Thidiazuron-Induced Somatic Embryogenesis in *Cymbidium bicolor* Orchid In Vitro

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ABSTRACT

Cymbidium bicolor is a highly hunted and traded orchid, leading to a decline in its wild population. Orchid conservation can be achieved through tissue culture, particularly via somatic embryogenesis. Thidiazuron (TDZ) is a growth regulator used to induce somatic embryogenesis. This study aimed to determine the optimal TDZ concentration for somatic embryo formation. Stem explants of C. bicolor were cultured on *Murashige Skoog* (MS) medium with TDZ concentrations of 0, 1, 2, and 3 ppm. Observations were conducted weekly for two months using a stereo microscope and OptiLab. Variables observed included the percentage of green explants, somatic embryo formation time, the number of explants forming somatic embryos, and the number and morphology of somatic embryos. The study was arranged in a Completely Randomized Design (CRD) with 14 replications. Results showed that TDZ addition influenced somatic embryo formation and maintained the green color of explants. Media with TDZ promoted faster growth and larger embryo size compared to media without TDZ. The optimal concentration was 1 ppm TDZ, which produced the highest number of embryos (172) and the fastest formation time compared to other concentrations (TDZ 0: 27, TDZ 2 ppm: 60, TDZ 3 ppm: 39).

Keywords: Clone; Differentiation; Plant propagation; Somatic embryo

INTRODUCTION

Indonesia is well-known to have a high diversity of orchids (Dewi et al., 2024). One of the orchids utterly popular in Indonesia is *Cymbidium bicolor,* whose flower characteristic resembles a boat (Pratama et al., 2021). The beauty of the *C. bicolor* flower causes this flower to be hunted from the forest so it can be traded, and its number in nature has begun to decrease. Yudaputra et al. (2024) state that the existence of orchids in the wild continues to decline, caused by habitat destruction and overexploitation.

Practical propagation efforts are needed to preserve *C. bicolor* orchid (Pratama et al., 2021). Conventional orchid propagation methods take a long time and a large area (Syamsiah et al., 2020). In addition, the natural propagation of orchids requires a suitable pollinator, even with the help of humans. Therefore, plant tissue culture was chosen as an effective method of propagation. Plant propagation through tissue culture can be done in three ways: adventitious shoot formation, lateral shoot proliferation, and somatic embryogenesis (Pardede et al., 2021). Propagation through somatic embryogenesis aims to form embryos from genetically identical somatic tissue (Kong et al., 2020).

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The success of regeneration through somatic embryogenesis is influenced by various factors, including the composition of the media and growth regulators (Ardiyani et al., 2020). The medium used in this study was Murashige Skoog (MS). This medium is often used because most plant cultures that use this medium produce an optimal response. Nutrients contained in MS media are thought to be able to optimize explants that have the potential for the formation of cell competence to form somatic embryos that occur when plant tissue is injured (Lopez et al., 2022).

PGR types and their concentrations in tissue culture depend on the desired goal or direction of plant growth (Murgayanti et al., 2020). One type of PGR widely used for somatic embryo induction is Thidiazuron (TDZ). This hormone is not structurally similar to other natural cytokinins, especially those based on purines. Furthermore, the action of TDZ to induce somatic embryogenesis sets it apart from other purine-based cytokinins (Pardede et al., 2021). The chemical structure of TDZ is stable, its degradation is slower than BAP, and its biological activity is higher to stimulate better embryogenesis growth (Rostiana, 2020). Pardede et al. (2021) stated that TDZ alone had been found to replace the role of auxin and other cytokinins in influencing the somatic embryogenesis process because TDZ can fulfill substantial prerequisites for inducing somatic embryos.

Thidiazuron controls many gene expressions, chloroplast development, secondary metabolite synthesis, cell specification, dedifferentiation, and differentiation. Growth in somatic embryos is triggered by TDZ activity during cell differentiation (Restanto et al., 2023). Pyati (2022) research on *Dendrobium ovatum* showed that 1 ppm TDZ combined with 0.5 ppm NAA resulted in the optimum somatic embryos. Thidiazuron with a concentration of 3 ppm combined with 1 ppm NAA showed the highest number of somatic embryos in *Phalaenopsis amabilis* cultured (Mose et al., 2020). Ghahremani et al. (2021) research on *Phalaenopsis amabilis* cv. Jihan cultured on medium supplemented with 3 ppm TDZ produced the highest number of somatic embryos. Mahendran & Bai (2012) conducted a study on the induction of somatic embryos in *C. bicolor* using explant sources in the form of Protocorm Like Bodies (PLB), where the best response was obtained at 1 ppm BAP and 2,4-D 2 ppm, which formed somatic embryos to the globular phase.

Thidiazuron diffuses into plants, and cellularly, it will act as a signal to increase the number of purines and convert adenine to adenosine. Adenosine will be converted into ribonucleotides (Pardede et al., 2021). Ribonucleotide activity will affect the synthesis of proteins that will act as enzymes isopentenyl transferase (IPT), nucleoside 5-monophosphate phosphoribohydrolase (LOG), and dehydrogenase (CKX), which are involved in the synthesis of cytokinins (Zhao et al., 2024). Nucleoside 5-monophosphate phosphoribohydrolase (LOG) is a regulator and second messenger that will affect cell division (Chen et al., 2022). In cell division, cytokinins (TDZ) signal to restructure the development towards the embryogenic pathway. This signal will trigger the embryogenic developmental path that leads to the formation of somatic embryos, where cells that initially do not have competence become embryonic competence due to the restructured pathway (Zhao et al., 2024).

In addition to adding TDZ to the culture media, it can also be supported by vitamins such as peptone, which is rich in nitrogen and amino acids. According to Carnelos et al. (2022), the presence of nitrogen is positively correlated with the concentration of cytokinins. Peptone is an additional supplement in tissue culture media (Krisdianto et al., 2020). Adding peptone can affect the composition of

organic nutrients in the media. Therefore, the induction of somatic embryos of *C. bicolor* orchids on MS media was carried out with various concentrations of TDZ to accelerate the induction response of somatic embryos to obtain a higher somatic embryo phase compared to somatic embryo studies with *C. bicolor* (Mahendran & Bai, 2012).

In this study, results were obtained in the form of intact plants, in contrast to Mahendran & Bai (2012) research, which had just reached the globular phase, which means that TDZ could spur the formation of Somatic Embryo to the coleoptile phase, exceeding the ability of BAP in Mahendran & Bai (2012) research, which only reached the globular phase.

