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Andriyana Setyawati

andriyanasetyawati@staff.uns.ac.id

Sebelas Maret University

Hibatulloh Azizi

Sebelas Maret University

M. Uddin

Texas A&M University

Nurashikin Kemat

Universiti Putra Malaysia

Research Article

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Posted Date: October 9th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10865690/v1>

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Additional Declarations: No competing interests reported.

Optimizing Thidiazuron and Carrot Extract for Micropropagation and Phytochemical Profiling of *Dendrobium macrophyllum* A.Rich

Andriyana Setyawati^{1*}, Hibatulloh Azizi², M. Nasir Uddin^{3,4}, Nurashikin Kemat⁵

¹⁻²Department of Agrotechnology, Faculty of Agriculture, Sebelas Maret University, Indonesia

³Texas A&M University School of Medicine, USA

⁴Baylor Scott & White Hospital

⁵Department of Agriculture Technology, Faculty of Agriculture, University of Putra Malaysia, Malaysia

*Corresponding Author:

E-mail: andriyanasetyawati@staff.uns.ac.id

ORCID: Andriyana Setyawati (0000-0003-2926-9372), Nurashikin Kemat (0000-0002-0654-8021)

ABSTRACT

Dendrobium macrophyllum is an orchid species native to Indonesia and other countries, playing a vital role in local ecosystems. Its classification under Appendix II of CITES highlights the need for preservation and sustainable management. Increasing *D. macrophyllum* populations requires effective methods like tissue culture techniques. This research evaluates the optimal Thidiazuron (TDZ) and carrot extract concentrations for enhancing *D. macrophyllum* multiplication. The study used a factorial Completely Randomized Design (CRD) with two factors: TDZ concentration (0, 0.5, 1, 1.5, and 2 ppm) and carrot extract concentration (0, 20, 40, 60, and 80 mL.L⁻¹). Data were analyzed using analysis of variance at a 5% level with the Duncan Multiple Range Test (DMRT). Results showed that TDZ at 1.5 ppm with carrot extract at 20 mL.L⁻¹ increased root numbers by 177% compared to the control. The application of TDZ at 1.5 ppm also increased leaf numbers by 69.5% and shoot numbers by 90.68%. Carrot extract at 20 mL.L⁻¹ accelerated shoot emergence by 9.28% and increased both leaf and root numbers. Phytochemical profiling via Gas Chromatography and Mass Spectrometry (GC-MS) identified five compounds in the plantlets, including two major (Octadecanoic acid, and cis-Vaccenic acid) and three minor compounds (cis-Vaccenic acid, Isopropyl linoleate, and 6-Octadecenoic acid, methyl ester, (Z)-). This research advances micropropagation protocols for *D. macrophyllum*, supporting conservation efforts and potential medicinal applications.

Keywords: cis-Vaccenic acid, GC-MS, indigenous orchid, MS modification, Octadecanoic acid

1. Introduction

Genus *Dendrobium* is one of the largest orchid genera with nearly 1,400 species (Jin et al. 2009), are known for their significant medicinal properties. Tissue culture and plant cell techniques are employed to generate secondary metabolites (Verpoorte et al., 2002). Orchids, as noted by Yuan et al. (2019), are highly valued for their diverse uses in traditional herbal medicine. *Dendrobium* as herb is rich in alkaloids, essential amino acids, polysaccharides, and various minerals. Phytochemical studies comparing wild and in vitro regenerated species have highlighted this genus as a source of nutraceuticals and antioxidants (Longchar and Deb, 2021). Extensive harvesting of therapeutic *Dendrobium* species has resulted in their scarcity in the wild (Teoh, 2016; Ninawe and Swapna, 2017). Moreover, factors such as degradation, fragmentation, habitat loss, and illegal trafficking have contributed to many of these species being classified as critically endangered or threatened (Pant, 2013).

Indonesia is one of the tropical countries that is very rich in plant diversity, including orchid plants. There are about 5,000 species of orchid plants that exist and are found in Indonesia (Jolman et al. 2022). According to Pammai et al. (2022), the best *Dendrobium* genus is found in many parts of eastern Indonesia, such as the *Dendrobium macrophyllum* orchid that it is classified as a native species in several countries including Indonesia and is an orchid in the appendix II category. *Dendrobium macrophyllum* is part of the country's biodiversity, playing a role in local ecosystems in its natural habitat in Indonesia. The appendix II status of *D. macrophyllum* means that this orchid needs to be preserved and can play a role in scientific research in Indonesia. *Dendrobium macrophyllum* is a unique and interesting orchid species due to its hairy flower morphology and pale green color.

Preserving natural orchids is important for biodiversity conservation, the horticultural potential of ornamental plants as an economic source, and opportunities for future research and innovation. According to Duadji et al. (2022), orchid development in Indonesia faces various problems, namely cultivation techniques that

have not been carried out properly, because they are carried out by separating tillers. Naturally, orchid seeds are difficult to germinate, because orchid seeds have a dependency and need assistance through symbiosis with mycorrhizal fungi to germinate well (Hossain, 2022). According to these various problems, there is a need for efforts to increase orchids in quantity using appropriate methods, such as using tissue culture techniques.

Orchid propagation through tissue culture can be done by planting parts of orchid plants such as leaves, callus, stems, and roots of orchid plants. Orchids can grow uniformly and obtain nutritional needs in large quantities when cultivated through in vitro culture. Orchids cultivated in vitro have the potential to multiply through the multiplication process. Growth of orchids in tissue culture is influenced by several factors, namely, media composition containing macro and micro nutrients, vitamins, and the addition of appropriate plant growth regulators (PGR) for the type of explant and culture purpose (Harahap et al. 2019).

Plant growth regulators (PGR) can be obtained from organic compounds as substitutes or inorganic. One source of organic PGR is phytohormones. Plants need natural plant growth regulators (phytohormones) for the growth process, namely auxin and cytokinin growth regulators. One of the phytohormones that can be used is carrot extract. According to Khadr et al. (2020), carrot extract contains the auxin hormone called Indole-3-acetic acid (IAA). One source of cytokinin growth regulator is Thidiazuron (TDZ). Thidiazuron is a compound that has the ability to stimulate shoot growth faster compared to other types of cytokinins (Karyanti et al. 2021). An application of TDZ to the tissue culture medium of *Phalaenopsis sp* were able to increase callus development (Restanto et al. 2023). Therefore, an alternative approach using natural material combined with inorganic phytohormones to support plant growth and secondary metabolite are necessary. This research investigated various levels of carrot extract and thidiazuron in providing good results on the growth and secondary metabolites of orchids (*Dendrobium macrophyllum*).

This research focuses on improving orchid tissue culture cultivation by adding the plant growth regulator Thidiazuron and carrot extract as phytohormones in an effort to optimize the growth, development, and secondary metabolite of *Dendrobium macrophyllum* under in vitro conditions. The combination of TDZ as a source of synthetic cytokinin and carrot extract rich in natural auxins is expected to create an ideal hormonal balance to stimulate cell proliferation, tissue differentiation, and organogenesis (Phillips and Garda, 2019). This study will develop more efficient and effective micropropagation protocols for *D. macrophyllum* orchids, which can contribute to the conservation efforts of this species as well as the development of better cultivation techniques for ornamental and medicinal purposes. The results of this research can also provide new insights into the interaction between synthetic and natural plant growth regulators in plant tissue culture, which may be applicable to other plant species in the future.

