the heat. 25 ± 1 °C , (2) compared Murashige and Skoog, Vacin and Went, Knudson C and New Phalaenopsis growth to get the best medium, (3) added PGR to medium (Thidiazuron (TDZ) 1 - 3 mg L^{-1} and Naphthalene-1-acetic acid (NAA) 1-3 mg L^{-1}), (4) isolate partial gen *DOH1* homologous by using primer of *DOH1*, (5) analyse sequence of PCR products. Optimal medium for callus embryogenesis production from leave was Knudson C + TDZ 1 mg L^{-1} + NAA 1 mg L^{-1}. Amplification of DNA fragments using degenerate primers of *DOH1* resulted 175 bp, indicating similarity about 88.64 % with between the *DOH1* gene structure in *E. hyacinthoides* and in *Dendrobium* 'Madame Thong-In'.

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INTRODUCTION

Orchids belong to the family Orchidaceae, one of the largest families of flowering plants with about 30,000 species worldwide, of which 5000 are distributed across almost all the islands of Indonesia (Semiarti 2018). One of them is the natural orchid *Eria hyacinthoides* (Blume) Lindl. *E. hyacinthoides* is a sympodial orchid with beautiful flowers. Fragrant flowers; therefore, they are thought to contain potential metabolites. The phytochemical content has been reported in *Eria stricta* (*phenanthrenes*) (Wang et al. 2012), and *Eria marginata* (*flavonone*) (Sun et al. 2014). Therefore, *E. hyacinthoides* orchids may contain chemical compounds that are useful for medicine, so it is necessary to propagate them in bulk and obtain uniform results by means of *in vitro* culture.

The population of *E. hyacinthoides* is not well known in its habitat, so its alleged high potential as a source of medicine needs to be known by propagating this orchid *in vitro* to provide plants. Therefore, it is important to identify the most suitable medium and PGR for *in vitro* propagation of this orchid. The combination of auxins and cytokinins plays an important role in the success of *in vitro* plant cultures. According to Mose et al. (2017), TDZ 3 mg L^{-1} was the most effective PGR for inducing direct somatic embryogenesis from various *Phalaenopsis amabilis* (L.) explants. Kasi and Semiarti (2016) reported that TDZ 10 mg L^{-1} combined with NAA 1 mg L^{-1} and NP + 15 % coconut water medium was the best treatment to induce somatic embryogenesis in *Phalaenopsis* 'Sogo Vivien' leaf explants.

The KNOX family is a well-defined homeobox transcription factor that is part of the TALE (three-amino acid loop extension) subclass. It is essential for leaf morphogenesis and apical meristem (SAM) formation (Gan et al. 2023). According to Ruben et al. (2022), shoot growth begins with the activation of homeobox genes in the *Shoot Apical Meristem* (SAM) which activates associated genes that control plant organ growth. *Homeobox* genes in orchids have been reported in *Dendrobium* and *Phalaenopsis*. *Dendrobium* 'Madame Thong-In' has *DOH1*, a *KNOTTED1-like homeobox* gene (Yu et al. 2000). The homologous of the *DOH1* gene in *P. amabilis* orchid was isolated by Semiarti and named *Phalaenopsis Orchid Homeobox 1* (*POH1*) (Semiarti et al. 2008). As a result, it is assumed that *Eria* has *homeobox* gene homology with *DOH1*, so it is necessary to isolate and characterize the *DOH1* gene homologous in the *E. hyacinthoides* orchid.