MATERIALS AND METHODS

Research Design

The research was conducted in January - May 2021 at the Tissue Culture Laboratory, Diponegoro University, Semarang. The research was arranged in a single-factor, completely randomized design (CRD), consisting of four treatments with fourteen replications, totaling 56 experimental units. The treatments were Thidiazuron with concentrations of 0 ppm (T0), 1 ppm (T1), 2 ppm (T2), and 3 ppm (T3). Murasige Skoog, with the addition of 1 g/L peptone, was used as basal media for all treatments.

Sterilization

Culture glassware, pipette, and scalpel were immersed in 5% natrium hypochlorite solution for ± 15 – 30 minutes, then sterilized in an autoclave at 121°C with a pressure of 15 Psi or 2 atm for 15 minutes.

Media Preparation

A 100 ppm TDZ stock solution was prepared by weighing 10 mg of TDZ and adding 3-4 drops of HCl. Then, it was shaken and dissolved with 100 ml of distilled water until evenly distributed. Ready-to-use MS media, sucrose, agar, and peptone were weighted according to their respective dosages on the analytical balance. 50 mL of distilled water was put into a beaker and heated on a hot plate. MS media of 4.43 g/L was put into a beaker and homogenized. 1 g/L peptone was put into a beaker and homogenized on a hot plate using a magnetic stirrer at 200-300 rpm. Sucrose 30 g/L was added, and the solution was left until homogeneous. 8 g/L agar powder was added. The concentrations of growth regulators used were TDZ 0 ppm, 1 ppm, 2 ppm, and 3 ppm. Thidiazuron hormone with various concentrations was added by taking several ml using a syringe, and the solution was allowed to boil. The distilled water was added until the volume reached 100 mL. Then, the media were poured into the Erlenmeyer, covered with aluminum foil, and the middle was pressed and sealed using plastic wrap. The media were sterilized by autoclaving for 15 minutes at 121°C with a pressure of 15 Psi or 2 atm. After sterilization, sterile media were poured into Petri dishes in LAF, then covered with plastic wrap and stored on a culture rack.

Somatic Embryo Induction

The explants used in this research were stems from the *C. bicolor* orchid, which grew from plantlets. The stems were cut into ±2 cm; in the middle of the stems, they were given a shallow wound

using a scalpel and placed horizontally on a petri dish containing MS+1 g/L peptone media with 4 TDZ treatments. Explants were planted under sterile conditions in the LAF.

Somatic Embryo Observation

Visual observations of the somatic embryo of *C. bicolor* were made weekly for two months using OptiLab and a stereo microscope. First, the petri dish containing the explant was put in the base of the stage plate of a stereo microscope and was observed with 4x10 magnification. The variables observed were the percentage of green explants, the formation time of somatic embryos, the number of explants that formed somatic embryos, somatic embryos, and somatic embryo morphologies.

Statical Analysis

All data were analyzed using IBM SPSS Statistics 25 software and Microsoft Excel. Variance (ANOVA) was analyzed on the somatic embryo percentage and continued with the Duncan Multiple Range Test (DMRT) at the 5% level.

RESULTS AND DISCUSSION

Explant's Response

This study showed that adding growth regulators (PGR) in the form of thidiazuron affected the explants to remain green. Browning often occurs in the tissue culture process, inhibiting vitro regeneration. Browning reduces growth rate, shoot, and root differentiation (Bariyyah & Putri, 2021) and can even cause death (Permadi et al., 2023).

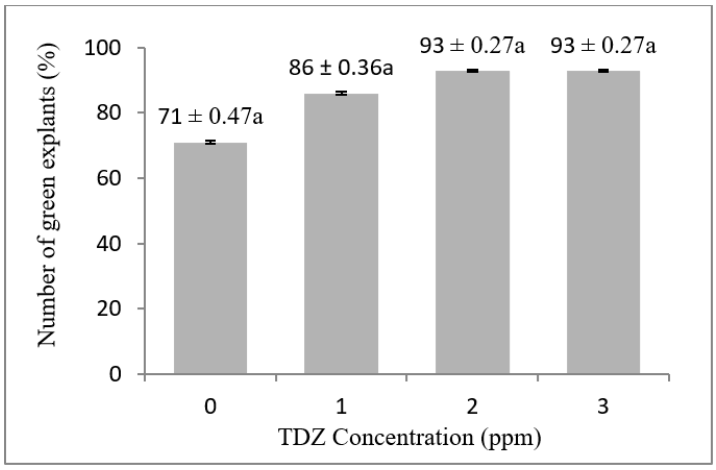

Figure 1. Percentage of green explants of *Cymbidium bicolor* orchid stems planted with TDZ treatment (0, 1, 2, 3 ppm) 8 weeks after planting (WAP) with fourteen replications. Values represent the percentage ± standard deviation. Values followed by the same letters are not significantly different according to the Duncan Test at a 0.05 significance level.

Based on the percentage of green explants (Figure 1), the addition of thidiazuron (TDZ) affected the greenness of the explants. The percentage of green explants indicated a tendency that with the addition of TDZ concentration, the browning of *C. bicolor* explants decreased (Figure 1). The percentage of green explants showed that the administration of TDZ increased the chlorophyll content produced by *C. bicolor* explants. Yuniati & Isda (2024) stated that TDZ could affect the formation/

synthesis of chlorophyll, increasing the amount of chlorophyll in explants. This statement is supported by Mihovilovic et al. (2020), showing that administering TDZ with the right concentration could increase the chlorophyll content in *Amelanchier alnifolia*.

According to Sumihar et al. (2021), explant color is one of the live indicators of explants in tissue culture propagation. In addition, the color of the explants also indicates maximum growth. The green color of the explants indicates that the plant cells are actively photosynthesizing and dividing, but if the explants are brown, it indicates that the explants are starting to become inactive and the cells are less viable or dying (Figure 2; A, C, E, G). Many factors affect brownings, including plant type and genotype, explant damage, media composition, and culture conditions (Amente & Chimdessa, 2021).

