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Optimizing in vitro multiplication of the Wonosobo local clone of ramie using apical shoots and thidiazuron

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Abstract

The availability of planting material becomes the most common problem in crop cultivation. Particularly, the crops such as ramie that planting material mainly come from vegetative part. Therefore, the advanced technique needed such as in vitro culture. The multiplication stage success in in vitro culture is influenced by factors such as planting material and plant growth regulators (PGRs). Apical shoots are commonly used as explants in in vitro culture, and thidiazuron, a cytokinin, is frequently used. This study aimed to determine the optimal concentrations of thidiazuron on in vitro ramie shoot multiplication. This study was conducted at the Seed Technology Tissue Culture Laboratory Universitas Padjadjaran. Apical shoots from the Wonosobo clone of ramie were used as explants and cultured in Murashige and Skoog medium. The culture was then stored in a culture room with 16 hours per day lighting. A Completely Randomized Design (CRD) with seven treatments and four replications was applied. The treatments consisted of thidiazuron concentrations: 0, 0.05, 0.075, 0.1, 0.125, and 0.175 ppm. The results showed that the addition of thidiazuron significantly affected bud break and the number of shoots. Thidiazuron accelerated shoot emergence by an average of 6 days and increased the number of shoots by 12.02% in the 0.075 ppm treatment. Thidiazuron significantly influenced the multiplication of ramie shoots. The optimal concentration for shoot multiplication was 0.075 ppm, which enhanced the number of shoots and accelerated shoot emergence.

Keywords: cytokinin, explant, in vitro, ramie, thidiazuron

Introduction

Ramie (*Boehmeria nivea*) is a plantation crop with multiple uses across its various parts. The leaves can be utilized as compost material and livestock feed, while the stems serve as a source of fuel (Trisiana et al., 2016) and fibers extracted from the inner bark are suitable as raw materials in the textile industry due to their similarity to cotton fibers (Mahendra & Risdianto, 2019). In 2020, Indonesia imported approximately 493,451 tons of cotton (Direktorat Jenderal Perkebunan, 2021). Given this context, ramie holds significant potential to be developed as an alternative raw material for the textile industry, thereby reducing the country's reliance on cotton imports.

The ramie cultivation must be supported by appropriate cultivation techniques to ensure optimal plant productivity. One of the main challenges in ramie cultivation is the availability of planting materials. These materials must exhibit uniform physical characteristics (such as size, color, and age), and must be free from damage caused by

pests, diseases, or improper handling during harvest and post-harvest processes (Purwati, 2010). Rhizomes are the most commonly used planting materials in ramie cultivation. However, their limited storage life presents a significant challenge, as the buds on the rhizomes begin to sprout within a short period, approximately 10 days (Nuraini et al., 2022; Mukherjee et al., 2018).

Other than rhizome, ramie can be propagated using other conventional methods, either vegetative such as air layering and stem cuttings or generative through seeds, as well as through in vitro (tissue culture) techniques. However, conventional propagation methods present several limitations. Air layering yields only a limited number of plantlets from a single mother plant (Lianah et al., 2022), while through stem cuttings often develop weak root systems (Kurniawan et al., 2021). Seed propagation may result in plants that differ from the parent due to genetic variation and also requires a longer germination period (Tanawani & Lengkong, 2020). Therefore, in vitro culture offers a promising alternative for the efficient and uniform propagation of ramie.

In vitro culture techniques involve the isolation of plant parts such as cells, protoplasts, tissues, or organs, which are then cultured in a sterile environment to regenerate into whole plants (Ziraluo, 2021). This method does not require large growing areas and enables the rapid production of a large number of plants (Sharma et al., 2015). Key factors influencing the success of in vitro culture include the type of explant used and the application of plant growth regulators (PGRs).

