In vitro propagation of *Vanda tricolor* Lindl. var. *suavis* protocorm on media containing liquid organic fertilizer as a substitute for MS media

A I Latunra, M Tuwo and N Rezky
Department of Biology, Faculty of Mathematics and Natural Science, Hasanuddin University, Jl. Perintis Kemerdekaan Km. 10, Tamalanrea, Makassar 90245, South Sulawesi, Indonesia

Email: mustikatuwo@gmail.com

Abstract. Vanda orchids have a high economic value in the flower industry, so it is necessary to have seeds available at all times. Tissue culture technology offers an important solution to produce plants in large numbers, but it is very costly in media preparation. Hence, it is necessary to have low-cost options for the application of planting media. One way of doing this is to substitute the composition of Murashige and Skoog (MS) media with alternative sources that are more affordable. Liquid organic fertilizer contains macro, micro, and good nutrients for the growth of explants. This study used explants from the protocorm of *Vanda tricolor* Lindl. var. *suavis* aged three months planted on media containing liquid organic fertilizer, namely Nasa, Bio88, and Fortune. The parameters observed were the number of shoots and the number of leaves in each treatment. The data were analyzed using the Kruskal-Wallis test at the 5% level, and if there was an effect, it was continued with the Mann-Whitney test. The results showed that liquid organic fertilizer gave different responses to the *Vanda tricolor* Lindl. var. *suavis* protocorm. Liquid organic fertilizer has a significant effect on the number of shoots and number of leaves.

1. Introduction
Orchids belong to the Orchidaceae family and are one of the most diverse flowering plants. Orchids can grow in lowland to highland areas [1]. Orchids on a global scale are estimated at 736-899 genera of 27,800 species. In addition, more than 100,000 are hybrid orchids [2]. Indonesia is the second country with orchid germplasm richness after Brazil. The number of orchid species in Indonesia continues to grow along with the discovery of new types of orchids, and there are even orchids endemic to Indonesia [3]. Orchids have the potential to be developed because they are unique and have high economic value, especially in Indonesia. One type of orchid that can be developed is *Vanda tricolor* Lindl. var. *suavis*. This orchid has the advantage of being resistant to heat because it has a heat-resistant gene, namely the HSP70 gene, and has a fragrant aroma. However, this orchid has seeds with a microscopic size of about ± 0.21 mm and does not have an endosperm as a food reserve. In addition, orchids are difficult to propagate in nature because orchids require the help of mycorrhizae to germinate [4].
In 2010, the existence of *Vanda tricolor* Lindl. var. *suavis* in the slopes of Mount Merapi has now been greatly reduced due to overexploitation and forest destruction due to natural disasters such as the eruption of Mount Merapi, which scorched 80% of the habitat of *Vanda tricolor* Lindl. var. *suavis* and erupted again in 2018 [5–7]. Orchid flowers are one type of ornamental plant that has a very beautiful charm in terms of shape and color pattern, so it is not surprising that the demand for orchids continues to increase every year [1]. This beautiful flower is also used as part of the national flower of Indonesia. One alternative that can be applied to compensate for the increasing demand for orchids is plant tissue culture that is able to produce large numbers of plants in a short time, has the same characteristics as the parent, and is free of pathogens [8]. The success of tissue culture depends on the media used, which consists of several components of macro and micronutrients, vitamins, and growth regulators for plant growth.

The media used in tissue culture can also be modified through the addition of cheaper organic materials as an alternative to expensive synthetic materials [9]. One of them is liquid organic fertilizer. According to [10], liquid organic fertilizer contains macro, micro, and good nutrients for the growth of explants. In addition, liquid organic fertilizer also contains growth regulators to stimulate growth if given in the right concentration [11]. The use of liquid organic fertilizers in tissue culture has been carried out in previous studies [10,12,13], so this study was conducted to obtain an alternative to MS media from liquid organic fertilizer given on the growth of the orchid protocorm *Vanda tricolor* Lindl. var. *suavis* in vitro.