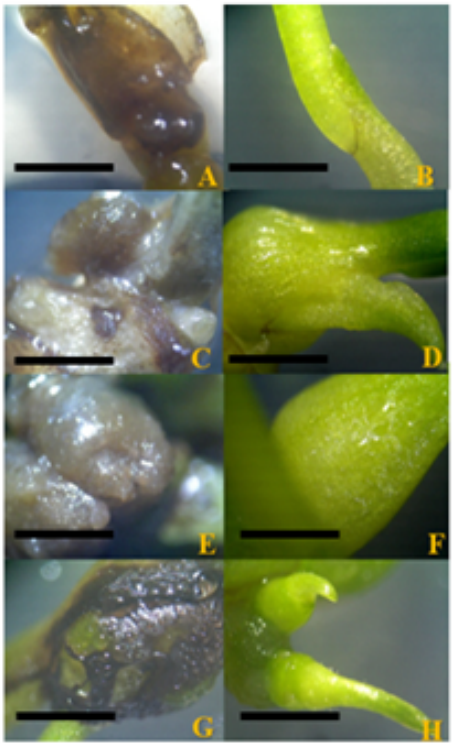

Figure 2. Explants of *Cymbidium bicolor* orchids that remained green (B, D, F, H) and those that experienced browning (A, C, E, G). A-B=TDZ 0, C-D=TDZ 1 ppm, F-E=TDZ 2 ppm, G-H=TDZ 3 ppm

The explants turned brown (Figure 2; A, C, E, G) due to the inhibition of nutrient absorption in the media, so it could be seen that the growth of the explants was disturbed (Figure 2; A, C, E, G). Growth and morphogenesis were not inhibited in explants that did not experience browning (Figure 2; B, D, F, H). According to Jaiswal et al. (2021), the browning condition of explants may also result from nutrient depletion, which halts their growth and necessitates periodic transfers to a fresh growing media. Browning can also occur due to stress caused by cutting and the release of phenol, which is then oxidized. Zhao et al. (2021) stated that the wound caused by cutting encouraged the release of phenol that will be oxidized to quinone by the Polyphenol Oxidase (PPO) enzyme. This irreversible growth inhibition occurs when the phenol is oxidized to the enzyme quinone, polymerizing and oxidizing the protein to an increasing amount of melanic compounds. Phenol that causes explants to brown and dry out can eventually lead to the death of the explants (Punja et al., 2019).

The growth and morphogenesis of explants in vitro are controlled by the balance and interaction of endogenous and exogenous PGRs. Genetic or endogenous traits originating from within the plant include the ability of cells to absorb nutrients available in the media. Meanwhile, exogenous properties can be in the form of technical influences on the implementation of culturing and the addition of PGR to the media.

The presence of TDZ in the media can stimulate the development of chloroplasts in explants (Rineksane et al., 2021). Thidiazuron can affect the color of explants to remain green. The use of TDZ at the right concentration can inhibit leaf discoloration (Bariyyah & Putri, 2021), maintain leaf functionality (Erland et al., 2020), reduce post-harvest browning of lychee fruit pericarp (Fahima et al., 2019), and inhibit leaf senescence (Wang et al., 2019b).

Somatic Embryo Formation

The induction of somatic embryogenesis in this study is direct embryogenesis, which is superior in terms of time reduction because it does not go through the callus stage (Adri, 2019). There is no dedifferentiation stage in direct embryogenesis, where there is little genetic reprogramming, and embryonic cells are formed directly from the explant surface (Xu et al., 2019). Direct embryogenesis minimizes the occurrence of genetic changes induced by tissue culture processes (Jayusman, 2021).

This study used Thidiazuron (TDZ) treatment because TDZ can induce somatic embryos (Restanto, 2023). Thidiazuron is an effective growth regulator in inducing somatic embryos, and it must be used at proper concentrations (Lizawati et al., 2023). The method used in this study was an induction of somatic embryogenesis with various concentrations of TDZ (0, 1, 2, 3 ppm) for eight weeks.

Administration of TDZ at low concentrations induces somatic embryogenesis faster than at high concentrations (Lizawati et al., 2023). Statistical analysis results on the formation of somatic embryos showed that the TDZ concentration of 1 ppm significantly differed from the treatment with TDZ 0, 2, and 3 ppm in *C. bicolor* explants, where 1 ppm TDZ was the treatment with the most optimal results. The results were obtained from the analysis of the number and percentage of somatic embryos formed from the four TDZ treatments, as shown in Table 1, where the values are presented in different letters (a/b) and have different significance at $p < 0.05$.

Table 1. Number and percentage of somatic embryos of *Cymbidium bicolor* Orchid explants with Thidiazuron treatment at concentrations of 0-3 ppm in 0-8 weeks after planting (WAP) with fourteen replications

TDZ Concentration (ppm)	Explants Forming Somatic Embryos (%)	Total Somatic Embryos	Average Somatic Embryos per Explant
0	93	27	1.93±1.27[a]
1	100	172	12.29±7.55[b]
2	100	60	4.29±6.32[a]
3	93	39	2.79±2.55[a]

Remarks: Values represent means ± standard deviations. Values followed by different letters show a significant difference based on the 95% Duncan test.

The findings of this investigation showed that 93% of the explants grown in a medium containing TDZ 0 and 3 ppm generated somatic embryos. All explants produced 100% somatic embryos when

cultivated on media treated with TDZ 1 and 2 ppm. The findings of this investigation showed that 93% of the explants grown in a medium containing TDZ 0 and 3 ppm generated somatic embryos. All explants produced 100% somatic embryos when cultivated on media treated with TDZ 1 and 2 ppm. A total of 172 somatic embryos were created at 1 ppm TDZ, 60 somatic embryos at 2 ppm TDZ, 39 somatic embryos at 3 ppm TDZ, and only 27 somatic embryos at 0 ppm TDZ (Table 1). The treatments between TDZ 0, 2, and 3 ppm were not significantly different (Table 1), so it was found that a concentration of 1 ppm TDZ induced the most optimal somatic embryos compared to other treatments. This follows previous studies on the induction of somatic embryos of *Dendrobium ovatum* by Pyati (2022), which found that 1 ppm TDZ was the most effective in increasing somatic embryo induction.

Based on the observations that have been made, the administration of TDZ in tissue culture media greatly affected the response of *C. bicolor* orchid stem explants, as indicated by an increase in the mean somatic embryos formed (Figure 3). At a concentration of 0 ppm, somatic embryos were formed because the explants of *C. bicolor* had endogenous hormones capable of inducing somatic embryos. The added thidiazuron can activate metabolic processes, such as carbohydrate metabolism, ROS (Reactive Oxygen Species) metabolism, photosynthesis, and protein synthesis, which are needed for the transformation process. This process causes somatic cells to dedifferentiate and then become meristems that trigger the induction of somatic embryos (Erland et al., 2020; Ali et al., 2022; Feher, 2019).