2. Materials and method

2.1 Preparation of planting material

The material used was explants of whole stem slices (1.5 - 2.0 cm) of orchid plants (*Dendrobium macrophyllum*) obtained from the orchid plant collection in the plant physiology laboratory of the Faculty of Agriculture, Sebelas Maret University. Explants from stem organs produce the highest percentage of embryogenic callus compared to explants from leaf and root organs. Stem explants (containing shoot primordia or apical meristems) give better results compared to leaves (Mostafa et al. 2020).

2.2 Sterilization of equipment

Equipment that needs to be sterilized includes culture bottles, dissection equipment (large tweezers, small tweezers, and scalpel knife). The sterilization process is carried out by washing the equipment thoroughly using dishwashing soap and followed by a drying process. Spatulas, tweezers, knives, and petri dishes are wrapped using brown paper and HVS paper before sterilization. Clean and dry culture bottles are sterilized using an oven for 2 hours at 170°C, while planting tools (scalpel, petri dishes, brown paper, and tweezers) are wrapped using paper and clear plastic, then sterilized using an autoclave for 30 minutes at 121°C. Sterilized equipment is placed in an incubation room with a temperature of 24-26°C.

2.3 Media preparation

The medium used were ½ MS (Murashige and Skoog) combined with organic material (coconut water, banana juice), and active charcoal. Media preparation begins by taking distilled water in a beaker glass on a magnetic stirrer and hot plate to make all materials homogeneous. Then rotate the magnetic stirrer at a low speed and add sugar 20 gram, micro nutrient 25 ml, macro nutrient 10 ml, Fe-EDTA 25 ml, coconut water 100 ml, banana juice 100 ml, vitamins 50 ml, active charcoal 1 gram, TDZ (without TDZ, 0.5, 1, 1.5, and 2 ppm) and carrots extract

(without carrot extract, 20, 40, 60, and 80 mL/L), active charcoal 1 gram, agar 7 gram. The required dose is 250 ml, if the dose has not reached 250 ml then distilled water is added until the volume becomes 250 ml. The pH level ranges from 5.5 where if the pH of the solution exceeds 5.5, HCl is added and if it is less than 5.5, NaOH is added. The next process is adding activated charcoal and agar then boiling using a hot plate. The boiled solution is then poured into culture bottles, tightly closed, labeled and sterilized using an autoclave for 30 minutes at 121°C then drying for 15 minutes. The treatment media were consisting several concentrations of TDZ (without TDZ, 0.5, 1, 1.5, and 2 ppm), carrot extract (without carrot extract, 20, 40, 60, and 80 mL/L) and combination both TDZ and carrot extract furthermore total treatments were 25 treatment. Each treatment repeated five times then total glass culture were 125 glass culture.

2.4 Planting process

About nine months old capsules orchid were collected from the *D. macrophyllum* growing in the Kemuning Orchid Garden, Karanganyar Regency, Central Java Province, Indonesia. After harvesting capsules were washing with a liquid detergent (1:100 v/v) followed by washing under running tap water. Dipping the capsule by 70% alcohol, then dried using sterilized tissue, brought to LAF then spray all of the capsule used 70% alcohol the flaming off for 2-3 second. The sterilized capsules were cut longitudinal then the powder seed were planting into MS (Murashige and Skoog) medium until growing the PLB (Plant Like Body) and being a plantlet (six-month-old). The plantlets used for propagation in the experiment were transferred to ½ MS medium supplemented with organic material (coconut water 100 ml, banana juice 100 ml, active charcoal 1 gram, TDZ (without TDZ, 0.5, 1, 1.5, and 2 ppm) and carrot extract (without carrot extract, 20, 40, 60, and 80 mL/L). The cultures were maintained in the culture room at 20 °C, 24 h light (cool white fluorescent lamp, TLD 30 W 84; Philip) then the experiments were under observation for 90 days.

2.5 Extraction

Plantlet obtained following culture (90 days after planting) all concentration of TDZ and carrot extract were washed thoroughly with running tap water and dried into oven by 40 °C, 1w. Similarly, to compare the result, orchid plant as a mother plant (1-year old) were washed thoroughly with running tap water and dried with oven (as above). Dried plantlet and mother plant were powdered using a mortar and pestle, 1 g of extract as sample was dissolved in 5 mL of 2-propanol alcohol and sterilized using an ultrasonic bath for 60 minutes. Sample was filtered with PTFE (polytetrafluoroethylene) 0.22 µm, then sample were injected to the GCMS system.

2.6 GC-MS analysis

Phytochemical compounds in the accumulation plantlet cultured as in vitro compared with mother plants from field of *D. macrophyllum* orchids were analyzed using Gas Chromatography - Mass Spectrometry (GC-MS) method. A diluted volume of 1.00 µL was injected into the system using an AOC-20i auto-injector with split injection mode (1:50); the injection temperature was 220 °C. The test was running using an GCMS-QP2010 Ultra, Shimadzu with an Agilent DB-5MS UI column of 30 m length. Helium gas was used as the carrier gas with total flow 75.9 mL.min⁻¹ (column flow 0.55 mL. min⁻¹). The oven temperature was programmed from 80 °C (4 min hold time) to 180 °C (6 min hold time) and then to 220 °C (6 min hold time). Solvent cut time was 2.00 min; the detector gain was 0.92 kV. Compound were identified by their mass spectra and retention indices using the NIST Mass Spectral Library and the Retention Index Database. The data were proseses using GC-MS Insight xcalibur soft-ware (Shwaish and Al-Imarah 2017)

2.7 Anatomy analysis

The leaf and root anatomy analysis were conducted by using interaction samples among the treatments after 90 days of culture. Samples were dipping to the methylene blue for 1 min. Samples were started manually by making thin leaf and root preparations using a sharp razor blade. Observation were performed using a light microscope (Olympus CX23) at various magnifications (4x and 10x; root and leaf, respectively). Images were captured to analyze mesophyll thickness in leaves and cortex thickness in roots. Measurements were carried out using ImageJ software.

2.8 Data analysis

The research design used a factorial Completely Randomized Design (CRD) with two factors. The first factor is the concentration of TDZ (without TDZ, 0.5, 1, 1.5, and 2 ppm) and the second factor is the concentration of carrot extract (without carrot extract, 20, 40, 60, and 80 mL/L). The variables observed were shoot emergence

time, number of leaves, number of roots, number of shoots, plantlet height, leaf and root anatomy, and phytochemical compounds then were collected after 90 days of culture (90 days after planting). Observations were conducted by directly counting the number of leaves, roots, and shoots per explant under aseptic conditions. Plantlet height was measured from the base of the stem to the tip of the longest leaf using a sterile ruler. The data collection was performed manually and recorded for each experimental unit to evaluate the growth parameters of *Dendrobium macrophyllum* under different treatments of TDZ and carrot extract. The data analysis used was analysis of variance (ANOVA) with F test at 5% level, and if there was a significant difference, it was continued with Duncan's Multiple Range Test (DMRT) at 5% level, in addition, a descriptive analysis was also carried out which was presented in the form of tables, figures, and conclusions will be drawn.