2. Materials and methods
The plant material used in this study was the protocorm of *Vanda tricolor* Lindl. var. *suavis* aged three months obtained from the Tissue Culture Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Hasanuddin. Protocorm was grown on MS media and modified media with the addition of liquid organic fertilizers that are commonly used, i.e., NASA, Bio88, and Fortune (Table 1).

| Liquid organic fertilizer | Concentration |
|---------------------------|---------------|
|                           | 0  | 1 ml/L | 1.5 ml/L | 2 ml/L | 2.5 ml/L | 3 ml/L |
| NASA                      | NA0 | NA1   | NA2      | NA3    | NA4      | NA5    |
| Bio88                     | Bio0 | Bio1  | Bio2     | Bio3   | Bio4     | Bio5   |
| Fortune                   | F0  | F1    | F2       | F3     | F4       | F5     |

All cultures were incubated in a culture room at a temperature of 26 ± 2°C with a relative humidity of 55 ± 5% and were exposed to a 16 h photoperiod. A number of shoots and leaves on each explant were recorded. Observation of protocorm is carried out every week for 3 months. The data were analyzed using the Analysis of Variance (ANOVA) test, and if there were differences, it was continued with the DMRT (Duncan Multiple Range Test) tests at a level of 5% for data with normal distribution. If the data is not normally distributed, a nonparametric statistical test is carried out using the Kruskal-Wallis test at a level of 5%, and if there is a difference, a further test is carried out using the Mann-Whitney test [13,14].

3. Results and discussion
Observations on the growth of the protocorm of *Vanda tricolor* Lindl. var. *suavis* was carried out after protocorm was planted on control media and liquid organic fertilizer (NASA, Bio88, and Fortune) treatment media with different concentrations (1 ml/L, 1.5 ml/L, 2 ml/L, 2.5 ml/L, and 3 ml/L) for 12 weeks. Protocorm cultured on the media showed different responses between the control and liquid
organic fertilizer treatments. This is probably due to the presence of endogenous hormone, which affects the growth of protocorm [15].

According to [16], orchid protocorms in their development will continue to grow until they pass the protocorm phase and turn into plantlets. Orchid protocorm is a unique structure that functions in a symbiotic process with mycorrhizae and forms shoot apical meristem (SAM) through cell division in the apical and basal parts of the protocorm. Observations showed that SAM was formed faster at 3 weeks after planting (WAP) in fortune treatment, followed by Nasa, Control, and Bio88 at 5, 6, and 7 WAP, respectively. Fortune's liquid organic fertilizer has a nitrogen content of 3.26%; P$_2$O$_5$ 4.11%; K$_2$O 3.45%; Fe 6.03 ppm; Mn 255.11 ppm; Cu 276.47 ppm; Zn 253.02 ppm; B 127.11 ppm; Co 5.16 ppm and Mo 3.48 ppm (Table 2). According to [13], the elements N, P, and K function in the formation of endogenous hormones in plants. In addition, phosphorus and potassium play a role in the division of meristem tissue in plants [17].

The first and second leaf primordial in all liquid organic fertilizer treatments were formed at 7 and 8 WAP, respectively, while in the Bio88 treatment, only SAM was formed because the protocorm survival percentage on Bio88 media was very low compared to other treatments. This might be due to the lower macro and micronutrient composition of Bio88 than Nasa and Fortune (Table 2).

Absorbing hair formed 11 WAP in the control treatment. Nasa formed roots without any absorbing hair, while in Fortune and Bio88 treatment, no protocorm survived until absorbing hair was formed. According to [18], absorbing hairs are formed on the surface of the protocorm and appear when leaf primordia are present. Absorbing hair is a structure that resembles fine hair that functions in nutrient absorption [19]. According to [13], the growth of absorbing hair and roots in orchid protocorms is influenced by the hormones ethylene and auxin. The hormone auxin in tissue culture causes the concentration of endogenous growth regulators in cells to increase. Auxin stimulates plant tissue growth by triggering the secretion of H$^+$ ions from cells [20,21], influencing protein metabolism.
reactions by influencing RNA metabolism through molecular transcription, and triggering apical dominance [22]. According to [23], the hormone auxin regulates almost all aspects of plant growth and development, including cell division, cell extension, and differentiation.

| Table 2. Composition of liquid organic fertilizer |
|-------------------------------------------------|
| **Element** | **Fortune** | **Nasa** | **Bio88** |
| Nitrogen (N) | 3.26% | 4.15% | 3.47% |
| P<sub>2</sub>O<sub>5</sub> | 4.11% | 4.45% | 4.10% |
| K<sub>2</sub>O | 3.45% | 5.66% | 3.53% |
| Fe | 6.03 ppm | 505.5 ppm | 575 ppm |
| Mn | 255.11 ppm | 1931.1% | 1187 ppm |
| Cu | 276.47 ppm | 1179.8% | 1026 ppm |
| Zn | 253.02 ppm | 1986.1% | 752 ppm |
| B | 127.11 ppm | 806.6% | - |
| Co | 5.16 ppm | 8.4 ppm | - |
| Mo | 3.48 ppm | 2.3 ppm | - |
| C Organic | - | 9.69% | 9.87% |
| pH | - | 5.61 | 6.24 |
| Pb | - | 4.7 ppm | 2 ppm |
| Cd | - | 0.1 ppm | Tt ppm |
| Hg | - | 0.03 ppm | Tt ppm |
| Growth regulator | - | Giberalin, Cytokinins, and Auxins | - |