MATERIALS AND METHODS

Plant Material, Medium Preparation and Growth Conditions

The explants utilised in this study were sourced from quality, disease-free in orchid *E. hyacinthoides* parent ± 8 years old. The research object used was the second leaf from inside the bud of an *E. hyacinthoides* orchid, which is about 20 - 30 days old and measures 6.3 - 6.7 cm in height shot in the Karangayam Research Station, Faculty of Biology, UGM, Yogyakarta. The orchid shoots of *E. hyacinthoides* were first cleaned with soap under running water before being dried. To sterilise the shoots, they were soaked in fungicide for 10 minutes and rinsed thrice using sterile distilled water in a laminar airflow (LAF) environment. They were then soaked in a bactericide for another 10 minutes, rinsed thrice with sterile distilled water, treated with a solution of 5.25 % NaOCl combined with Tween 20 for 3 minutes, and finally rinsed three times with sterile distilled water. Shoots were immersed in 70 % alcohol for 30 s and washed three times in deionized water 3 times. The shoots are immersed in a PPM solution for 15 min. The 2nd leaf from the tip was selected as an explant for *in vitro* culture. Explants were cut using a 1 × 1 cm scalpel and planted on a growth medium. Explants were grown on basic mediums such as MS (Murashige & Skoog), NP (New Phalaenopsis), KC (Knudson C), and VW (Vacin and Went) medium. The best medium was treated with the addition of

growth regulators (TDZ 1 - 3 mg L^{-1} + NAA 1 - 3 mg L^{-1}) (Table 1). Explants were derived from the leaf bases. Explants were cultivated in petridish (16 x 2.5 cm) with five repetitions. Subculture was once a month. The culture was placed in an incubation room maintained at a temperature of 25 ± 1 °C, featuring 16 hours of light and 8 hours of darkness. Growth and development of explants were observed for 12 weeks.

Table 1. The treatment of NAA and TDZ into medium for somatic embryo induction of *E. hyacinthoides*.

Treatments*	T0	T1	T2	T3
N0	N0T0	N0T1	N0T2	N0T3
N1	N1T0	N1T1	N1T2	N1T3
N2	N2T0	N2T1	N2T2	N2T3
N3	N3T0	N3T1	N3T2	N3T3

Anatomical preparation

A paraffin embedding method with a single staining method of safranin was used by Ruzin (Schmid 1999) to confirm the anatomical structure of the cross leaf and nodular masses of *in vitro* cultured leaf explants. The preparation was observed under a light microscope (Olympus, Jepang) using an Optilab 2.2 (MICONOS, Indonesia).

Detection of *DOH1* homologous in the genome of *E. hyacinthoides*

The genome DNA was isolated by using Murray and Thompson's (1980) method, with modification by adding 1 % PVP to the CTAB 3 %. The gene was amplified by using Polmerase Chain Reaction (PCR) with *DOH1* and *ACTIN* primers (Table 2). PCR amplifications were performed using MyTaq™ Red Mix protocols (Bioline). The results were seen by using a UV transilluminator and 1 % agarose gel. The confirmed PCR results containing the *DOH1* target gene were sequenced. Sanger sequencing was conducted. The samples were analyzed using an Applied Biosystems 3500 Genetic Analyzer 2550 equipment (Hitachi, Japan) at the Integrated Research and Testing Laboratory of Universitas Gadjah Mada (LPPT, UGM).

Table 2. List of primers used in this study.

Primer	Sequence		Product length
DOH1	F	5'- CACCAACGATGGATGAGATG -3'	
			175bp
	R	5'- CGAGAAGATGGGGATAACG -3'	
ACTIN	F	5'- GTATTCCCTAGCATTGTTGGT -3'	
			114bp
	R	5'- CAGAGTGAGAATACCTCGTTTG -3'	

Data Analysis

In vitro culture data obtained from explant growth and development for 12 weeks were analysed using Microsoft Excel 2010. Furthermore, the mean values of the parameters were analyzed by ANOVA 95 % degree of confidence, followed by Duncan's multiple range test was carried out whenever there were significant differences between the treatments. Molecular analysis of the sequencing results used BioEdit to help edit and prepare the target sequences and analysed it using the Basic Local Alignment Search Tool (BLAST). Nucleotide sequences were translated into amino acids using The Expasy-translate website (https://web.expasy.org/). Protein motifs were searched on the MotifFinder website (https://www.genome.jp/tools/). Sequence align-

ment was done using the MultAlin website (http://multalin.toulouse.inra.fr/). Exploration of reference sequences were obtained from NCBI (National Center for Biotechnology Information) and OrchidBase 6.0 websites.