Various plant parts can be used as explants for in vitro culture, including shoot tips, nodes, leaves, and seeds (Deli et al., 2015). In the case of ramie, apical shoots are suitable explants for in vitro propagation. Apical shoots refer to the buds that grow at the tip of the plant. They are particularly effective as explants because cell division and the formation of new cells are highly active in the region (Putriana et al., 2019). Moreover, in vitro culture using apical shoots has the potential to produce virus-free plants (Hasan et al., 2019).

Plant growth regulators (PGRs) are used in in vitro culture to control and promote plant growth and development. Cytokinins, a group of PGRs, play a key role in cell division and enlargement, as well as in stimulating shoot formation (Hidayati, 2014). Thidiazuron (TDZ) is a widely used synthetic cytokinin, as a urea-type synthetic cytokinin, TDZ is not degraded by cytokinin oxidase, making it more stable within plant tissues (Guo et al., 2011). TDZ has proven effective for in vitro culture due to its ability to accelerate cell division and stimulate shoot multiplication (Saputro et al., 2020).

The application of TDZ in culture media using apical shoot explants is expected to enhance shoot multiplication in ramie plants. Therefore, research is needed to determine the optimal TDZ concentration for effective apical shoot multiplication in ramie.

Research Method

Place and Time of Research

The research was conducted from September 2022 to May 2023 at the Tissue Culture and Seed Technology Laboratory, Faculty of Agriculture, Universitas Padjadjaran, Jatinangor, Sumedang Regency, West Java, Indonesia.

Experimental Procedure

Equipment preparation and sterilization

All equipment was washed with antibacterial soap, rinsed thoroughly with running water, and then sterilized using an autoclave at a pressure of 17.5 psi and a constant temperature of 121°C for 20 minutes. Other tools such as petri dishes, scalpels, forceps, and jars were sterilized in a dry oven at 121°C for 1 hour before planting.

Preparation of TDZ stock solution

A 50 mL stock solution of thidiazuron was prepared at a concentration of 50 ppm. TDZ powder (Phytotech) was weighed at 0.0025 g, then dissolved in 0.1 mL of 1 N HCl. Sterile distilled water was added to bring the total volume to 50 mL, and the solution was homogenized thoroughly. The TDZ stock solution was transferred into a bottle, sealed tightly, and stored in a refrigerator.

Media preparation and sterilization

treatments, each with 120 mL. The media were prepared by dissolving 25.2 g sucrose in 200 mL sterile distilled water with a magnetic stirrer on a hot plate, followed by the addition of stock solutions (MS macronutrients, micronutrients, iron, and vitamins). The solution volume was adjusted to 350 mL, then divided into seven 250 mL beakers (50 mL each). TDZ was added to each beaker at concentrations of 0; 0.05; 0.075; 0.1; 0.125; 0.15; and 0.175 ppm, then topped up with sterile distilled water to 120 mL per treatment. The pH of each solution was adjusted to 5.6–5.8 using 1 N NaOH or HCl as needed. Next, 0.24 g agar gelzan was added to each treatment, stirred until dissolved, and heated to boiling. Media were poured into culture bottles (10 mL per bottle), sealed, and sterilized in an autoclave at 17.5 psi and 121°C for 15 minutes.

Explant Subculture

Subculturing was carried out in a laminar airflow cabinet (LAF). Each culture bottle contained one apical shoot explant. All equipment were sprayed with 95% alcohol, and tools such as scalpels, forceps, and scissors were dipped in 95% alcohol and flame-sterilized using a Bunsen burner before use. Explants were removed from the culture bottles and placed in petri dishes. The apical shoots were trimmed using a scalpel and transferred onto culture media according to the treatment, using sterile forceps. Each bottle was sealed with plastic, secured with a rubber band, and sealed with plastic wrap. The cultures were maintained in a growth room at approximately 22°C, 60% relative humidity, and under 16-hour daily illumination using fluorescent lights.

Experimental Design

The experiment was conducted using a Completely Randomized Design (CRD) consisting of seven treatments and four replications, resulting in a total of 28 experimental units. Each unit contained three explant samples. The treatments involved the application of Thidiazuron (TDZ) at different concentrations, as follows: control (0 ppm), 0.05 ppm, 0.075 ppm, 0.1 ppm, 0.125 ppm, 0.15 ppm, and 0.175 ppm.