The composition contained in the Nasa showed the presence of auxin growth regulators. One of the functions of auxin is to stimulate root growth. According to [24], the hormone auxin functions to control leaf initiation, stimulate root formation, and regulate tissue patterns in the developing embryo. In addition, Nasa contains P elements that function in root development and branching [25]. Therefore, the presence of roots can be used as a growth parameter of the protocorm of *Vanda tricolor* Lindl. var. *suavis*. Observations of the number of shoots and number of leaves were carried out at the end of the observation and then analyzed using the Kolmogorov-Smirnov normality test where the results showed a value of 0.000 < 0.05, which means the data was not normally distributed. Therefore, the parametric test cannot be carried out, so it is replaced with a nonparametric test, namely the Kruskal-Wallis test.

| Table 3. Kruskal-Wallis test results for the effect of liquid organic fertilizer treatment |
|-------------------------------------------------|
| **Number of shoots** | **Number of leaves** |
| Chi-Square | 14.493 | 13.925 |
| Df | 3 | 3 |
| Asymp.Sig. | 0.002 | 0.003 |

The results of the Kruskal-Wallis test (Table 3) showed that the significant value for the number of shoots was 0.002, and the significant value for the number of leaves was 0.003. This value is less than 0.05, which means that there are differences in the treatment of the number of shoots and the number of leaves. Based on these results, further tests were carried out using the Mann-Whitney test to determine the difference in treatment based on the liquid organic fertilizer used.
Table 4. Mann-Whitney test results for the effect of a number of shoots and leaves

| Treatments                  | Number of shoots | Number of leaves |
|-----------------------------|------------------|------------------|
| Bio88 : Fortune             | 1000             | 1000             |
| Bio88 : NASA                | 0.094            | 0.173            |
| Bio88 : Control (MS)        | 0.002            | 0.005            |
| Fortune : NASA              | 1000             | 0.700            |
| Fortune : Control (MS)      | 0.108            | 0.035            |
| NASA : Control (MS)         | 1000             | 1000             |

Based on the results of the Mann-Whitney test (Table 4), there was one treatment that was significantly different, namely the Bio88 treatment and the control with a value of 0.002 < 0.05 for the number of shoots. As for the number of leaves, there were two significantly different comparisons, namely Bio88 treatment and control with a value of 0.005 < 0.05 and Fortune treatment and control with a value of 0.035 < 0.05.

Table 5. Kruskall-Wallis test results for the effect of concentration

|                      | Number of shoots | Number of leaves |
|----------------------|------------------|------------------|
| Chi-Square           | 1.541            | 1.398            |
| Df                   | 4                | 4                |
| Asymp.Sig.           | 0.819            | 0.845            |

Based on the results of the Kruskall-Wallis test (Table 4) for the effect of concentration, it showed that the significant value for the number of shoots was 0.819, and the significant value for the number of leaves was 0.845. The significant value is greater than 0.05, which means that there is no significant difference between the concentration given to the number of shoots and the number of leaves.

4. Conclusion

There is a significant effect between the addition of liquid organic fertilizer on the number of shoots with a value of 0.002 and the number of leaves with a value of 0.003. However, there was no significant effect between concentration on the number of shoots and number of leaves with significant values of 0.819 and 0.845, respectively.