RESULTS AND DISCUSSION

Orchid *E. hyacinthoides* has sympodial growth type with pseudobulb, from one pseudobulb there are 2 - 3 leaves attached to the end of *pseudobulb*. The average pseudobulb height of *E. hyacinthoides* is ± 8 cm with a width of ± 2.5 cm. The leaves of *E. hyacinthoides* are lanceolate with fingered leaf veins and unsymmetrical pointed leaf tips, with an average leaf length of ± 26.6 cm and leaf width of ± 4.5 cm. *E. hyacinthoides* flowers grow from the leaf axils, clustered white and contain ± 28 flowers with a diameter of ± 1.5 cm with a flower stalk length of ± 23.4 cm. *E. hyacinthoides* flowers bloom for ± 7 days. According to Nursanti et al. (2020) *Eria* orchids like shady and humid places, usually grows well at an altitude of 250 - 1000 m above sea level. Buds are the parts for somatic embryo induction in this study (Figure 1). Leaf number 2 from the inside is used as an explant measuring 1 x 1 cm.

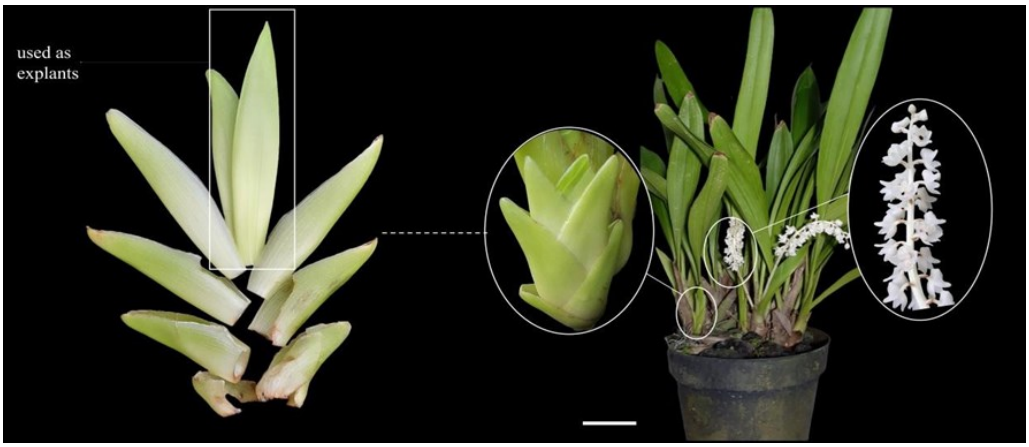

Figure 1. Morphology of *E. hyacinthoides*. Leaf number 2 from the inside of the shoots was used as an explant with a size 1 x 1 cm. (Scale bar 1 cm)

The Best Medium for *In vitro* Growth and Development of *E. hyacinthoides* cultures

Based on the results, after 15 days of explant inoculation on basal medium, the best medium for *in vitro* explant growth of *E. hyacinthoides* is a KC medium as shown in Figure 2. KC medium shows the best medium because it has reached phase 2. The development of *E. hyacinthoides* orchid *in vitro* explants is categorised into 4 phases modified from the research of Dewi et al. (2012), including: phase 1 the explants responded by showing indentations on the wounded part; phase 2 the indentations on the explants thickened (bubbling); phase 3 the thickening on the explants is getting bigger or there is a sign that there will be a protrusion; phase 4 the appearance of nodular masses protruding from the surface of the epidermis.