3. Result and discussion

3.1 Shoot emergence time

Shoot emergence time is one of the indicators of success in in vitro culture or multiplication. Shoot emergence time in tissue culture is influenced by environmental factors or related to genetics such as plant type, age and condition of plantlets, and growth regulators.

Table 1. The application of TDZ and carrot extract on the emergence time of *D. macrophyllum* orchid shoots at 90 Days After Plant (DAP)

Concentration TDZ (ppm)	Shoot emergence time
Without TDZ	40.87 ^a ± 15.54
0.5	43.93 ^a ± 18.10
1	43.33 ^a ± 18.98
1.5	45.83 ^a ± 13.04
2	44.40 ^a ± 20.29
Concentration carrot extract (mL/L)	
without carrot extract	40.87 ^{ab} ± 19.58
20	37.40 ^a ± 8.70
40	38.60 ^{ab} ± 6.73
60	53.13 ^a ± 15.12
80	48.41 ^{bc} ± 19.71
interaction	-

Note: Numbers followed by the same letter for each treatment indicate that they are not significantly different at 5% DMRT, the – sign indicates there is no interaction

Table 1 shows that the application of carrot extract at concentrations of 20 and 40 mL/L was significantly different from the treatment without carrot extract, with the fastest shoot emergence time found in the 20 mL/L carrot extract treatment at 37.40 days after planting (DAP), while the longest shoot emergence time was in the 60 mL/L carrot extract treatment at 53.13 DAP. Furthermore, there was no interaction between TDZ and carrot extract in accelerating shoot emergence time. The application of TDZ alone was not able to increase shoot emergence time.

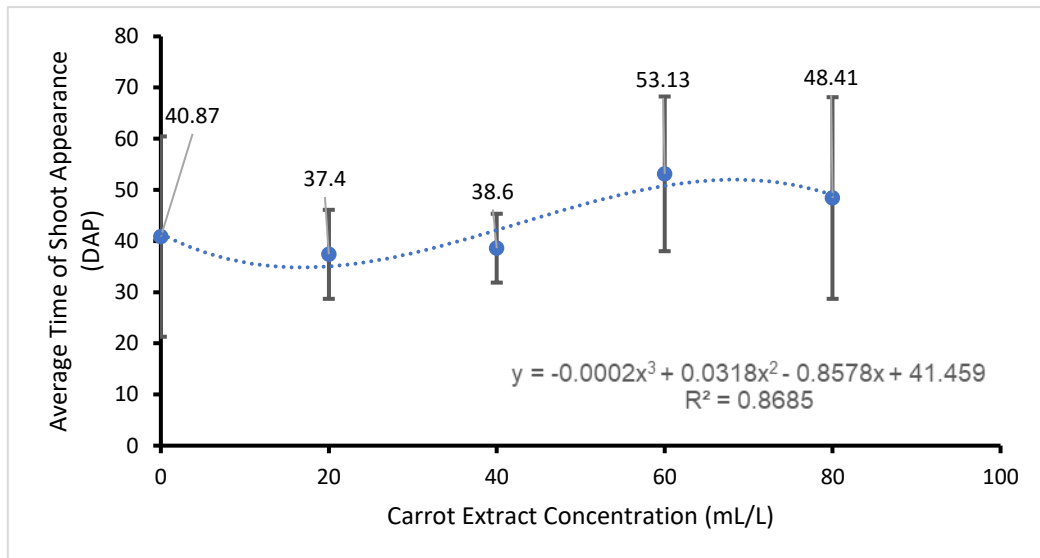


Fig 1 Regression graph of the effect carrot extract on the shoot emergence time of *D. macrophyllum*

The follow-up test results showed that the application of carrot extract at concentrations of 20 and 40 mL/L was significantly different from the treatment without carrot extract, with the fastest shoot emergence time found in the 20 mL/L carrot extract treatment at 37.40 DAP, while the longest shoot emergence time was in the 60 mL/L carrot extract treatment at 53.13 DAP (Fig 1). This research shows that the moderate concentrations (20-40 mL/L) significantly accelerated shoot emergence, while higher concentrations (60-80 mL/L) delayed the shoot emergence. Li et al. (2024), Carrot extracts have been identified to contain auxins, particularly indole-3-acetic acid (IAA), which play an important role in root development and overall plant growth but at high concentrations can cause disturbances in leaf cell formation and development. Sherif et al (2016) revealed that application of IAA with low concentration induced the callus initiation and proliferation using various explant. Pant and Thapa (2012) confirmed that the supplemented IAA on various concentration effective to induce root formation. Al-Jubori and Al-Amery (2022) found that the presence of auxin will affect the work of cytokinins. If auxin is at the right concentration, then cytokinin transport according to its function to initiate shoots will emerge. If the amount of auxin is too high, then the shoot differentiation process will be inhibited, because the ability of meristem cells to divide is higher than the differentiation process into shoots.

3.2 Number of leaves

The number of leaves is one of the parameters that needs to be observed in plant growth because leaves are the site of photosynthesis, so the more leaves there are, the better the plant growth will be. Table 2 shows that the results of analysis of variance through the 5% F test of TDZ and carrot extract application had a significant effect on the number of leaves of *D. macrophyllum* orchids, but the interaction of the two factors had no significant effect on the number of leaves of *D. macrophyllum* orchids.

Table 2. The number leaves of TDZ and carrot extract of *D. macrophyllum* leaves at 90 Days After Plant (DAP)

Concentration TDZ (ppm)	Number leaves
Without TDZ	6.53 ^a ± 2.17
0.5	7.87 ^{ab} ± 2.39
1	9.13 ^b ± 3.11
1.5	11.07 ^c ± 3.33
2	8.93 ^a ± 2.79
Concentration carrot extract (mL/L)	
without carrot extract	7.00 ^a ± 1.96
20	9.87 ^b ± 3.80
40	9.00 ^b ± 2.24
60	9.40 ^b ± 3.42
80	8.27 ^{ab} ± 3.26
interaction	-

Note: Numbers followed by the same letter for each treatment indicate there is no significant effect on 5% DMRT, the - sign indicates there is no interaction

Table 2 showed the results of the application carrot extract at concentrations of 20 to 60 mL/L were significantly different from the treatment without carrot extract in terms of number of leaves. Moreover, there was no interaction between TDZ and carrot extract in increasing the number of leaves. Based on observations, the significant and highest number of leaves were found in the 1.5 ppm TDZ treatment with 11.07 leaves.