Observation Parameter

Observations were conducted from 1 to 12 weeks after planting (WAP). The main non-destructive parameters observed included the time to shoot emergence (days after planting), number of shoots, culture height (cm), number of leaves, and visual appearance. In addition to these, dry weight was measured as a destructive parameter to assess plant biomass.

Data Analysis

Data analysis was performed using statistical methods. The significance of treatment effects was assessed through Analysis of Variance (ANOVA) at a 5% significance level, using the F-test. If significant differences were detected, post-hoc analysis was conducted using Duncan's Multiple Range Test (DMRT) at a 5% significance level to determine specific group differences. All analyses were performed using SPSS software version 25. For qualitative data, including visual observations, a descriptive analysis method was applied to summarize and interpret the results.

Results and Discussion

Effect of thidiazuron concentration on shoot emergence time

The average time to shoot emergence for each treatment is shown in Table 1. The emergence of shoots from explants is an indication of successful growth, as it proven cell division capable of producing new organs (Murgayanti et al., 2020). The results showed that the application of TDZ significantly enhanced shoot emergence in ramie. This may be due to the fact that cytokinin application at the given concentration enhances the concentration of endogenous hormones in the explant, then triggering cell division and differentiation (Praseptiana et al., 2017). Lestari (2018), also stated that the concentration of endogenous PGRs within cells increases when cytokinin is added to the culture medium, stimulating tissue growth and development. The condition of the explant and the use of the appropriate concentration of plant growth regulators (PGRs) will affect the time of shoot emergence (Yulia et al., 2020). Cytokinin PGRs, such as TDZ, can be used to accelerate shoot emergence in cultures. Shoot emergence is marked by the appearance of protrusions at the apical and axillary regions of the culture

In the control treatment, shoot emergence occurred 11 days after planting, which was significantly slower compared to the treatments with TDZ application. The control culture was able to produce shoots, even though at a slower rate. The presence of endogenous hormones in the explant allows for shoot emergence even without the application of exogenous PGRs (Praseptiana at al., 2017). However, it is assumed that the endogenous hormones in ramie cultures were not sufficient to accelerate shoot emergence, leading to slower growth compared to cultures supplemented with TDZ.

Table 1. Effect of thidiazuron concentration on average shoot emergence time

TDZ Concentration Treatment	Average shoot emergence time (DAP)
0.000 ppm (control)	11,00 [a]
0.050 ppm	7,83 [b]
0.075 ppm	6,75 [b]
0.100 ppm	6,00 [b]
0.125 ppm	6,00 [b]
0.150 ppm	6,00 [b]
0.175 ppm	7,00 [b]

Note: Numbers followed by the same letter in each column are not significantly different according to Duncan's Multiple Range Test at the 5% significance level.
- TDZ = Thidiazuron, DAP = Days After Planting, ppm = parts per million.

Effect of thidiazuron concentration on shoot number over time

The average number of shoots for each treatment is presented in Table 2. The number of shoots reflects the success of the multiplication stage, as a greater number of shoots enhances the potential for plant propagation. The results showed that from the fourth week after planting (WAP), the supplementation of TDZ in the culture medium significantly increased the number of apical shoots in ramie explants. In contrast, the control treatment produced only a single shoot throughout the entire observation period (up to 12 WAP). Shoot counts were performed by quantifying both apical and axillary shoots that developed from the explants.

The treatment with 0.075 ppm TDZ produced the highest number of shoots. This treatment showed a 12.02% increase compared to 0.05 ppm. Meanwhile, treatments with TDZ concentrations of 0.05, 0.075, 0.1, and 0.125 ppm did not significantly differ from one another. However, further increases in TDZ concentration resulted in a gradual decline in shoot numbers. For instance, shoot numbers slightly decreased when TDZ concentration was increased from 0.075 ppm to 0.1 ppm, and continued to decline with further increases up to 0.15 ppm. Interestingly, a minor increase was observed at 0.175 ppm, although this value remained lower than that observed at 0.075 ppm. These results suggest that 0.075 ppm is the optimal TDZ concentration for shoot proliferation in ramie under the conditions of this study.

