Utilization of Tempeh Extract as an Organic Supplement Alternative for Banana Tissue Culture

Alexander Willy Dimaswarabrata, Anastasia Tatik Hartanti,

Listya Utami Karmawan*

Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia, Jakarta 12930, Indonesia
**Corresponding author. Email:* listya.utami@atmajaya.ac.id

ABSTRACT

The addition of organic materials to tissue culture media has been known to have a positive impact on plant growth. However, a tissue culture medium utilizing organic supplements originating from Indonesia as its specialty, such as tempeh, has not been discovered. This study aims to determine the effect of adding tempeh extract to the tissue culture medium on the growth of Pisang Ambon Kuning. The media was supplemented with tempeh extract at concentrations of 5%, 15%, 25%, and 50% v/v. The banana plantlets used were 8 months old. In the initiation stage, explants inoculated in media supplemented with tempeh extract did not show any shoot growth. Similar results were shown in the subculture stage where the tempeh extract treatment demonstrated poor growth compared to the control treatment. Despite its potential to support growth, tempeh extract can hinder culture growth. The suboptimal growth results are suspected to be a stress response to the high copper content in tempeh extract, which is approximately 0.24 ± 0.02 mg.L^{-1}.

Keywords: *banana tissue culture, growth, medium, organic supplementation, tempeh.*

1. INTRODUCTION

The Banana (*Musa* sp.) is a fruit tree classified under the Musaceae family, which is extensively cultivated in tropical regions such as Southeast Asia and Africa [1]. Indonesia is one of the nations that produce the most bananas globally, with 8.741.147 tons of fruits produced [2]. One of the many tropical fruit cultivars that are extensively grown in Indonesia is Musa acuminata cv. Pisang Ambon Kuning AAA group. However, the main obstacle to the production of Pisang Ambon Kuning is the limited availability of genetically uniform superior banana seedlings, which has resulted in many farmers being unable to cultivate them in large quantities [3].

The tissue culture of bananas has been widely conducted to achieve high multiplication rates, virus-free status, and genetic uniformity, resulting in a higher banana yield [4]. In tissue culture, the media used for explant growth contains vitamins vital in culture growth, such as accelerating cell division in root meristems and serving as coenzymes in carbohydrate energy production [5]. Adding organic ingredients to tissue culture media has been known to positively impact plant growth, such as coconut water, yeast extract, and potato extract [6]. Until now, a tissue culture medium utilizing organic supplements derived from Indonesia as its specialty, such as tempeh, has yet to be discovered.

Tempeh is a traditional food made from fermented soybeans using Rhizopus oligosporus. Tempeh has been recognized by the Indonesian community as one of the highly nutritious foods [7]. Tempeh contains various amino acids and is rich in vitamin B. In 100 grams of tempeh, there are 18 types of essential amino acids, 0.08 mg of Vitamin B1, 0.36 mg of Vitamin B2, 2.64 mg of Vitamin B3, 0.28 mg of Vitamin B5, 0.22 mg of Vitamin B6, and 0.08 µg of Vitamin B12 [8]. Knowing this, tempeh extract has the potential to be added to tissue culture medium due to its amino acids and B vitamins content. This study aims to determine the effect of adding tempeh extract to tissue culture media on the growth of Pisang Ambon Kuning.

2. MATERIALS AND METHODS

This research was done at the Tissue Culture Laboratory, Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia, from October 2022 to July 2023. The primary materials for this research consist of Pisang Amon Kuning planlet that were obtained from PT D aFa Teknoagro Mandiri Bogor, Tempeh that was

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obtained from Pasar Modern Intermoda BSD, Murashige & Skoog (MS) media (Caisson MSP09-50LT), 6-Benzylaminopurine (BAP) (#B3408-5G) and 1-Naphthaleneacetic Acid (NAA) (#N0640-25g) as growth regulators. The main steps in carrying out this research are tempeh extraction, preparation of tempeh extract medium, initiation, subculture, observation, and analysis of macronutrient, micronutrient, and B vitamins content in tempeh extract.

2.1 Tempeh extraction

Tempeh extraction was done by weighing tempeh and distilled water with a ratio of 1:1 w/v. It is then finely ground in a food processor, and the solution is filtered through filter paper [9]. Afterward, it is stored at a cold temperature of -20°C in a sealed container. The extracted tempeh will be utilized throughout the research period.

2.2 Supplementation of tempeh extract

The media used is MS [10] medium, with a sucrose content of 3% w/v and agar of 1% w/v in the medium. Then, the media was supplemented with tempeh extract as a treatment, with different concentrations of 5%, 15%, 25%, and 50% v/v, and the pH was adjusted to 5.8. Thereafter, it was sterilized through autoclaving at 121°C for 15 min. The following is the experimental design of the media treatment used.

Table 1 Experimental design of media used

	Tempeh Extract				
Media	**0%**	**5%**	**15%**	**25%**	**50%**
MS	Control	ET 5%	ET 15%	ET 25%	ET 50%

ET: Tempeh Extract

Initiation media used were supplemented with growth regulators BAP at 2 ppm and NAA at 0.5 ppm, whereas subculture media used was MS medium without the addition of any growth regulators. The addition of growth regulators is given due to their capability to induce shoot growth [11].