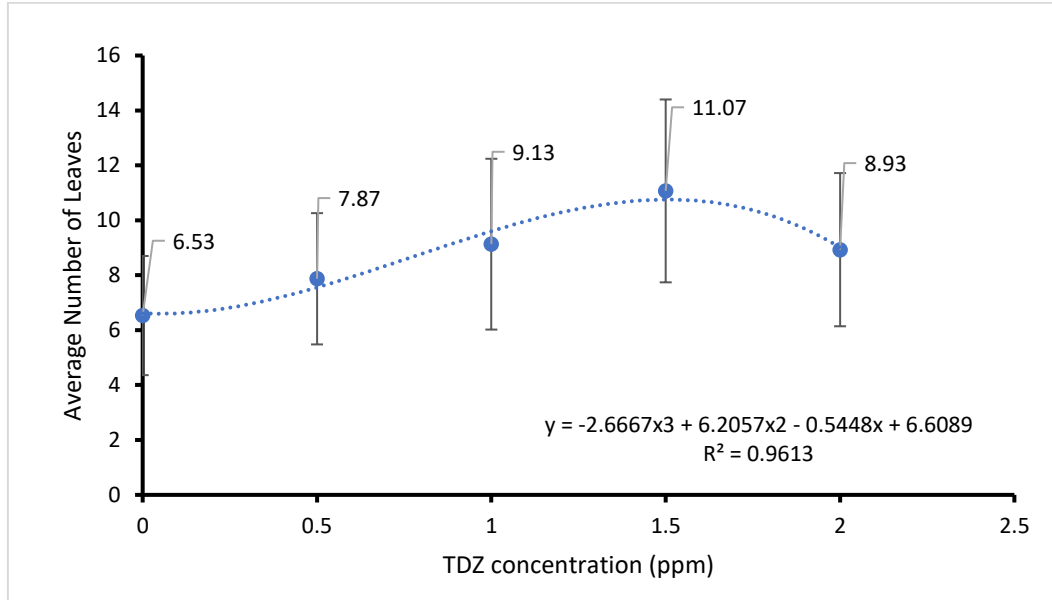


Fig 2 Regression graph of the effect TDZ on the numbers leave of *D. macrophyllum*

Table 2 showed that the 1.5 ppm TDZ treatment obtained a leaf number value by 11.07, significantly different from the control level by 6.53 leaves. This treatment increased by 69.5% from the control treatment. The application of TDZ by 0.5 - 1.5 ppm are able to accelerate the average number of leaves. Moreover, the high concentration (2 ppm) decreases the average number of leaves. This occurs because of low TDZ concentration could be absorbed by *D. macrophyllum*. According to Aggarwal et al (2020) the application of low concentration TDZ beneficial for shoot regeneration on *Ocinum tenuiflorum*. TDZ plays a role either directly or indirectly in various morphological and physiological responses in plant tissue (Guo et al. 2011). Moreover, TDZ increased the availability of purines required for cellular proliferation and the accumulation of purin metabolites, which are essential for the rapid protein synthesis and cell division involved in somatic embryo development (Ali et al. 2022). Previous studies have demonstrated that a TDZ-enriched medium is effectively promoting shoot bud induction in *Ajunga parviflora* (Kumari et al. 2024) *Capparis decidua* (Ram et al. 2022), *Gymnocladus assamicus* (Gupta et al. 2020a) and *Mertensia maritima* (Park et al. 2019). Deepa et al. (2018) revealed that TDZ is effective in promoting callus formation and shoot regeneration, its concentration must be carefully controlled to avoid inhibitory effects on other beneficial growth regulators. It can be concluded that TDZ in high concentrations will inhibit the work of carrot extract if it is not balanced in the administration needed by plants.

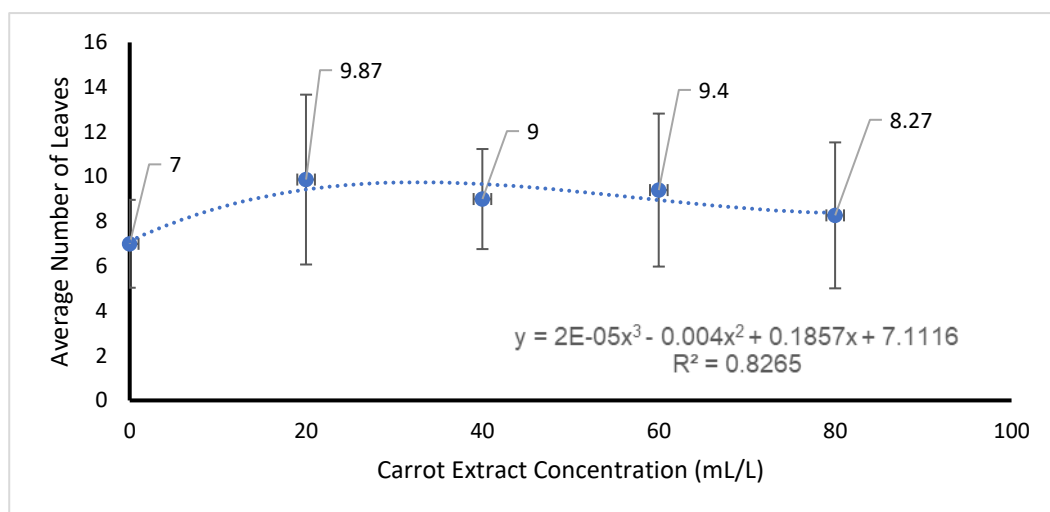


Fig 3 Regression graph of the effect carrot extract on the number leaves of *D. macrophyllum*

Table 2 showed that the 20 mL/L carrot extract treatment obtained a leaf number value of 9.87, significantly different from the control level of 7 leaves. This treatment increased by 41% from the control treatment. The graph explains that the application of 20 mL/L carrot extract showed an increase in the average number of leaves up to a concentration of 40 mL/L of carrot extract but it was not significantly different from the 80 mL/L of carrot extract treatment (Fig 3). This research resulted that the application of higher of carrot extract given could inhibit or have no effect on plant growth. According to Zenda et al. (2021), when the concentrations carrot extracts higher than the optimum concentration will promote growth or can interfere with plant metabolism and development. This is because at high auxin concentrations effect on the cell enlargement process then can take place quickly and cells will become large. This condition will cause the cell's turgid reaction to be disturbed and the cell will experience dryness.

3.3 Number of roots

The roots of *D. macrophyllum* orchid plants consist of two types of roots namely adhesive roots and air roots. Air roots function for plant growth and development while adhesive roots function to hold the plant in place. According to Verma et al. (2021), plant roots directly mediate the absorption of nutrients and water and thus affect the overall development and productivity of plants, so the more roots there are, the more nutrients the plant can absorb.

Table 3. Combination TDZ and carrot extract against the number of roots of *D. macrophyllum* 90 Days After Plant (DAP)

TDZ (ppm)	Carrot Extract (mL/L)					TDZ average
	0	20	40	60	80	
0	4.33 ^{a-d} ± 2.51	11.33 ^{c-g} ± 5.51	9.67 ^{d-g} ± 2.31	2.67 ^{a-c} ± 0.58	5.33 ^{a-g} ± 3.22	6.73 ^a ± 4.45
0.5	4.67 ^{a-c} ± 3.79	6.33 ^{a-g} ± 0.58	6.00 ^{a-g} ± 4.36	8.00 ^{b-g} ± 1.73	5.33 ^{a-g} ± 3.22	6.07 ^a ± 2.84
1	8.00 ^{b-g} ± 3.61	3.67 ^{a-c} ± 2.89	1.00 ^a ± 0.00	9.00 ^{c-g} ± 6.56	5.00 ^{a-f} ± 3.46	5.33 ^a ± 4.47
1.5	5.67 ^{a-g} ± 1.53	12.00 ^g ± 7.21	10.0 ^{d-g} ± 4.00	4.67 ^{a-c} ± 2.31	4.67 ^{a-c} ± 2.52	7.40 ^a ± 4.64
2	9.67 ^{d-g} ± 1.15	11.33 ^{c-g} ± 0.58	8.33 ^{b-g} ± 5.77	6.00 ^{a-g} ± 2.65	2.00 ^{abv} ± 1.00	7.47 ^a ± 4.17
Average carrot extract	6.47 ^{ab} ± 3.14	9.00 ^b ± 5.03	7.00 ^{ab} ± 4.72	6.07 ^a ± 3.73	4.47 ^a ± 2.72	+

Note: Numbers followed by the same letter for each treatment indicate there is no significant effect on 5% DMRT. the + sign indicates an interaction.