Thidiazuron has been shown to be effective at relatively low concentrations, as it promotes high cell division activity at such levels (Huetteman & Preece, 1993). Previous studies have also confirmed that low concentrations of TDZ can effectively induce shoot proliferation (Sajid & Aftab, 2009). According to Huetteman and Preece (1993), TDZ concentrations below 0.2 ppm are generally more effective for shoot induction compared to other cytokinins type. Conversely, higher concentrations of TDZ may inhibit shoot development. This is likely due to structural changes in the adenine moiety of cytokinins, leading to forms that are less biologically active (Nielsen et al., 1995). Chang et al. (2003) also stated that excessive cytokinin levels reduce the rate of shoot proliferation and suppress overall growth. Supporting this, Murgayanti et al. (2023) reported that high concentrations of TDZ inhibited shoot formation in *Curcuma zedoaria*.

Table 2. Effect of thidiazuron concentration on average number of shoot over time (2, 4, 6, 8, 10, and 12 WAP)

TDZ Concentration Treatment	Average number of shoots					
	2 WAP	4 WAP	6 WAP	8 WAP	10 WAP	12 WAP
0.000 ppm (control)	1,00 a	1,00 b	1,00 c	1,00 c	1,00 c	1,00 c
0.050 ppm	1,42 a	1,58 a	1,67 ab	1,75 ab	2,00 ab	2,08 ab
0.075 ppm	1,58 a	1,67 a	2,00 a	2,17 a	2,33 a	2,33 a
0.100 ppm	1,58 a	1,67 a	1,75 a	1,75 ab	1,92 ab	2,17 ab
0.125 ppm	1,42 a	1,50 a	1,67 ab	1,75 ab	2,00 ab	2,00 ab
0.150 ppm	1,25 a	1,25 ab	1,17 c	1,33 bc	1,50 bc	1,58 b
0.175 ppm	1,25 a	1,25 ab	1,25 bc	1,42 bc	1,58 b	1,67 b

Note: Numbers followed by the same letter in each column are not significantly different according to Duncan's Multiple Range Test at the 5% significance level.
- TDZ = Thidiazuron, WAP = Weeks After Planting, ppm = parts per million.

Effect of thidiazuron concentration on plantlet height over time

The results showed that the average height did not differ significantly until 10 WAP, but became significantly different in 12 WAP (Table 3). The highest average culture height was observed in the control treatment, which was 2.32 cm. This may be due to the lower number of shoots in the control treatment compared to the others. The number of shoots directly impacts culture height. Height increases when fewer shoots are present, while growth is hindered when more shoots are produced. This occurs because the energy required for shoot elongation is redirected toward the formation of additional shoots, thus slowing overall height growth (Murgayanti et al., 2020).

Treatments with TDZ application showed lower culture heights compared to the control treatment. This can be indicate the fact that TDZ promotes shoot formation, which restricts height growth. Thidiazuron can stimulate the production of endogenous ethylene, which inhibits stem elongation. However, the short length of the shoots formed can be alleviated by transferring the planlets to a medium without TDZ supplementation (Murgayanti et al., 2023). In a study by Wang et al. (2007), ramie explants supplemented with 0.1 ppm TDZ exhibited short shoots, and transfer to a TDZ-free medium was necessary for shoot elongation.