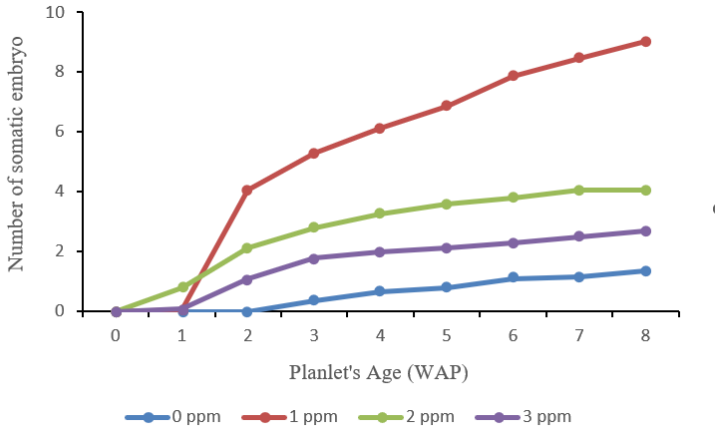

Figure 3. Average number of somatic embryos of *Cymbidium bicolor* formed after TDZ treatment (0,1,2,3 ppm) from 0-8 weeks after planting (WAP).

Table 1 shows that TDZ 2 and 3 ppm concentrations were less than optimal due to a decrease in the yield of somatic embryos compared to TDZ 1 ppm because the concentrations were thought to be too high. Thidiazuron concentrations that are too high can cause abnormalities and inhibit somatic embryo growth. Thidiazuron is a herbicide that can kill tissue and cause deviations in plant tissue development (Pardede et al., 2021). This is related to the absorption and transfer of nutrients and the metabolism of TDZ. Suppose the concentration of TDZ given to plants is appropriate. In that case, it will increase the overall absorption of sugar from the culture media, increase primary metabolism, transfer terpene metabolism, and mediate stress metabolism through indoleamine and phenylpropanoid metabolism (Erland et al., 2020).

The average induction time in TDZ treatment of 0-3 ppm was 2 WAP (Figure 3). *C. bicolor* explants grown on TDZ-added media showed a faster induction time (1 WAP) (Figure 3). This is because the hormone spreads in the tissue quickly. Devireddy et al. (2020) stated that the spread of the hormone could be through the intercellular space/cytoplasm, not necessarily through the vascular system. Therefore, the induction of somatic embryos in *C. bicolor* orchids occurs rapidly.

Thidiazuron in tissue culture media at the right concentration will stimulate somatic embryogenesis (Budi, 2020). Figure 3 also shows that TDZ works at low concentrations; this is proved by the response to the emergence of the most optimal somatic embryos shown in the 1 ppm TDZ treatment, namely the emergence of somatic embryos in the first week after planting (WAP), and the mean somatic embryos formed were more significant than other treatments. These results significantly differed from the treatment of 0, 2, and 3 ppm, which indicated that against *C. bicolor* explants, TDZ worked at low concentrations.

Thidiazuron works at low concentrations, where the medium can quickly transfer and metabolize TDZ because the higher the concentration, the more complex the distribution and metabolism process. As a result, somatic embryo growth remains at an early, globular stage. In another sense, besides being able to induce somatic embryogenesis, the TDZ hormone can also inhibit the regeneration of somatic embryos, depending on its concentration and the type of plant being cultured (Pardede et al., 2021). Budi (2020) also reinforces this statement, stating that the hormone concentration will inhibit the plant's growth if it is too high.

Table 1 shows that the endogenous hormones in the explants have a role in forming somatic embryos. It is proven that somatic embryos were still formed even at 0 ppm TDZ treatment. Non-specific tissues, such as meristematic tissue, produce endogenous hormones that can be produced when stimulated (Hong et al., 2019). Stimuli that can affect hormone production are growth media and environmental conditions (Hong et al., 2019). When the hormone has reached a specific concentration, the previously inactive gene will begin to express (Fadón et al., 2020).

Apart from endogenous hormones, somatic embryos can be induced at 0 ppm media because *C. bicolor* already has cell competence to form somatic embryos. The media already contained nutrients such as nitrogen, amino acids, and vitamins and was also supported by peptone added to the media, which increased the nitrogen content (Krisdianto et al., 2020). MS media added with peptone and supported by endogenous hormones, as well as other environmental factors such as the appropriate temperature (±25°C) and sufficient light, worked synergistically in influencing and activating the formation of a cell's competence in forming somatic embryos.

The addition of exogenous PGR affects the activity of endogenous hormones. This activity is a factor that stimulates further growth and development (Burgel et al., 2020). The interaction between TDZ added to the media and endogenous hormones determines the direction of the development of an explant (Rahmah et al., 2020). This will trigger physiological changes and the formation of somatic embryos because cells that initially do not have embryonic capabilities become embryonic competencies. Narváez et al. (2019) stated that some cells acquired embryogenic abilities after adding TDZ hormone in culture media. This is proved by the study of Vallado et al. (2022), reporting the success of increasing the induction rate of somatic embryos in *Hippeastrum hybridum* using low

concentrations of TDZ (0, 0.5, 1, 1.5, and 2 ppm), which in that study, the TDZ 0 ppm also produced somatic embryos. However, with the addition of TDZ, even at low concentrations, the number of somatic embryos increased. Low TDZ concentrations have affected the formation of somatic embryos in *C. bicolor* orchids.

Morphology of Somatic Embryo of *C. bicolor*

The results of the qualitative analysis of this study were obtained by observing the morphological structure formed on the explants of *C. bicolor* for eight weeks. The observation of the growth of somatic embryos in vitro showed a growth pattern of somatic embryos directly from explant cells without going through the callus phase (Figure 4). Somatic embryos were formed in the wounded area of the *C. bicolor* explant. However, if the area was browning, somatic embryos were formed near the wounded area (Figure 4). The incision wounds encourage the wounded explant cells to become meristematic and then actively divide, allowing the previously differentiated explants to generate totipotent somatic embryos (Wehbi et al., 2022). The wound causes TDZ to diffuse into plant tissues more easily so that TDZ will stimulate and induce somatic embryogenesis.