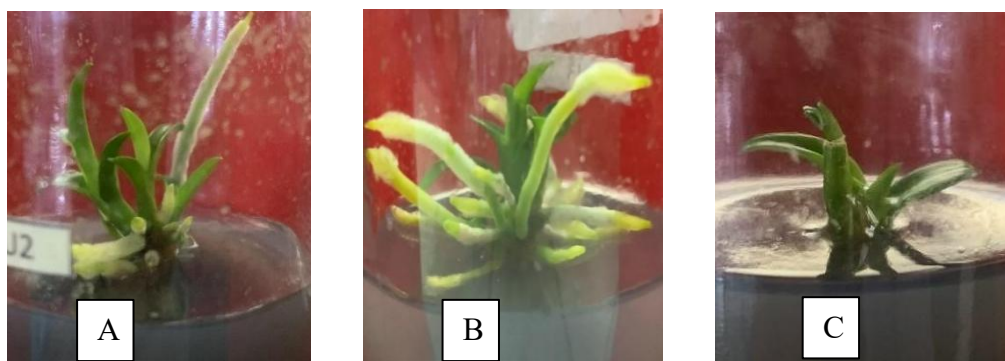
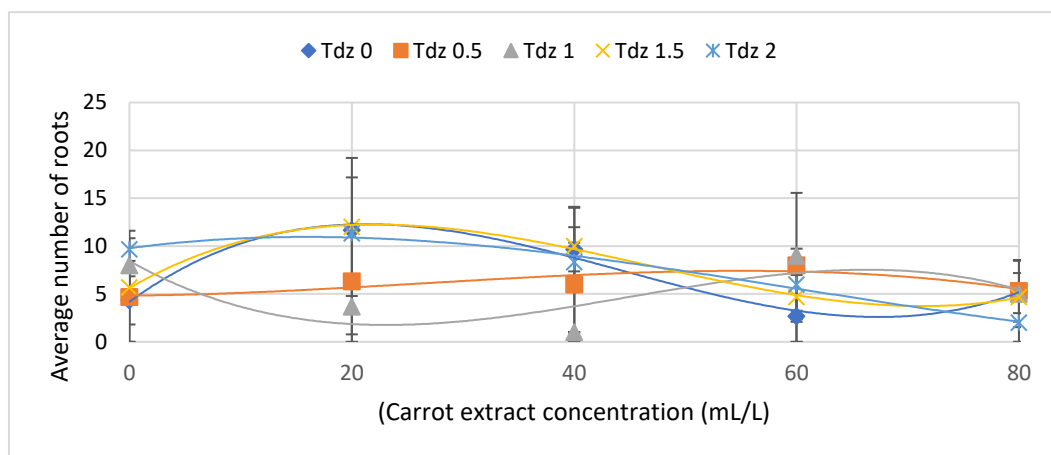


Fig 4 Root growth on *D. macrophyllum* orchid plantlets (A) carrot extract concentration 40 mL/L. (B) carrot extract concentration 20 mL/L. and (C) treatment without carrot extract

Table 3 shows the interaction between TDZ and carrot extract in increasing the number of roots. Based on the observations resulted that the analysis of variance through the 5% F test showed that the application of carrot extract and the interaction between TDZ had a significant effect on the number of roots of *D. macrophyllum* orchids. The average roots production were around 1.00-12.00 roots. The highest production was pointed out on the carrot extract by 20 mL/L combined with TDZ by 1.5 ppm (12.00 roots). Enkhbileg et al. (2019) stated that every concentration of cytokinin can induce secondary root propagation of all types of explants. This phenomenon can occur due to interactions between organic materials in the media or endogenous hormones and exogenous additive growth regulators or other secondary metabolic effects on hormone sensitivity or cell reactions. Moreover, the combination of carrot extract includes auxin and detoxifying agents called indoles as well as amino acids, proteins and fibers that stimulate growth and are used as bio-stimulants in increasing plant growth (Gergis Daw et al. 2019). Emenecker and Strader (2020) stated that low levels of auxin concentration can stimulate root growth and higher concentrations of auxin inhibit root growth and high levels of exogenous auxin can inhibit root elongation.



Note:

y (Without TDZ)	$= 0.0002x^3 - 0.0263x^2 + 0.851x + 4.183$	$R^2 = 0.973$
y (TDZ 0.5 ppm)	$= -3E-05x^3 + 0.0022x^2 + 0.0093x + 4.832$	$R^2 = 0.709$
y (TDZ 1 ppm)	$= -0.0001x^3 + 0.0191x^2 - 0.655x + 8.452$	$R^2 = 0.660$
y (TDZ 1.5 ppm)	$= 0.0001x^3 - 0.0199x^2 + 0.671x + 5.618$	$R^2 = 0.995$
y (TDZ 2 ppm)	$= 3E-05x^3 - 0.0056x^2 + 0.156x + 9.779$	$R^2 = 0.984$

Fig 5 The Regression of carrot extract and TDZ on the number roots of *D. macrophyllum*

Fig 5 showed that the combination of TDZ by 1.5 ppm with carrot extract are resulted the optimal point compared to other treatments. The application of TDZ by 1.5 ppm followed by an enhancement of carrot extract concentration up to 20 mL/L was able to produce the highest roots numbers by 12 roots. Furthermore, the lowest

roots number was found in the concentration by 1 ppm TDZ and 40 mL/L carrot extract with 1 root. According to Sosnowski et al. (2023) the use of carrot extract as auxin and TDZ as exogenous cytokinin in appropriate concentrations increases the yield of plant dry matter and also increases the stability of plant root growth. This is reinforced by the research results of Sitingjak and Pratomo (2019) that cytokinin and auxin regulate each other's synthesis showing feedback mechanisms to align plant hormone levels in developing meristems from roots and shoots including in regulating apical meristems, root patterns, and organogenesis. Kumari (2024) revealed that the combination of TDZ and NAA successfully inducing for direct root regeneration and petiole explant. As a potential cytokinin, TDZ promote lateral bud dormancy, stimulates the synthesis and accumulation other endogenous cytokinin, increase nutrient uptake and absorption plant tissue (Hussain *et al.* 2019). Moreover, Fig 5 indicated that single application of carrot extract without TDZ shows the strong positive trend ($R^2 = 0.973$), it was suggested that the addition of carrot extract to the medium could promote root formation. Previous studi on different species reported that the medium enrichment with carrot extract significantly induced for plantlet height, root length, and number of roots (Latifah et al. 2017; Galib et al. 2024).

3.4 Number of shoots

The number of shoots is an important index for assessing plant health and has potential in ecological succession which produces many seedlings. Table 4 shows that the results of analysis of variance through the 5% F test of TDZ application had a significant effect on the number of shoots of *D. macrophyllum* orchids. Otherwise, carrot extract application and their interaction had no significant effect on the number of shoots of *D. macrophyllum* orchids.