Finding a suitable culture medium is the most important aspect of culture conditions. In plant tissue culture, a good culture medium will determine the results that will be obtained. Good medium is not only able to support the life of explants but also can increase the growth of explants optimally (Semiarti et al. 2020). Leaf number 2 from the inside of *E. hyacinthoides* orchid buds were used as explants in *in vitro* culture on KC medium with pH 5.8. Explants were maintained in an incubation room with photoperiod of 16 hours of light and 8 hours of dark light and temperature set at 25 ± 1 °C. Growth was observed for 3 months. Based on the observations in Table 3, it is known that the combination treatment of TDZ 1 mg L^{-1} + NAA 1 mg L^{-1} stimulated the

formation of nodular mass by 20 % at the tip of the explant after three months of inoculation.

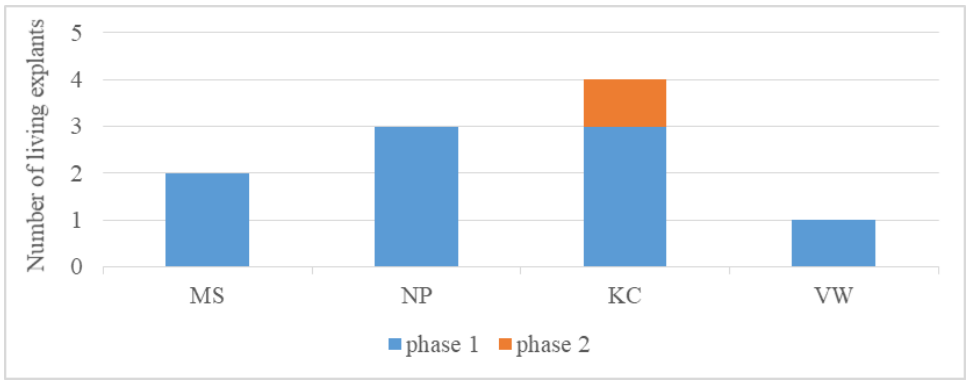

Figure 2. Growth frequency of explants on basal medium assessed after 15 days in culture. MS = Murashige and Skoog; VW = Vacin and Went; KC = Knudson C; NP = New Phalaenopsis.

The optimum amount of TDZ plays an important role in the translocation and distribution of NAA, so that it will accumulate in cells that have high totipotency (Ningrum et al. 2017). When plants are grown in a medium containing auxin without cytokinin, their size increases and the cells do not divide. Adding cytokinin to the medium causes cell division and differentiation. The presence of auxin and cytokinin in the same quantity causes the formation of callus without growth (Bhatla & Lal 2018). Several previous studies have found that leaf blowers respond well to somatic embryo initiation in several orchid species, including *P. amabilis* (Mose et al. 2017) and *Tolumnia* (Chookoh et al. 2019). In general, it is thought that somatic embryogenesis occurs in response to changes in PGR levels, especially auxin and cytokinin in tissue culture systems. Embryonic tumors appear as nodular masses appearing on the surface of the injured tissue (Mose et al. 2017).

The application of TDZ in the *in vitro* culture of *E. hyacinthoides* produced a positive response at 1 mg L^{-1} TDZ, with surviving explant growth reaching 80 %. Adding hormones into the medium is an important step in *in*

Table 3. The Growth percentage of *in vitro* explants of orchid *E. hyacinthoides* on KC medium with various PGR and their concentrations.

Treatments	Percentage of living explants	Percentage of embryogenic explants	Percentage of explants that formed embryogenic callus
N0T0	80%ab	60%ab	0%a
N1T0	60%ab	60%ab	0%a
N2T0	0%a	0%a	0%a
N3T0	0%a	0%a	0%a
N0T1	20%ab	20%ab	0%a
N0T2	20%ab	20%ab	0%a
N0T3	0%a	0%a	0%a
N1T1	80%b	60 %ab	**20%b**
N2T1	80%b	80%b	0%a
N3T1	80%b	80%b	0%a
N1T2	40%ab	20%ab	0%a
N2T2	60%ab	20%ab	0%a
N3T2	60%ab	40%ab	0%a
N1T3	40%ab	40%ab	0%a
N2T3	60%ab	60%ab	0%a
N3T3	60%ab	60%ab	0%a