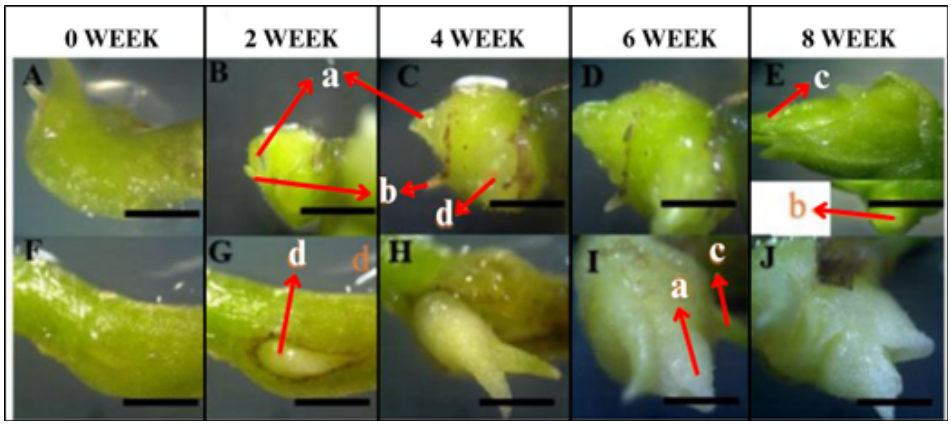

Figure 4. Development of *Cymbidium bicolor* Orchid Somatic Embryo with TDZ at concentrations of 1 ppm (A-E); 3 ppm (F-J). a: Shoot Apical Meristem (SAM), b: Root Apical Meristem (RAM), c: leaf, d: globular phase.

The development of somatic embryos of *C. bicolor* in vitro in the first week started with swelling of the embryo and some color changes to become brighter. In the second week (Figure 3 G), the embryo swelled and began to make a round shape, called the globular phase. The stages of the formation of somatic embryogenesis start from cell division into a collection of cells that form globular (Hernandez et al., 2019). TDZ treatment of 1 ppm at 2 WAP (Figure 4 B) formed a bipolar structure characterized by the appearance of SAM and RAM at the heart phase stage. In the 3 ppm TDZ treatment, SAM was formed first, then after 4 WAP, RAM began to appear. In Figure 4D, SAM and RAM continue to grow lengthwise and enlarge; this is the torpedo phase. SAM then developed into leaves at the coleoptile phase (Figure 4 E, J). Agustin et al. (2020) stated that SAM was formed before leaf primordia and would develop into mature leaves. The development of somatic embryos (Figure 4) corresponds to the development of *P. amabilis* orchid seeds that have been reported. In the first phase, the embryo is yellow; then, it will turn green, forming a bipolar structure, then leaf primordia, and the leaves will continue to form (Gulzar et al., 2020).

The explants used in this study were stems of the *C. bicolor* orchid plantlet. Orchid stems were chosen because they contain floral meristems (Wang et al., 2019a), which have high meristematic abilities. Loyola-Vargas et al. (2022) also stated that cells with high meristematic ability showed high regeneration potential and embryogenesis induction ability. Therefore, the induction of somatic embryogenesis is easier on stem explants. Somatic embryos of *C. bicolor* orchids growing on media containing TDZ experienced faster growth. Their size automatically became larger (Figure 5 B) compared to somatic embryos grown on media without the addition of TDZ (Figure 5 A, C, D); the number of somatic embryos produced was even higher (Figure 14 B, C, D). Thidiazuron can increase the growth rate of somatic embryos because TDZ is one of the most active cytokinins that induces more significant proliferation than other cytokinins. This hormone does more vigorous activity than other types of cytokinins.

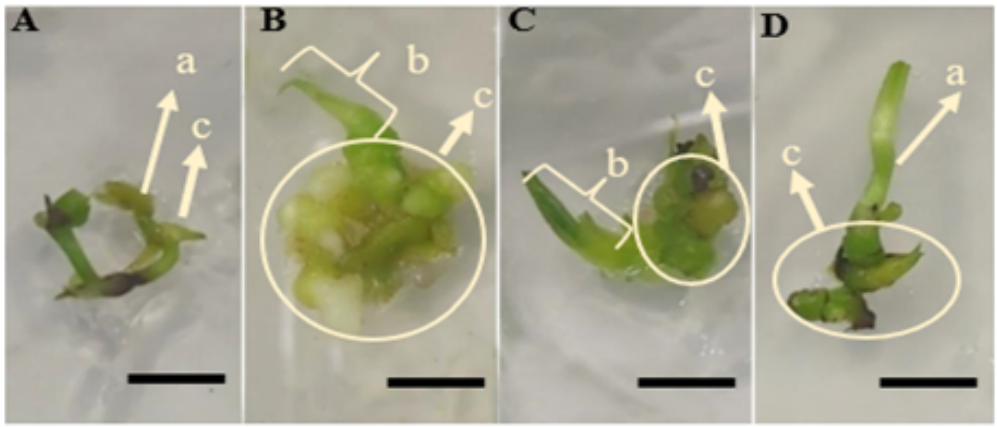

Figure 5. Morphology of Somatic Embryo of *Cymbidium bicolor* Orchid at 8 WAP with various concentrations of TDZ: 0 ppm (A), 1 ppm (B), 2 ppm (C), 3 ppm (D), showing shoot with leaf primordia (a), leaf primordia (b), and somatic embryo (c) (Scale bar = 5 mm)

Thidiazuron diffuses into plants and cellularly will act as a signal that will increase the number of purines and convert adenine to adenosine, then adenosine will be converted into ribonucleotides (Chandel, 2024; Erland et al., 2020). Ribonucleotide activity will affect the synthesis of proteins that will act as enzymes isopentenyltransferase (IPT), nucleoside 5-monophosphate phosphoribohydrolase (LOG), and dehydrogenase (CKX), which are involved in the synthesis of cytokinins (Chen et al., 2022). Nucleoside 5-monophosphate phosphoribohydrolase (LOG) is a regulator and second messenger that will affect cell division (Zhao et al., 2024). In the process of cell division, cytokinins (TDZ) signal to restructure development towards the embryogenic pathway. This will trigger the embryogenic developmental pathway that leads to the formation of somatic embryos, where cells that initially do not have competence become embryonic abilities due to the restructuring of the pathway (Zhao et al., 2024).