Table 4. The application of TDZ and carrot extract on the number of shoots of *D. macrophyllum* 90 DAP

Concentration TDZ (ppm)	Number of shoots
Without TDZ	1.61 ^a ± 0.74
0.5	1.81 ^a ± 0.98
1	2.93 ^b ± 1.16
1.5	3.07 ^b ± 1.53
2	2.28 ^{ab} ± 1.19
Concentration carrot extract (mL/L)	
without carrot extract	1.93 ^a ± 0.96
20	2.53 ^a ± 1.55
40	2.35 ^a ± 0.96
60	2.67 ^a ± 1.40
80	2.23 ^a ± 1.49
interaction	-

Note: Numbers followed by the same letter for each treatment indicate there is no significant effect on 5% DMRT. the - sign indicates there is no interaction.

Table 4 shows that the application of TDZ at concentrations by 1 to 1.5 ppm was significantly different from the treatment without TDZ with the highest number of shoots found at a concentration by 1.5 ppm with 3.07 shoots. While the lowest number of shoots was found in the treatment without TDZ with 1.61 shoots. It was proven that TDZ significantly inducing shoot proliferations on *D. macrophyllum* on dose dependent manner. Existing studies have noted that TDZ the highest axillary shoot multiplication (Siddique et al. 2015; Silue et al. 2016; Dewir 2018). Furthermore, TDZ is well-known as activating inactive cytokinin form, elevate endogenous cytokinin levels by inhibiting catabolism and enhancing synthesis cytokinin (Dinani et al. 2018). In contrast, the addition of carrot extract to the medium did not significantly different resulting shoots number of *D. macrophyllum*. It is possibly due to the auxin content in carrot extract insufficient to promote the number of shoot formation in the orchid. The minimum threshold and an appropriate ratio of exogenous and endogenous hormones are essential for effective shoot regeneration (Gupta et el. 2020b). Moreover, the effect of auxin biosynthesis could inhibit of shoot regeneration, it was negatively affected on shoot regeneration in several plants under experimental conditions (Ohbayashi et al. 2022). In other hand, the biosynthesis and transport of auxin plays a key role in regulating of root initiation and development (Saini et al 2013).

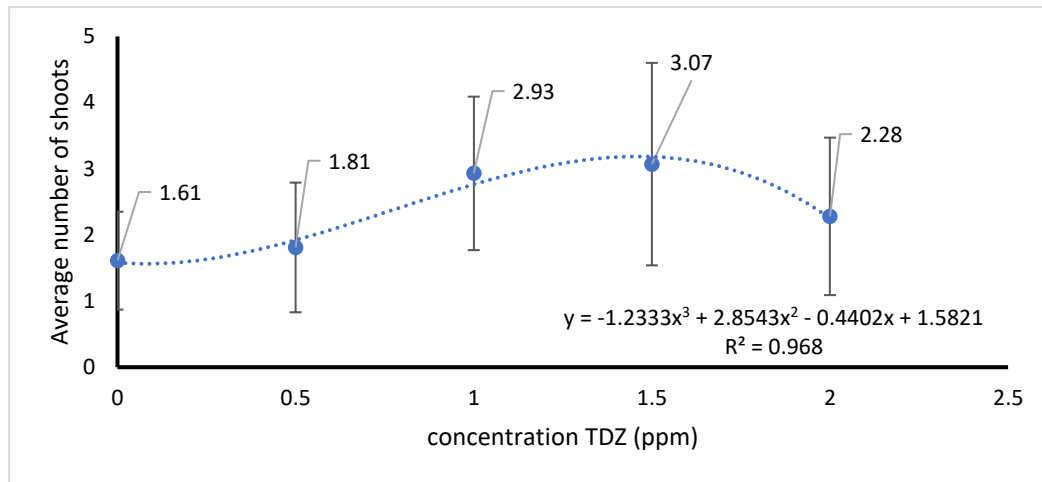


Fig 6 Regression graph of the effect TDZ on the number shoots of *D. macrophyllum*

The regression test graph shows that the 1.5 ppm TDZ treatment obtained a shoot number value of 3.07, significantly different from the control level of 1.61. This treatment increased by 90.68% from the control treatment. The graph explains that the application of 0.5 ppm TDZ showed an increase in the average number of shoots until it reached the highest point at a TDZ concentration of 1.5 ppm. This explains that the application of TDZ up to a concentration of 1.5 ppm showed a positive effect to increase the average number of shoots of *D. macrophyllum* orchid plants, but at concentrations above 1.5 ppm showed a decrease in the average number of shoots of *D. macrophyllum* orchids. According to Abdalla et al. (2022), the use of low levels of TDZ concentration is an effort to avoid abnormal shoot growth such as a small number of shoots. This is reinforced by the statement of Talla et al. (2019) where lower levels of cytokinin promote shoot multiplication and elongation.

3.5 Leaf and root anatomy

Leaf anatomy is one of the important factors to identify factors that affect photosynthesis that occurs in the leaf parts of plants. Amitrano et al. (2021) stated that leaf anatomy and epidermal cell structure determine the conductance of carbon dioxide and water vapor to enter and exit leaves. Mitu et al. (2020), stated that species adapt to different environmental growth conditions must change leaf properties to provide optimal function. The thickness of *D. macrophyllum* leaves formed is an adaptation to the environment and the effect of giving TDZ growth regulators and carrot extract with different concentrations.

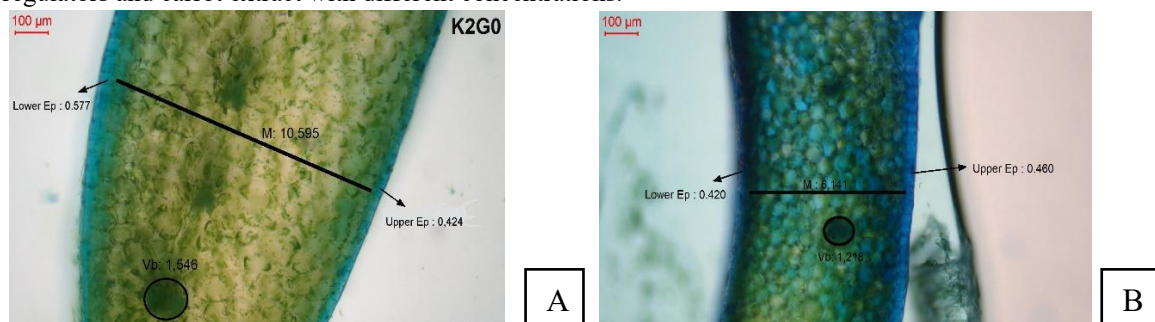


Fig 7 Comparison of leaf anatomy in treatments (a) 1 ppm TDZ and without carrot extract, and (b) 1 ppm TDZ and 60 mL/L carrot extract

Fig 7 showed that the largest mesophyll thickness was found by 10.595 µm in the treatment of TDZ by 1 ppm without carrot extract. The application of TDZ possibly promotes the formation of cell density on *D. macrophyllum*. In line with Manokari et al. (2022) that exogenous cytokinin stimulate cell differentiation and expansion then resulting in increased cell density and providing in the development of structurally stable foliage under in vitro conditions. Moreover, Sosnowski et al. (2023) explained that TDZ stimulates stomatal opening, resulting in thickening of plant cell tissue, especially in adult plants and leaf senescence. In comparison to the treatment TDZ by 1 ppm combined with extract carrot by 60 mL/L resulting the smallest mesophyll thickness by 6.141 µm. It may occur because the concentration between cytokinin and auxin was not balanced. In line with Hussain et al. (2021) that the administration of cytokinin and auxin in high concentrations shows the size of leaf meristem tissue that changes an abnormal phyllotaxis. Dewir et al (2018) explained that the application TDZ in

high concentration stimulate undesirable morphological change in plant, such as fasciated shoots, abnormal leaf morphology and swollen shoot bases in several kind plant.