Table 3. Effect of thidiazuron concentration on average plantlet height over time (2, 4, 6, 8, 10, and 12 WAP)

TDZ Concentration Treatment	Average plantlet height (cm)					
	2 WAP	4 WAP	6 WAP	8 WAP	10 WAP	12 WAP
0.000 ppm (control)	0,85 a	0,95 a	1,07 a	1,72 a	1,98 a	2,32 a
0.050 ppm	0,88 a	0,99 a	1,06 a	1,07 a	1,09 a	1,12 b
0.075 ppm	0,90 a	0,99 a	1,08 a	1,13 a	1,13 a	1,15 b
0.100 ppm	0,93 a	1,01 a	1,08 a	1,11 a	1,13 a	1,13 b
0.125 ppm	0,97 a	1,05 a	1,14 a	1,17 a	1,20 a	1,22 b
0.150 ppm	0,87 a	0,99 a	1,09 a	1,11 a	1,13 a	1,13 b
0.175 ppm	0,89 a	1,00 a	1,07 a	1,09 a	1,14 a	1,15 b

Note: Numbers followed by the same letter in each column are not significantly different according to Duncan's Multiple Range Test at the 5% significance level.
- TDZ = Thidiazuron, WAP = Weeks After Planting, ppm = parts per million.

Effect of thidiazuron concentration on number of leaves over time

The results showed significant differences in the average number of leaves at 6 WAP, but no significant differences were observed in the following weeks (Table 4). The inability of cytokinins to increase the number of leaves may be due to each explant having a specific concentration threshold for growth, with high concentrations of plant growth regulators (PGRs) potentially inhibiting explant growth (Popilla et al., 2021).

While there were no significant differences overall, increases in number of leaves were observed at lower TDZ concentrations. Specifically, treatment with 0.05 ppm TDZ resulted in a 29.07% increase compared to the control, while a further 17.72% increase was seen with 0.075 ppm TDZ. At higher concentrations, such as 0.15 ppm and 0.175 ppm TDZ, there was a 19.38% increase, but these higher concentrations

also led to a decline in leaf number at 0.1 ppm (decrease of 17.09%), 0.125 ppm (decrease of 6.46%), and 0.15 ppm (decrease of 15.13%).

The observed changes in leaf number are likely influenced by the number of shoots formed, as the number of leaves increases with the number of shoots (Demissie, 2013). Cytokinins promote cell division and enhance cell expansion during the proliferation and expansion stages of leaf cell development (Wu et al., 2021).

Table 4. Effect of thidiazuron concentration on average number of leaves over time (2, 4, 6, 8, 10, and 12 WAP)

TDZ Concentration Treatment	Average number of leaves					
	2 WAP	4 WAP	6 WAP	8 WAP	10 WAP	12 WAP
0.000 ppm (control)	0,42 a	0,67 a	1,08 c	1,92 a	2,42 a	2,58 a
0.050 ppm	0,67 a	1,42 a	2,08 ab	2,42 a	3,00 a	3,33 a
0.075 ppm	0,33 a	1,67 a	2,25 a	3,08 a	3,33 a	3,92 a
0.100 ppm	0,42 a	1,42 a	2,25 a	2,58 a	3,00 a	3,25 a
0.125 ppm	0,75 a	1,42 a	2,00 ab	2,08 a	2,30 a	3,04 a
0.150 ppm	0,25 a	0,83 a	1,25 bc	1,67 a	2,25 a	2,58 a
0.175 ppm	0,25 a	0,75 a	1,67 abc	2,25 a	2,58 a	3,08 a

Note: Numbers followed by the same letter in each column are not significantly different according to Duncan's Multiple Range Test at the 5% significance level.
- TDZ = Thidiazuron, WAP = Weeks After Planting, ppm = parts per million.

Effect of thidiazuron concentration on plantlet dry weight

The dry weight of the culture reflects the accumulation of carbohydrates, proteins, and organic materials derived from the media. The result showed that there were no significant differences in dry weight among the treatments (Table 5). In this experiment, no significant differences in dry weight were observed across treatments. This result is likely correlated with the culture height, leaf number, and callus formation, which also showed no significant differences. According to Yanto (2016), dry weight can be an indicator of vegetative organ growth, such as roots, leaves, and stems, which contribute to carbohydrate content.