2.3 Effect of tempeh extract on initiation of banana in vitro culture

Initiation of Pisang Ambon Kuning was done using shoot tips from young offspring as explant sources. The explants are sterilized according to Mekonen et al. [12], with modification. In the initiation stage, the explants used are derived from shoots of banana seedlings. The explants are then trimmed to a size of 3-4 cm, removing the stem, bulb, and roots. Subsequently, the explants are soaked with running water and liquid detergent for 30 min. Afterward, the explants are soaked in a solution of fungicide and bactericide with a concentration of 2g/L for 1 h. Then, the explants are soaked in sodium hypochlorite (NaOCl) solution with concentrations of 10%, 5%, and 1% v/v, respectively for 10 min, 5 min, and 1 min. After that, the explants are rinsed with sterile distilled water three times. After completing the surface sterilization stage, the explants are trimmed to approximately 2 cm in size and inoculated into the media.

2.4 Effect of tempeh extract on banana plantlet growth during subculture

Alongside the initiation, subculture was performed using a tissue culture plantlet of banana plants obtained from PT DaFa Teknoagro Mandiri Bogor. In the beginning, the utilized plantlets were 8 months old, approximately 3.2 – 6.7 cm in height, and had a total leaf count of approximately 2-5 leaves. Subculture was done by trimming the explant's roots and brown leaves and then transferring them to the media according to the treatment.

2.5 Statistical analysis on banana growth parameter

Observations were conducted every 4 weeks and documented once every 1 week, including measuring the number of shoots produced in the initiation stage. In the subculture stage, measurements were taken for changes in biomass, height, and total leaf count. To measure all changes in the subculture stage is done by subtracting the final measurement from the initial measurement (1).

$$Changes\ (\Delta M) = Final\ measurement\ (Mtn) - Initial\ measurement\ (Mt0) \quad (1)$$

The data was processed using IBM® SPSS® for Windows software (Version 27). The normality of the data distribution was analyzed using the Kolmogorov-Smirnov test, and the homogeneity of variance between groups was analyzed using the Levene test. Data that passed the normality and homogeneity tests were then analyzed using one-way ANOVA, followed by the Scheffe test as a post-hoc analysis. On the other hand, data that do not follow a normal distribution and/or have significantly different variances will be analyzed using the Kruskal-Wallis test, followed by Stepwise Stepdown Multiple Comparisons as post-hoc analysis.

2.6 Analysis of macronutrients, micronutrients, and B vitamins in tempeh extract

Analysis of macro-, micronutrients, and B vitamins in tempeh extract was done by PT Saraswanti Indo Genetech to support the resulting data.

3. RESULTS

3.1 Explants' condition after initiation

Explants inoculated in media without the addition of tempeh extract have shown shoot growth, while other explants did not show any shoot growth (Figure 1). none of the other treatments resulted in any observable shoot growth. This implies that the applied treatments had inhibitory effects on shoot development.

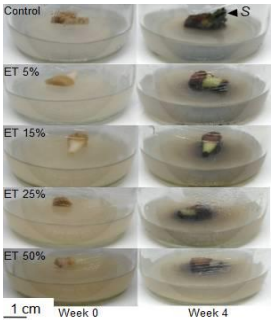

Figure 1 Explants condition 4 weeks after initiation, tempeh extract (ET), arrow indicated Shoot growth(S)

Some explants have been shown to be contaminated or browning 12 days after initiation (Figure 2). The contamination rate of this experiment is 20%, on the other hand, the browning rate of this experiment is 13.34%. The contamination that occurred at this stage is suspected to be from inadequate surface sterilization rather than the media used. The types of contaminants that were predominantly observed included bacterial contamination, with an additional occurrence of fungal contamination which grew around the explants.

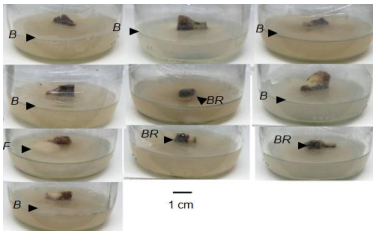

Figure 2 Explants that undergoes browning or contaminated 12 days after initiation. Arrow indicated Fungal contamination (F), bacterial contamination (B), and browning (BR)

The observed contamination during the initiation stage implies a reduced availability of banana explants suitable for further processing. To address this, we are using banana explants that have been previously subcultured, ensuring that the minimum sample quantity still aligns with the Federer formula calculation standards. The same applies to explants experiencing browning. Nevertheless, this has no impact on the subculture stage, as the quantity of samples used adheres to the Federer formula.

3.2 Planlets condition after subculture

The inoculated plantlets (Figure 3) on tempeh extract media showed symptoms of necrosis 4 weeks after subculture, while the control group did not show any necrosis symptoms.