D. macrophyllum is an epiphytic orchid that lives attached to other plants. Regarding to, Baishnab et al. (2023), *D. macrophyllum* an orchid species indeed has two types of roots aerial roots and clinging roots. It, namely adhesive roots found at the bottom of the media and air roots found at the top of the media. Galindo-Castañeda et al. (2022) stated that root anatomy and architecture affect the ability of plants to absorb nutrients or nutrients that are sustainable and tolerant to plant stress. The thickness of *D. macrophyllum* roots formed is an adaptation to the environment and the effect of giving TDZ growth regulators and carrot extract with different concentrations.

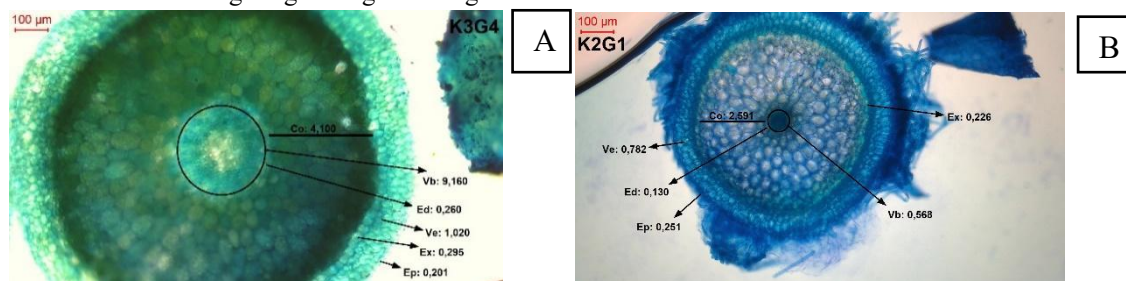


Fig 8 Comparison of root anatomy in treatments (a) 1.5 ppm TDZ and 80 mL/L carrot extract. and (b) 1 ppm TDZ and 20 mL/L carrot extract

Fig 8 shows that the largest cortex thickness (4.100 µm) was observed in the treatment with 1.5 ppm TDZ combined with 80 mL/L carrot extract. In contrast, the lowest cortex thickness (2.591 µm) was recorded in the treatment with 1 ppm TDZ and 20 mL/L carrot extract. These results suggest that a medium enriched with high level of carrot extract and TDZ can enhance cortex thickness in plant roots. Viana et al. (2022) stated that auxins such as carrot extract is needed for gradient formation and auxin accumulation in vascular bundles and basal meristem cells adjacent to the peripheral vascular cylinder and induces cell dedifferentiation and root primordium formation. Based on research Çelikel et al. (2021) TDZ shows inhibition of root elongation, hypocotyl elongation, and cotyledon tightening swelling. Rivas et al. (2022) stated that cytokinin reduces cell elongation and consequently inhibits elongation in roots. Giving cytokinin in optimal conditions able to inhibit plant root growth. Increasing exogenous cytokinin levels results in a reduction in lateral root density, while decreasing cytokinin activity increases lateral root organogenesis.

3.6 Identification Bioactive compound in in vitro-derived plantlets across all treatments and mother plant of *D. macrophyllum*

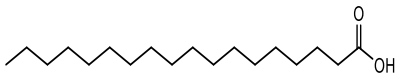
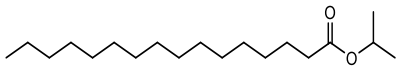
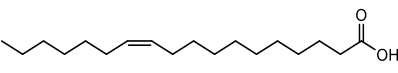
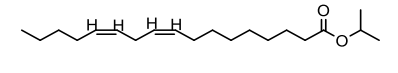
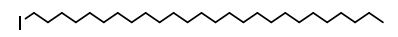
Dendrobium is one of the orchid species that has potential as medicine because it contains phytochemical compounds. Yuan et al. (2019) stated that orchids are known for their diverse roles in various traditional herbal medicines. *Dendrobium* is an eternal herb that containing polysaccharides, alkaloids, essential amino acids and several mineral elements. The GC-MS test (Table 5) results presents the identification of phytochemical compounds by GC-MS for *D. macrophyllum* plantlet and mother plant extract were showing the existence of 5 and 7 bioactive compounds, respectively, that possessing several activities like environmental stress responses, antimicrobial, anti-inflammatory and antioxidant. Among the bioactive compounds were detected important compound in both plantlet and mother plant extract, Octadecanoic acid ($C_{18}H_{36}O_2$) and cis-Vaccenic acid ($C_{18}H_{34}O_2$) were found to have anti-bacterial activity and antioxidant property (Sudharsan et al. 2011; Ali et al. 2020) anti-inflammatory and antioxidant, respectively. The highest peak area percentage of bioactive compound was identified as Octadecanoic acid (52.11%), in the plantlets across all treatments, whereas the mother plant exhibited a lower percentage of 27.25%. This difference may be attributed to the synergistic effect of auxin (carrot) and cytokine (TDZ), which could enhance bioactive compound productivity (Reddy et al. 2013). Izabela et al. (2015) revealed that the application 0.5 µM of TDZ the most concentration that induced the polyphenol production on *Scutellaria alpina*. Moreover, octadecanoic acid also known as stearic acid belongs to the fatty acid compounds that including stearic acid can play a role in regulating plant responses to environmental stress (Ghavam et al., 2021).

Additionally, cis-vaccenic acid was detected in both plantlet and mother plant extract. Cis-vaccenic acid belongs to the fatty acid group that it is known to possess anti-inflammatory and natural antioxidant properties in plants (Deora and Deora, 2021). Moreover, several compounds were detected such as Isopropyl palmitate (5.00%), Isopropyl linoleate (8.76%), 6-Octadecenoic acid, methyl ester (Z)- (3.43%) that found in the in vitro-derived

plantlets across all treatments. Besides, sample from the mother plant were indicated several compounds like Tetracosanoic acid, methyl ester (16.17%), Kaur-16-ene (11.10%), 9-Octadecenoic acid (Z)-, methyl ester (8.55%), Hexacosane, 1-iodo- (5.69%), and 9,12-Octadecadien-1-ol, (Z,Z)- (1.34%).