Therefore, this study stated that TDZ at a concentration of 1 ppm successfully induced somatic embryos in *C. bicolor*. The effectiveness of the research is highlighted by the ability to produce somatic embryos with low TDZ concentrations, eliminating the need for higher concentrations.

CONCLUSION

Thidiazuron treatment stimulated the formation of somatic embryos. The optimal concentration of TDZ to stimulate the induction of somatic embryos in *C. bicolor* was 1 ppm, which induced somatic embryos the fastest (one week after implantation) and produced the highest number of somatic embryos.

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AUTHORS CONTRIBUTIONS

NS and YN designed and conceived the experiments. AMI and NS experimented. AMI, NS, and YN contributed to the preparation of samples and interpretation of the results. The manuscript was primarily composed by AMI. All authors provided critical feedback and contributed to developing the research, analysis, and manuscript.

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.

REFERENCES

Adri, R. F. (2019). Induksi Kalus Theobroma cacao sebagai Tahap Awal Pengembangan Tanaman Melalui Embriogenesis Somatik. *Menara Ilmu, 13*(8), 69–73. https://doi.org/10.33559/MI.V13I8.1484

Agustin, R., Suharsono, & Rizal Putra, R. (2020). Pengaruh Ekstrak Tomat Terhadap pertumbuhan Embrio Anggrek *Phaius tankervilleae* Khas Gunung Galunggung Kabupaten Tasikmalaya. *Bioma : Jurnal Ilmiah Biologi, 9*(2), 264–279. http://103.98.176.9/index.php/bioma/article/view/7064

Ali, H. M., Khan, T., Khan, M. A., Ullah, N. (2022). The Multipotent Thidiazuron: A Mechanistic Overview of its Roles in Callogenesis and Other Plant Cultures in vitro. *Biotechnology and Applied Biochemistry, 69*(6), 2624-2640. https://doi.org/10.1002/bab.2311

Amente, G & Chimdessa, E. (2021). Control of Browning in Plant Tissue Culture: A Review. *Journal of Scientific Agriculture, 5*, 67-71. https://doi.org/10.25081/jsa.2021.v5.7266

Ardiyani, F., Utami, E. S. W., Purnobasuki, H., Paramita, S. A. (2020). Development and regeneration of somatic embryos from leaves-derived calli of *Coffea liberica*. *Biodiversitas, 21*(12), 5829-5834.

Bariyyah, K., & Putri, I. (2021). Kajian Kombinasi Perlakuan Zat Pengatur Tumbuh TDZ dan Benzil Adenin Terhadap Perkembangan Kalus Durian Merah. *Agroekoteknologi, 13*(1), 52–60. https://doi.org/http://dx.doi.org/10.33512/jur.agroekotetek.v13i1

Budi, R. S. (2020). Uji Komposisi Zat Pengatur Tumbuh Terhadap Pertumbuhan Eksplan Pisang Barangan (*Musa paradisiaca* L.) Pada Media MS Secara in vitro. *BEST Journal (Biology Education, Sains and Technology), 3*(1), 101–111. https://doi.org/10.30743/best.v3i1.2475

Burgel, L., Hartung, J., Schibano, D., & Graeff-Hönninger, S. (2020). Impact of Different Phytohormones on Morphology, Yield and Cannabinoid Content of *Cannabis sativa* L.. *Plants, 9*(725), 1–16. https://doi.org/10.3390/plants9060725

Carnelos, D., Miglioli, J. L., Giardina, E., Tognetti, J., Benedetto, A. H. (2022). Cytokinin Action Revisited: Leaf Anatomical Changes Play a Key Role in 6-Benzilaminopurine-driven Growth in Pot-grown Lettuce. *Revista Chapingo Serie Horticultura, 28*(2), 109-133. https://doi.org/10.5154/r.rchsh.2021.07.015

Chandel, N. S. (2024). Nucleotide Metabolism. *Cold Spring Harb Prespect Biol, 13.* https://doi:10.1101/cshperspect.a040592

Chen, L., Jameson, G. B., Guo, Y., Song, J., Jameson, P. E. (2022). The Lonely Guy Gene Family: From Mosses to Wheat, The Key to the Formation of Active Cytokinins in Plants. *Plant Biotechnol J., 20(*4), 625-645. https://doi.org/10.1111/pbi.13783

Devireddy, A. R., Zandalinas, S. I., Fichman, Y., & Mittler, R. (2020). Integration of reactive oxygen species and hormone signaling during abiotic stress. *The Plant Journal*, 105(1), 459–476. https://doi.org/10.1111/tpj.15010

Dewi, N. P. Y. A., Angket, S., Ledheng, L., Sele, Y., Weisillana. (2024). Diversity of Orchid Types in the Oeluan Tourism Forest, Bijeli Village, Noemuti District, North Central Timor Regency. *Interdiciplinary International Journal of Conservation and Culture, 2*(1), 31-38. https://doi.org/10.25157/iijcc.v2i1.3906

Erland, L., Giebelhaus, R., Victor, J. M. R., Murch, S. J., & Saxena, P. K. (2020). The morphoregulatory role of thidiazuron: Metabolomics-guided hypothesis generation for mechanisms of activity. *Biomolecules, 10*(9), 1–34. https://doi.org/10.3390/biom10091253

Fadón, E., Fernandez, E., Behn, H., & Luedeling, E. (2020). A Conceptual Framework for Winter Dormancy in Deciduous Trees. *Agronomy, 10*(241), 1–20. https://doi.org/10.3390/agronomy10020241

Fahima, A., Levinkron, S., Maytal, Y., Hugger, A., Lax, I., Huang, X., Eyal, Y., Lichter, A., Goren, M., Stern, R. A., & Harpaz-Saad, S. (2019). Cytokinin treatment modifies litchi fruit pericarp anatomy leading to reduced susceptibility to post-harvest pericarp browning. *Plant Science, 283*(10), 41–50. https://doi.org/10.1016/j.plantsci.2019.02.006

Feher, A. (2019). Callus, Dedifferentiation, Totipotency, Somatic Embryogenesis: What These Terms Mean in the Era of Molecular Plant Biology?. *Front Plant Sci, 10*. https://doi.org/10.3389/fpls.2019.00536