Table 5. Effect of thidiazuron concentration on average plantlet dry weight

TDZ Concentration Treatment	Average plantlet dry weight (g)
0.000 ppm (control)	0,0266 a
0.050 ppm	0,0184 a
0.075 ppm	0,0076 a
0.100 ppm	0,0192 a
0.125 ppm	0,0542 a
0.150 ppm	0,0153 a
0.175 ppm	0,0143 a

Note: Numbers followed by the same letter in each column are not significantly different according to Duncan's Multiple Range Test at the 5% significance level.
- TDZ = Thidiazuron, ppm = parts per million.

Effect of thidiazuron concentration on plantlet visual appearance

The visual appearance of ramie explants was observed descriptively from 1 to 12 MST. The growth of explants was assessed based on shoot formation, leaf development, stem elongation, and root growth. The visual presentation of the explants over time is shown in Figure 1. Leaf appearance is shown in Figure 1. In the control treatment, the leaves were round, dark green, with serrated edges. In contrast, leaves formed in treatments supplemented with TDZ were lighter in color compared to the control. As the concentration of TDZ increased, the leaf size tended to decrease (Saputro et al., 2020). In treatment TDZ 0.175 ppm, the leaves were oval-shaped, unlike the rounded leaves observed in other treatments. This could be attributed to the high concentration of TDZ. High TDZ concentrations may lead to abnormal leaf morphology (Lu, 1993).