Description: The frequency of explants forming embryogenic callus was assessed after 84 days in culture. N = NAA; T = TDZ; 0 = Single; 1 = 1 mg L^{-1}; 2 = 2 mg L^{-1}; 3 = 3 mg L^{-1}, n = 5

vitro propagation to increase explant growth, which the amount and type of PGR used must be in accordance with the objectives to be achieved. TDZ is often used alone or in combination with other PGRs to induce somatic embryogenesis in orchids (Balilashaki & Ghehsareh 2016). Cytokinins are a class of plant growth hormones that promote cell division (Bhatla & Lal 2018). According to Feng and Chen (2014), *Phalaenopsis aphrodite* orchid plants can produce somatic embryos when TDZ is used alone. TDZ is an important PGR factor in the *in vitro* micropropagation of some orchids such as *Dendrobium aqueum* Lindl. (Parthibhan et al. 2015), *Cypripedium lentiginosum* (Jiang et al. 2017), *Gastrochilus japonicus* (Kim et al. 2019a), *Ansellia africana* Lindl. (Bhattacharyya et al. 2019), and *Pecteilis* radiate to produce protocorms (Kim et al. 2019b).

The use of a single 1 mg L^{-1} NAA is the best single NAA concentration which the percentage of surviving explants reaches 60%. One characteristic of cell walls in growing cells is that they stretch faster at acidic pH than at neutral pH. This phenomenon is called acid taste. Cleland and Rayle proposed a hypothesis to explain auxin-stimulated cell elongation. According to them, auxin causes acidification of the cell wall through the release of protons in the cell. The low pH of apoplastic (acidic) produced causes the process of loosening the cell wall through enzymatic activity. Another German scientist named Hager also suggested the role of plasma membrane-bound H+-ATPases in auxin-stimulated proton release. These two strategies, formerly known as the Cleland-Hager strategy, are now known as the acid growth factor for cell proliferation (Bhatla & Lal 2018).

Based on anatomical analysis of 3-month-old *E. hyacinthoides in vitro* culture explants, it is known that meristematic cells were still actively dividing, indicating that secondary somatic cells continued to grow (Figure 3e-f) in contrast to explant cells at the age of 0 days (Figure 3d). Staining with safranin reacted well to the part of the cell that had thickened lignin. Lignin was thought to be a tissue containing phenolic compounds. Nodular mass formation began with the development of embryogenic cells at the wound site. The visible stages show the formation of early globular embryos characterised by a rounded structure at the explant wounding site (Figure 3b-c). Multiple layers of embryogenic cells are characterised by densely arranged cells with dense cytoplasm and clearly visible nuclei. Explants on day 0 of inoculation are shown in Figure 3a.