Ghahremani, R., Daylami, S. D., Mirmasoumi, M., Askari, N., Vahdati, K. (2021). Refining a Protocol For Somatic Embryogenesis and Plant Regeneration of *Phalaenopsis amabilis* cv. Jinan from Mature Tissues. *Turkish Journal of Agriculture and Forestry, 45*(3), 356-364. https://doi.org/10.3906/tar-2004-107

Gulzar, B., Mujib, A., Qadir Malik, M., Sayeed, R., Mamgain, J., & Ejaz, B. (2020). Genes, proteins, and other networks regulating somatic embryogenesis in plants. *Journal of Genetic Engineering and Biotechnology, 18*(31), 1–15. https://doi.org/10.1186/s43141-020-00047-5

Hernandez, H. A., Rodriguez, M. L., Montalvo, R. N. A., Gomez, Y. L. J., Skeete, A., Montalvo, J. A., Pena, C. D. L., & Vargas, V. M. L. (2019). Signaling Overview of Plant Somatic Embryogenesis. *Front. Plant Sci., 10*,77. https://doi.org/10.3389/fpls.2019.00077

Hong, Lee, Z., Hirakawa, T., Yamaguchi, N., & Ito, T. (2019). Molecular Sciences The Roles of Plant Hormones and Their Interactions with Regulatory Genes in Determining Meristem Activity. *International Journal of Molecular Sciences, 20*(4065), 1–19. https://doi.org/10.3390/ijms20164065

Jaiswal, N., Verma, Y., & Misra, P. (2021). High frequency in vitro callogenesis and plant regeneration of *Glycyrrhiza glabra* L.. *Vegetos, 34*(3), 495–504. https://doi.org/10.1007/s42535-021-00219-9

Jayusman. (2021). Biotechnologi Propagasi Vegetatif Tanaman Hutan: Keuntungan dan Risiko. *Prosiding SNPBS (Seminar Nasional Pendidikan Biologi Dan Saintek), 6*(1), 248–257. https://proceedings.ums.ac.id/index.php/snpbs/article/view/40

Kong, E. Y. Y., Biddle, J., Foale, M., & Adkins, S. W. (2020). Cell suspension culture: A potential in vitro culture method for clonal propagation of coconut plantlets via somatic embryogenesis. *Industrial Crops and Products*, 147(1), 1–19. https://doi.org/10.1016/j.indcrop.2020.112125

Krisdianto, A., Saptiningsih, E., Nurchayati, Y., & Setiari, N. (2020). View of Growth of *Phalaenopsis amabilis* (L.) Blume Orchid Plantlet on Subculture Stage by Difference of Media

Types and Pepton Concentrations. *Journal of Biological Sciences, 7*(2), 39–47. https://doi.org/10.24843/metamorfosa.2020.v07.i02.p06
Lizawati, L., Zulkarmain, Z., Antony, D., Purnamaningsih, R. (2023). The effect of 2,4-D, BA and Thidiazuron on somatic embryo induction of liberica coffee of Tungkal Composite from Jambi. *E3S Web of Conferences 373*, 03012. https://doi.org/10.1051/e3sconf/202337303012
Lopez, R. A., Rodriguez, M. L., Contreras, A. S., Avelizapa, L. I. R., Coello, N. G. S., Collado, N. M., Pastrana, R. N. (2022). Inorganic Compounds that Aid in Obtaining Somatic Embryos. *Methods Mol Biol, 2527*, 203-221. https://doi.org/10.1007/978-1-0716-2485-2_15
Loyola-Vargas, M., Ochoa-Alejo, N., Elhiti, M., & Stasolla, C. (2022). Transduction of Signals during Somatic Embryogenesis. *Plants, 11*(178), 1–16. https://doi.org/10.3390/plants11020178
Mahendran, G., & Bai, V. N. (2012). Direct somatic embryogenesis and plant regeneration from seed-derived protocorms of *Cymbidium bicolor* Lindl. *Scientia Horticulturae, 135*(1), 40–44. https://doi.org/10.1016/j.scienta.2011.12.003
Mihovilovic, A. B., Jercic, I. H., Prebeg, T., Pavicic, I. A., Baric, M., Keresa, S. (2020). Light Source and Cytokinin Type Affect Multiplication Rate, Chlorophyll Content and Stomata Formation of *Amelanchier alnifolia* Shoots in Vitro. *Journal of Central European Agriculture, 21*(4), 826-838. https://doi.org/10.5513/JCEA01/21.4.2909
Mose, W., Daryono, B. S., Indrianto, A., Purwanto, A., Semiarti, E. (2020). Direct Somatic Embryogenesis and Regeneration of an Indonesian orchid *Phalaenopsis amabilis* (L.) Blume under a Variety of Plant Growth Regulators, Light Regime, and Organic Substances. *Jordan Journal of Biological Sciences, 13*(4), 509-518.
Murgayanti, M., Sumadi, S., & N, R. F. (2020). Multiplikasi tunas kunyit putih (*Kaempferia rotunda* L.) pada jenis dan konsentrasi sitokinin secara in vitro. *Kultivasi, 19*(3), 1230–1236. https://doi.org/10.24198/kultivasi.v19i3.29469
Narváez, I., Martín, C., Jiménez-Díaz, R. M., Mercado, J. A., & Pliego-Alfaro, F. (2019). Plant Regeneration via Somatic Embryogenesis in Mature Wild Olive Genotypes Resistant to the Defoliating Pathotype of *Verticillium dahliae*. *Frontiers in Plant Science, 10*(1), 1–11. https://doi.org/10.3389/fpls.2019.01471
Pardede, Y., Mursyanti, E., & Sidharta, B. R. (2021). Pengaruh Hormon terhadap Induksi Embrio Somatik Kacapiring (*Gardenia jasminoides*) dan Potensi Aplikasinya dalam Pembuatan Benih Sintetik. *Biota : Jurnal Ilmiah Ilmu-Ilmu Hayati, 6*(3), 162–177. https://doi.org/10.24002/biota.v6i3.4093
Permadi, N., Nurzaman, M., Alhasnawi, A. N., Doni, F., Julaeha, E. (2023). Managing Lethal Browning and Microbial Contamination in *Musa* spp. Tissue Culture: Synthesis and Perspectives. *Horticultrae, 9*(4), 453. https://doi.org/10.3390/horticulturae9040453
Pratama, F. F., Setiari, N., Nurchayati, Y. (2021). Growth of *Cymbidium bicolor* Lindl. Plantlet During Subculture With Variation of Media. *Jurnal Biologi Udayana, 25*, 71-77. https://doi.org/10.24843/JBIOUNUD.2021.v25.i01.p08
Punja, Z. K., Collyer, D., Scott, C., Lung, S., Holmes, J., & Sutton, D. (2019). Pathogens and Molds Affecting Production and Quality of *Cannabis sativa* L. *Frontiers in Plant Science, 10*(1120), 1–23. https://doi.org/10.3389/fpls.2019.01120
Pyati, A. N. (2022). In vitro Propagation of orchid (*Dendrobium ovatum* (L.) Kraenzl.) through Somatic Embryogenesis. *Plant Tissue Cult. & Biotech., 32*(1), 53-6. https://doi.org/10.3329/ptcb.v32i1.60472
Rahmah, M., Anwar, A., & Swasti, E. (2020). Karamunting (*Rhodomyrtus tomentosa*) Callus Induction In Vitro. *International Journal of Environment, Agriculture and Biotechnology, 5*(2), 459–465. https://doi.org/10.22161/ijeab.52.20
Restanto, D. P., Felayati, I., Fanata, W. I. D., Dewanti, P., Kriswanto, B., Khozin, M. N., Prayoga, M. C. (2023). Optimization of TDZ Hormone on the Formation of Somatic Embryogenesis in *Dendrobium Orchids* (D.50TH Stage Beauty X D. Bobby Mesina). *Jurnal Natur Indonesia, 21*(1), 42-46. https://doi.org/10.31258/jnat
Rineksane, I. A., Juliarachmi, A. F., Putri, R. K., Astuti, A., & Samidjo, G. S. (2021). Optimization of 2,4-D and Cytokinin Combination for the Growth of *Vanda tricolor* in Solid and Liquid Medium. *IOP Conference Series: Earth and Environmental Science, 752*(1), 1–7. https://doi.