Isopropyl palmitate, Tetracosanoic acid, methyl ester and Isopropyl linoleate are classified as ester compounds and are used in cosmetics and pharmaceuticals. Barsé et al. (2019) stated that Isopropyl palmitate is an ester commonly used as a solvent, emollient, and in cosmetic and pharmaceutical products. Milivojević et al. (2019) stated that Isopropyl linoleate is a compound used in cosmetic products because it has good solvent and emollient properties. Kaur-16-ene belongs to the terpenoid compounds that functionalized diterpenoid compounds have been established as local antimicrobial phytoalexins in plants (Murphy and Zerbe, 2020). 9-Octadecenoic acid (Z)-, methyl ester was found in field plant samples and belongs to the ester compounds is an important compound useful as a perfume ingredient and has antioxidant, anti-cancer, and antidiabetic properties (Alwasia et al., 2023). 6-Octadecenoic acid, methyl ester, (Z)- found in the plantlet sample that classified as an ester compound. Agbeke Iyun et al. (2022) stated that 6-octadecenoic acid, methyl ester plays a role in antimicrobial activity that is very effective in suppressing the growth of *Aspergillus terreus*. 9,12-Octadecadien-1-ol, (Z, Z)- was found in the field plant sample and is a Linoleyl alcohol that it could be used as an ideal cancer drug-like compound and has potential as an antioxidant (Niveshika et al., 2020).

Table 5. The compound in the plantlets and field plants of *D. macrophyllum* extract using GC-MS as shown in Figure 9

No.	Compound Name	Molecule Formula	Structure	Peak Area (%)		Function
				Plantlets	field plants	
1	Octadecanoic acid	C ₁₈ H ₃₆ O ₂		51.11	27.25	Environmental stress responses (Ghavam et al., 2021)
2	Isopropyl palmitate	C ₁₉ H ₃₈ O ₂		5.00	-	Cosmetic product (Barsé et al. 2019)
3	cis-Vaccenic acid	C ₁₈ H ₃₄ O ₂		23.20	27.22	Anti-inflammatory and antioxidant (Deoram and Deora, 2021).
4	Isopropyl linoleate	C ₂₁ H ₃₈ O ₂		8.76	-	Cosmetic product (Milivojević et al. (2019)
5	Hexacosane, 1-iodo-	C ₂₆ H ₅₃ I		-	5.69	Protective layer on the surface of leaves, stems, and fruits (Salleh et al., 2021)

6	Tetracosanoic acid. methyl ester	$C_{25}H_{50}O_2$	<chem>CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC(=O)OC</chem>	-	16.17	Cosmetic product (Barsé et al. 2019)
7	Kaur-16-ene	$C_{20}H_{32}$	<chem>CC1=C(C)CCC2=C(C)CCC3=C(C)CCC4=C(C)CCC1234</chem>	-	11.10	Antimicrobial (Murphy and Zerbe, 2020)
8	9-Octadecenoic acid (Z)-. methyl ester	$C_{19}H_{36}O_2$	<chem>CCCCCCCC=CCCCCCCCCCCCCCCC(=O)OC</chem>	-	8.55	Perfume ingredient, antioxidant, anti-cancer, and antidiabetic (Alwasia et al., 2023)
9	6-Octadecenoic acid, methyl ester, (Z)-	$C_{19}H_{36}O_2$	<chem>CCCCCCCC=CCCCCCCCCCCCCCCC(=O)OC</chem>	3.43	-	Antimicrobial Agbeke Iyun et al. (2022)
10	9,12-Octadecadien-1-ol, (Z, Z)-	$C_{18}H_{34}O$	<chem>CCCC=CC=CCCCCCCCCCCCCO</chem>	-	1.34	Antioxidant (Niveshika et al., 2020)

The GC-MS test results showed that there were the compounds found in plantlets and field plants of *D. macrophyllum* orchids (Table 5).

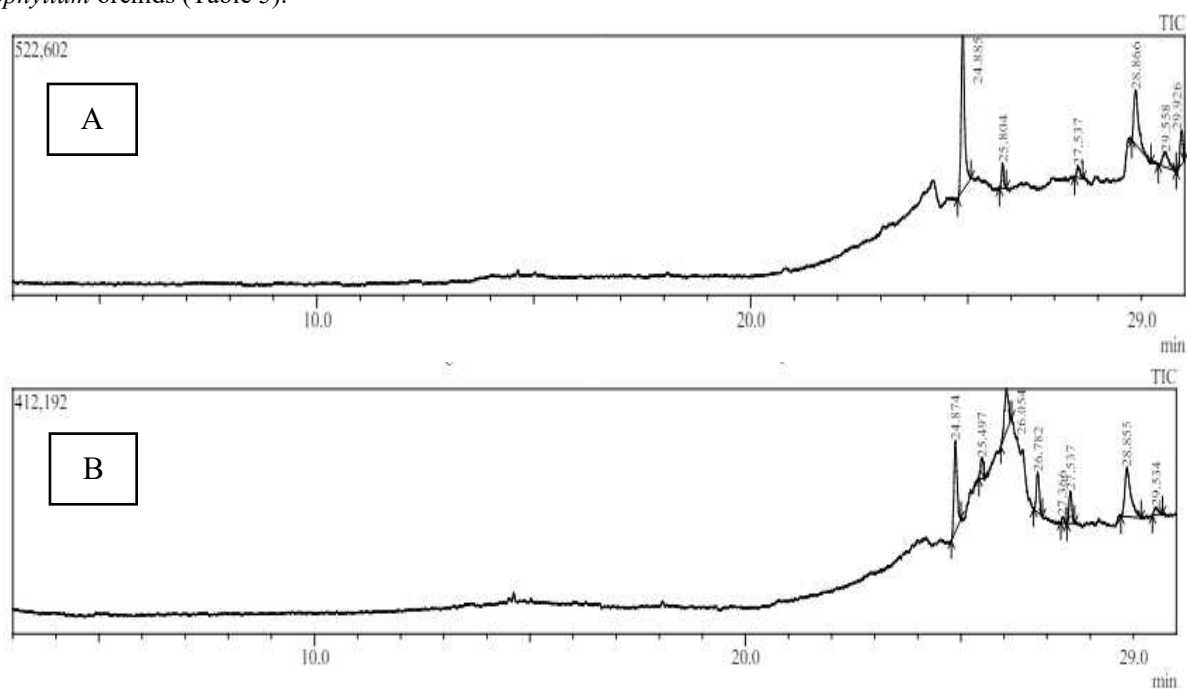


Fig 9 GCMS Chromatograms of *D. macrophyllum* orchids. (A) accumulation plantlets and (B) field plants

4. Conclusion

This study demonstrates the efficacy of combined TDZ and carrot extract in micropropagation, with optimal growth achieved using 1.5 ppm TDZ and 20 mL/L carrot extract. These treatments enhance leaf and shoot development, root formation, and influence anatomical structures and revealed differences in phytochemical composition between in vitro and field-grown plants. These findings contribute to the optimization of micropropagation protocols for *D. macrophyllum*. Supporting both conservation efforts and potential medicinal applications. The study underscores the synergistic effects of synthetic and natural growth regulators in orchid tissue culture, providing a basis for further research in this field as habitat loss threatens biodiversity and optimizing such micropropagation techniques for endangered species becomes increasingly crucial.

5. Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

6. Acknowledgement

The authors gratefully acknowledge Universitas Sebelas Maret for its institutional support and the Indonesian Endowment Fund for Education (LPDP) for the financial assistance.

7. Funding Declaration

This research is funded by the Indonesian Endowment Fund for Education (LPDP) on behalf of the Indonesian Ministry of Higher Education, Science and Technology and managed under the Sabbatical Leave (Outbound Staff Mobility) Program (No. 1309/UN27/TU/2024).

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