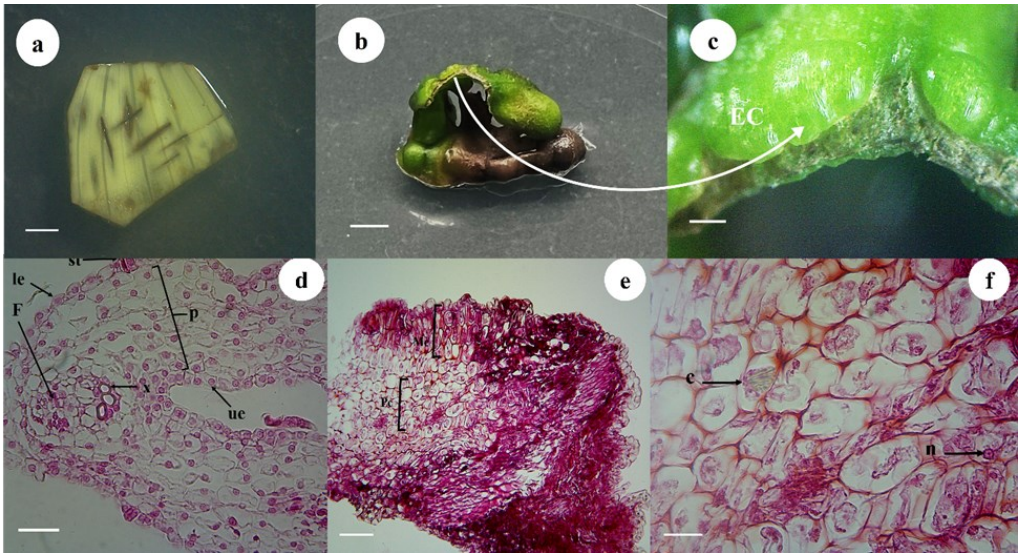

Figure 3. Development of nodular mass (arrowheads) in *in vitro* culture of *E. hyacinthoides*. (a) Leaf spreading and shoot of *E. hyacinthoides* aged 0 days; (b-c) embryonic cells formed in leaf explants in *E. hyacinthoides* shoots treated with TDZ 1 mg L^{-1} and NAA 1 mg L^{-1} after 3 months of inoculation, the explants showed early globular-like

structures derived from the epidermal cell layer. Cross section of (d) 0-month-old explants (e) nodular mass (arrow) Mc indicates meristematic part, Pc stands for parenchyma cells and the darker colored part is dead sclerenchyma tissue. (f) polarized embryogenic suspensor mass. Abbreviations: ec (embryonic cells); ue (upper epidermis); le (lower epidermis); st (stomata); p (parenchymal cells); f (phloem); x (xylem); c (oxalate crystals); n (cell nucleus). Barres a-b = 100 µm; c = 400 µm; d-e = 200 µm; f = 300 µm.

In general, it is thought that somatic embryogenesis will occur in response to changes in PGR levels, especially auxin and cytokinin in tissue culture systems. The first embryo appears as a nodular figure emerging from the surface of the infected material (Mose et al. 2017). The effectiveness of plant tissue culture is greatly influenced by the interaction between auxin and cytokinin. In this study, NAA alone did not induce morphogenetic reactions in explants. The inhibitory activity of exogenous auxin on leaf regeneration was also observed in *Dendrobium* cv. Chiengmai Pink (Chung et al. 2007).

Analysis of the sequence *DOH1* homologous gene in *E. hyacinthoides*

The results of genomic DNA isolation of *E. hyacinthoides* are shown in Figure 4. The amplification of the *ACTIN* gene in the *E. hyacinthoides* genome of 114 bp, this is indicating that the genomic DNA is in good condition and can be utilised to control the amplified gene. *ACTIN* is a housekeeping gene and the most stable reference gene for all plant tissues and leaves, therefore it might be utilised as an internal/positive control for further amplification (Yuan et al. 2014).

The amplification product of the *homeobox* gene in *E. hyacinthoides* using the *DOH1* primer was found to have a length of 175 bp (Figure 5). The genomic sample obtained was then amplified using the *DOH1* gene to see its homology with *E. hyacinthoides* and the *ACTIN* gene which is a housekeeping gene used as a positive control.

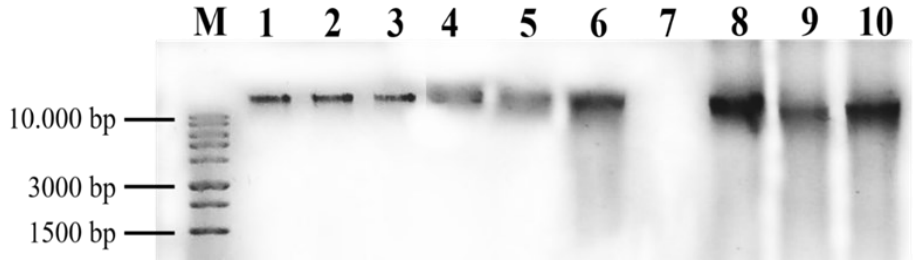

Figure 4. Visualisation of genomic DNA of *E. hyacinthoides*. Lane descriptions: M = Markers; 1-3 = shoots; 4-5 = roots; 6-8 = leaves; 9-10 = flowers.