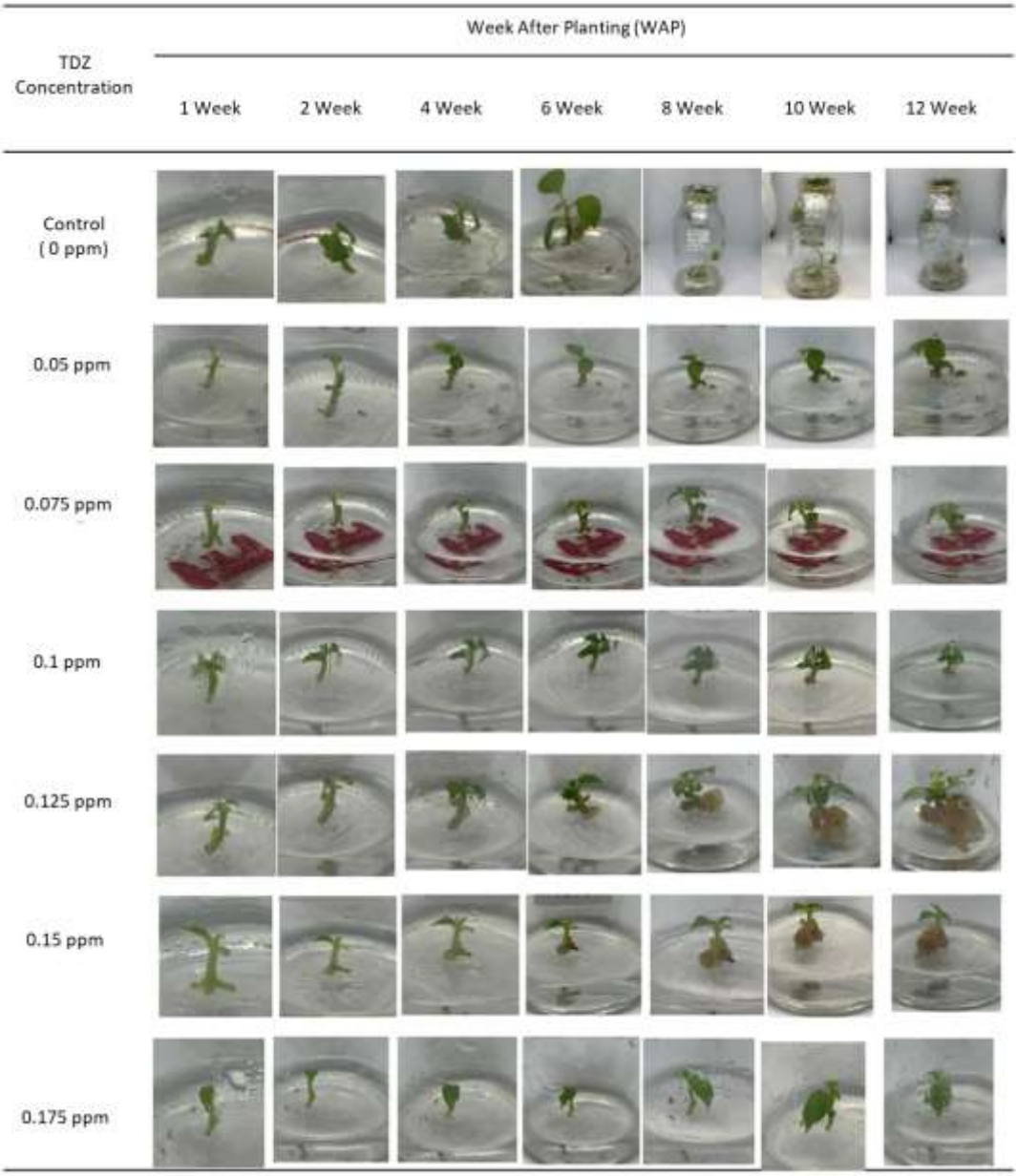

Figure 1. Visual appearance of plantlets over time (1, 2, 4, 6, 8, 10, and 12 WAP)

The percentage of rooted explants in this experiment was 13.09%, with 11 out of 84 explants forming roots (Figure 2). Rooting was observed only in the control and treatment with TDZ 0.1 ppm. The highest number of rooted explants was found in control treatment with 10 rooted explants (83%), while treatment D had only 1 rooted explant (8%). Root formation is influenced by auxins and cytokinins. Cytokinins can inhibit the action of endogenous auxins during root formation (Su et al., 2011). The high number of rooted explants in the control treatment is likely due to the promotion of root formation by endogenous auxins, which regulate root growth (Saini et al., 2013).

In treatments with TDZ, nearly all explants failed to form roots. This could be attributed to TDZ inhibiting root formation in the culture medium. Thidiazuron is a cytokinin that inhibits root development (Lu, 1993). Root inhibition is a stress response in cultures. According to Murthy et al. (1998), TDZ application causes the accumulation of ions and metabolites such as proline and abscisic acid, which induce stress and inhibit root formation.

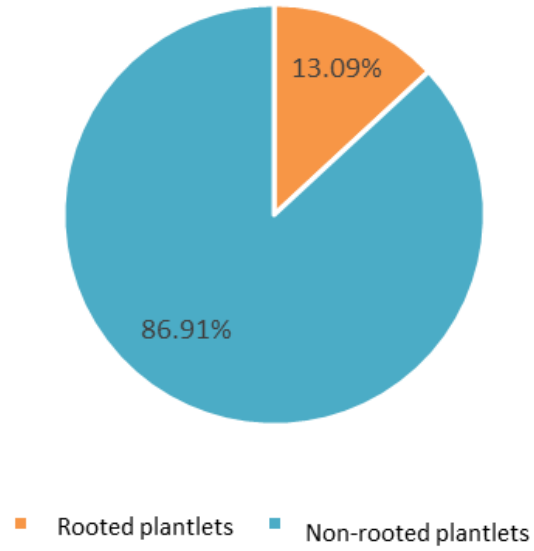

Figure 2. Percentage of plantlets with roots

Conclusion

The conclusion of this study is: 1). The application of various concentrations of TDZ has an effect on increasing apical shoot multiplication, as seen from the time of shoot emergence and the number of shoots in Wonosobo clone ramie; 2). The use of 0.075 ppm TDZ increases apical shoot multiplication, as indicated by the number of shoots formed. The control treatment resulted in a higher culture height compared to the TDZ treatments.

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