References

[1] Sadili A and Siti S 2017 Keanekaragaman, sebaran, dan pemanfaatan jenis-jenis anggrek (Orchidaceae) di Hutan Bodogol, Taman Nasional Gede Pangrango, Jawa Barat Widyariset 3 95–106
[2] Cardoso J C, Cesar A Z and Jen-Tsung C 2020 An Overview of orchid protocorm-like bodies: mass propagation, biotechnology, molecular aspects, and breeding Int. J. Mol. Sci. 21 1–32
[3] Djufri, Hasanuddin and Fauzi 2015 Orchidaceae pulau Rubiah Kota Madya Sabang Provinsi Aceh J. Biot. 3 1–8
[4] Pusapasari R R, Ikhsanudin N R, Eka F C N and Endang S 2018 Pengaruh pepton terhadap pertumbuhan embrio anggrek Vanda tricolor Lindley var. suavis asal merapi secara in vitro Scr. Biol. 5 47–50
[5] Dwiyani R 2014 Anggrek Vanda tricolor Lindl. var. suavis (Denpasar: Udayana University)
[6] Rinekstone I A and Masrukhani S 2015 Regenerasi anggrek Vanda tricolor pasca erupsi merapi melalui kultur in vitro Seminar nasional universitas PGRI Yogyakarta pp 378–84
[7] Widodo E and Hastuti 2019 Riwayat aktivitas gunung merapi: potensi dan ancamannya bagi sektor pariwisata Geodemia 17 21–34
[8] Oseni O M, Veena P and Tapan K N 2018 A review on plant tissue culture, a technique for propagation and conservation of endangered plant species Int.j.curr.Microbiol.app.sci. 7 3778–86
[9] Meilani S N, Septarini D A and Fatimatuz Z 2017 Efektifitas penambahan media organik ekstrak ubi jalar (Ipomoea batatas L.) pada pertumbuhan subkultur anggrek Cattleya sp J. florea 4 5–11

[10] Melisa A O 2018 Pemberian kombinasi 2,4-D dan kinetin terhadap induksi protokorm like bodies (PLB) anggrek Grammatophyllum scriptum secara in vitro J. Biol. Educ. 1 34–46

[11] Nirmala R and Ratna S 2019 Pertumbuhan dan ketahanan penyakit kejut “kuning” pisang paska aklimatisasi bIBUT di pembibitan dengan pupuk organik nasa cair dan Trichoderma J. agroteknologi Trop. lembab 1 77–87

[12] Hayanti I P, Retna B A and Praswanto 2012 2012 Pengaruh pupuk organik cair dan ekstrak rumput mutiara terhadap pertumbuhan tunas pegagan (Centella asiatica) secara in-vitro Biofarmasi 10 54–60

[13] Yusuf Y and Ari I 2017 Pengaruh medium pupuk organik cair (poc) terhadap karakter morfologi dan jumlah tunas protokorm anggrek Vanda limbata Blume x Vanda tricolor Lindl. J. bionature 17 14–23

[14] Mendrofa F N E, Nurcahyo W S and Lutfi A 2021 2021 Introduction shoots from callus of cucumber apple (Cucumis sp.) using a combination of benzyl amino purine and naphthalene acetic acid concentrations in vitro J. mangifera edu. 5 103–20

[15] Chen J, Bo Y, Yanjing T, Yongmei X, Yang L, Dongyu Z and Shinxing G 2020 symbiotic and asymbiotic germination of Dendrobium officinale (orchidaceae) respond differently to exogenous gibberellins Int. J. Mol. Sci. 21 1–23

[16] Yeung E 2017 A perspective on orchid seed and protocorm development Bot. Stud. 58 1–14

[17] Madusari S 2019 Processing of fibre and its application as liquid organic fertilizer in oil palm (Elaeis quineensis Jacq.) seedling for sustainable agriculture J. Appl. Sci. Adv. Technol. 1 81–90

[18] Utami E S W and Sucipto H 2019 In vitro seed germination and seedling development of a rare indonesian native orchid Phalaenopsis amabilis J.J.Sm. Hindawi 1–7

[19] Ningrum E F C, Ikhsanudin N R, Riza R P and Endang S 2017 Perkembangan awal protokorm anggrek Phalaenopsis amabilis secara in vitro setelah penambahan zat pengatur tumbuh α-naphtaleneacetic acid dan thidiazuron Biosfera 34 9–14

[20] Batti J R, Larekeng S H, Arsyad M A, Gusmiaty and Restu M 2020 In vitro growth response on three provenances of Jabon Merah based on auxin and cytokinin combinations IOP Conf. Ser. Earth Environ. Sci. 486 1–16

[21] Paembonan S, Umar A and Halimah Larekeng S 2020 Techniques For Mass Development Of Bamboo Betung (Dendrocalamus Asper Back.) Using Branch Cuttings vol 20

[22] Asra R, Ririn A S and Mariana S 2020 Hormon Tumbuhan (Jakarta: UKI Press)

[23] Lee Z H, Takeshi H, Nobutoshi Y and Toshiro I 2019 The roles of plant hormones and their interactions with regulatory genes in determining meristem activity Int. J. Mol. Sci. 20 1–19

[24] Novak S D, Lila J L and Roshan N G 2014 Role of auxin in orchid development Plant Signal. Behav. 9 1–8

[25] Razaq M, Peng Z, Hai-long S and Salahuddin 2017 Influence of nitrogen and phosphorus on the growth and root morphology Acer mono PLoS One 12 1–13