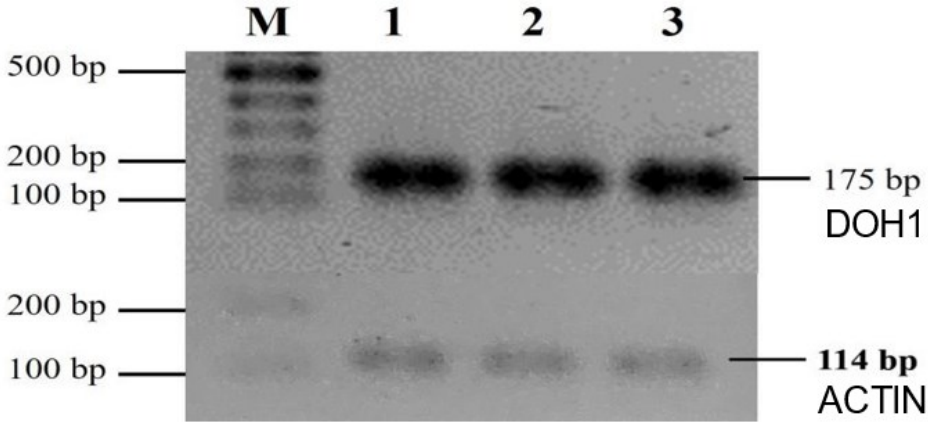

Figure 5. Detection of *DOH1* gene in *E. hyacinthoides* genomic DNA samples. A) Bands of 175 bp amplification of the *DOH1* gene. B) Bands of 114 bp amplification of the *ACTIN* gene. Lane descriptions: M = Markers 100bp; 1-3 = sample 1-3.

Homology analysis between the amplified DNA fragment of the *DOH1* gene in *E. hyacinthoides* and the database using BLAST NCBI database found that the *DOH1* gene in *E. hyacinthoides* revealed similarities with *DOH1* gene in *D.* 'Madame Thong-In' in 88.64 %. Therefore, based on the similarity results of the homologous *DOH1* gene in *E. hyacinthoides*, it may be stated to be *Eria Orchid Homeobox1 (EOH1)*. The sequence of *EOH1* also showed similarity with other *KNOX* genes in orchid species, including *Homeobox Protein Knotted-1-Like 6* in *Dendrobium catenatum* with 90.34 % similarity and *Class 1 KNOX* in *Dendrobium nobile* with similarity of 90.34 %. This similarity needs to be followed up for longer amplification.

The alignment results (Figure 6) showed the location of the *DOH1* gene in *E. hyacinthoides* with *DOH1* gene in *D.* 'Madame Thong-In' in the exon region. The encoded amino acids indicated the *KNOX1* protein motif. The function of the *KNOX1* gene was associated with tissue proliferation and the preservation of the meristematic potential of moss sporophytes and blooming plants. The diversity of leaf shape observed in flowering plants were partly attributed to the control of *KNOX1* activity (Furumizu et al. 2015). Based on motif analysis conducted between *E. hyacinthoides* and other orchids (Figure 7), it showed a representation of motif images that was not full. This was be-