org/10.1088/1755-1315/752/1/012025
Rostiana, O. (2020). Aplikasi Sitokinin Tipe Purin Dan Urea Pada Multiplikasi Tunas Anis (*Pimpinellla anisum* L.) In Vitro. *Jurnal Penelitian Tanaman Industri, 13*(1), 1–7. https://doi.org/10.21082/jlittri.v13n1.2007.1-7
Sumihar, S. T. T., Siahaan, F. R., Pujiastuti, E. S., & Laia, D. A. S. (2021). Leaf Fertilizer as a Source of Nutrients for In Vitro Propagation Culture Media of Banana Raja Bulu (*Musa paradisiaca* L. cv. Raja Bulu). *Jurnal Ilmu Pertanian, 9*(2), 89–94.
Syamsiah, M., Imansyah, A. A., Suprapti, H. K., & Badriah, D. S. (2020). Respon Multiplikasi Anggrek Bulan (*Phalaenopsis* sp.) Terhadap Penambahan BAP(Benzyl Amino Purine) pada Media in vitro. *Agroscience (Agsci), 10*(2), 148. https://doi.org/10.35194/agsci.v10i2.1157
Vallado, N., Almeida, D., Borges, E., & Carlos, J. (2022). Plant Science Somatic embryogenesis from flower tepals of *Hippeastrum* aiming regeneration of virus-free plants. *Plant Science*, 317, 111191. https://doi.org/10.1016/j.plantsci.2022.111191
Wang, S.-L., Kasi Viswanath, K., Tong, C.-G., Ryun An, H., Jang, S., & Chen, F.-C. (2019a). *Floral Induction and Flower Development of Orchids*. *Frontier in Plant Science*, *10*(1258). https://doi.org/10.3389/fpls.2019.01258
Wang, W., Hao, Q., Wang, W., Li, Q., Chen, F., Ni, F., Wang, Y., Fu, D., Wu, J., & Wang, W. (2019b). The involvement of cytokinin and nitrogen metabolism in delayed flag leaf senescence in a wheat stay-green mutant, tasg1. *Plant Science, 278*(8), 70–79. https://doi.org/10.1016/j.plantsci.2018.10.024
Wehbi, H., Soulhat, C., Morin, H., Bendahmane, A., Hilson, P., & Bouchabké-Coussa, O. (2022). One-Week Scutellar Somatic Embryogenesis in the Monocot *Brachypodium distachyon*. *Plants, 11*(1068), 1–17. https://doi.org/10.3390/plants11081068
Xu, J., Su, Y. H., & Fehér, A. (2019). Callus, Dedifferentiation, Totipotency, Somatic Embryogenesis: What These Terms Mean in the Era of Molecular Plant Biology? *Frontiers in Plant Science, 10*(536), 1–11. https://doi.org/10.3389/fpls.2019.00536
Yudaputra, A., Munawaroh, E., Usmadi, D., Purnomo, D. W., Astuti, I. P., Puspitaningtyas, D. P., Handayani, T., Garvita, T. R., Aprilianti, P., Wawangningrum, H., Renjana, E., Handini, E., Angio, M. H., Firdiana, E. R., Witono, J. R., Juswara, L. S., Fijridiyanto, I. A., Ariati, S. R., Yuzammi, Sudarmono, Wanda, I. F., Wibowo, A. R. W., Wati, R. K., Hutabarat, P. W. K., Raharjo, P. D., Solihah, S. M., Saputra, R., Cropper, W. P. (2024). Vulnerability of Lowland and Upland Orchids in Their Spatially Response to Climate Change and Land Cover hange. *Ecological Informatics, 80*, 102534. https://doi.org/10.1016/j.ecoinf.2024.102534
Yuniati & Isda, M. N. (2024). Combination of Thidiazuron and Basal Media Type on Optimizing in vitro Growth of *Grammatophyllum stapeliiflorum* Orchid. *Jurnal Ilmiah Pertanian, 21*(1), 21-32. https://doi.org/10.31849/jip.v21i1.12829
Zhao, J., Wang, J., Liu, J., Zhang, P., Kudoyarova, G., Liu, C. J., Zhang, K. (2024). Spatially distributed cytokinins: Metabolism, signaling, and transport. *Plant Communications, 5*(7), 2590-3462, https://doi.org/10.1016/j.xplc.2024.100936
Zhao, S., Wang, H., Liu, K., Li, L., Yang, J., An, X., Li, P., Yun, L., & Zhang, Z. (2021). The role of JrPPOs in the browning of walnut explants. *BMC Plant Biology, 21*(1), 1–12. https://doi.org/10.1186/s12870-020-02768-8