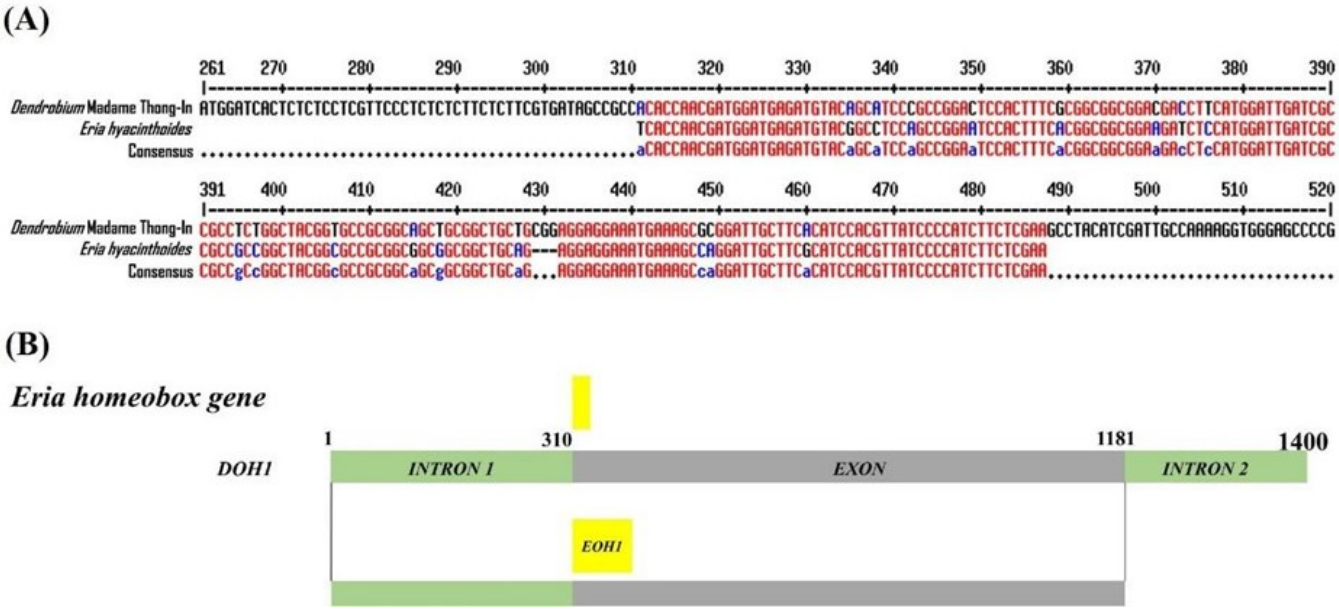

Figure 6. Sequence analysis of *DOH1* homologous genes in *E. hyacinthoides* with *DOH1* in *D.* 'Madame Thong-In' (A) Sequence alignment between *DOH1* and *EOH1*, (B) Schematic representation of *EOH1* fragment aligned with *DOH1*.

Figure 7. Schematic representation of *DOH1* protein motifs homologous to *E. hyacinthoides* and other orchids. Color description: Green = *KNOX1*; Blue = *KNOX2*; Purple = *ELK*; Red = *Homeobox KN*.

cause the amplification of the *DOH1* gene in *E. hyacinthoides* obtained only 175bp.

The functions of most *homeobox* genes acted as transcriptional regulators, especially of developmental processes (Viola & Gonzalez 2016). The activation of the *KNOX1* homeobox gene which caused tissue proliferation, may be linked to nodular masses with constantly dividing meristematic cells in *in vitro* cultures of *E. hyacinthoides* orchids.

CONCLUSIONS

The conclusion obtained from this study is the best explant for the growth of *in vitro* culture of *E. hyacinthoides* orchids is the youngest leaf from shoot. The best medium for the *in vitro* culture of the orchid *E. hyacinthoides* is KC medium, and the optimal concentration of PGR to induce the development of somatic embryos in *E. hyacinthoides* is TDZ 1 mg L^{-1} + NAA 1 mg L^{-1}. The *EOH1 homeobox* gene structure in *E. hyacinthoides* showed 88.64 % homology to the *DOH1* gene of *D.* 'Madame Thong-In'.

AUTHOR'S CONTRIBUTION

ES designed and managed the research process. RF performed the analysis and data analysis. KNP maintained the mother plant for *in vitro* culture.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in this research.

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