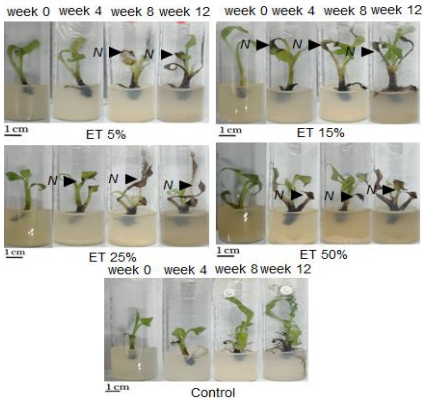

Figure 3 Planlet condition 12 weeks after subculture. Tempeh extract (ET), arrow indicated symptoms of necrosis (N)

3.3 Observation of the subculture stage

All changes in Figures 4, 5, and 6 were calculated against data in week 0. The biomass change (Figure 4) in the fourth week was not significantly different (p: 0.05, one-way ANOVA followed by Scheffé's post-hoc test).

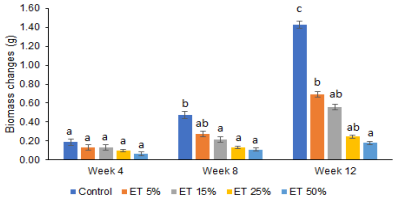

Figure 4 The biomass changes in the subculture stage. Different letters within the same time point indicate a significant difference with a $p < 0.05$. Data was presented in the form of the average ± SD

The height change (Figure 5) in the fourth week was not significantly different (p: 0.77, one-way ANOVA followed by Scheffé's post-hoc test). Starting from the eighth week, there was a significant difference (p: 0.00, one-way ANOVA followed by Scheffé's post-hoc test), and also in the twelfth week, there was a significant difference (p: 0.00, Kruskal-Wallis followed by Stepwise Stepdown Multiple Comparison as post-hoc test).

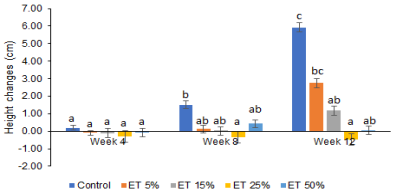

Figure 5 The height changes in the subculture stage. Different letters within the same time point indicate a significant difference with a $p < 0.05$. Data was presented in the form of the average ± SD

The change in total leaf count (Figure 6) in the fourth week was not significantly different (p: 0.17, Kruskal-Wallis followed by Stepwise Stepdown Multiple Comparison as a post-hoc test). Starting from the eighth week, there was a significant difference (p: 0.04, Kruskal-Wallis followed by Stepwise Stepdown Multiple Comparison as a post-hoc test), and also in the twelfth week, there was a significant difference (p: 0.01, Kruskal-Wallis followed by Stepwise Stepdown Multiple Comparison as a post-hoc test). The most optimal growth for the banana culture was observed at the control treatment, which shows the highest biomass and height changes compared to other treatments. Although the control treatment showed a lesser change in the total number of leaves (Figure 6) compared to ET 5% 12 weeks after to subculture, it shows no significant difference.

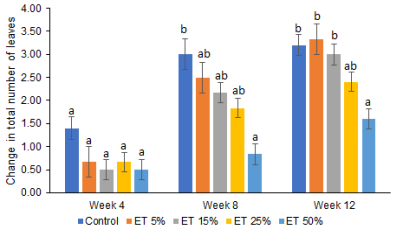

Figure 6 Change in the total leaf count in the subculture stage. Different letters within the same time point indicate a significant difference with a $p < 0.05$. Data was presented in the form of the average ± SD

3.4 Analysis of macro-, micronutrients, and B Vitamins of tempeh extract

The macro- and micronutrient content in tempeh extract, when compared to the macro and micronutrients present in MS media, was known that there were elements concentrations exceeding those in MS media, such as phosphorus with a content ranging from 1.09 - 1.90 times higher in media treated with ET, and copper with a content ranging from 1.48 - 5.80 times higher in media treated with ET (Table 2).

Table 2 Content of macro- and micronutrients in tempeh extract compared to Murashige & Skoog media

Element	Tempeh extract (ppm)	MS Media (ppm)	MS + ET 5% (ppm)	MS + ET 15% (ppm)	MS + ET 25% (ppm)	MS + ET 50% (ppm)
Nitrogen (N)	80.00 ± 0.00	3550	3554.00 (1.00×)	3562.00 (1.00×)	3570.00 (1.01×)	3590.00 (1.01×)
Phosphorus (P)	305.37 ± 1.05	170	185.27 (1.09×)	215.81 (1.27×)	246.34 (1.45×)	322.69 (1.90×)
Potassium (K)	96.60 ± 0.57	2070	2070.05 (1.00×)	2070.16 (1.00×)	2070.26 (1.00×)	2070.53 (1.00×)
Calsium (Ca)	109.00 ± 0.99	440	445.45 (1.01×)	456.35 (1.04×)	467.25 (1.06×)	494.50 (1.12×)
Magnesium (Mg)	42.85 ± 0.07	370	372.14 (1.01×)	376.43 (1.02×)	380.71 (1.03×)	391.43 (1.06×)
Sulphur (S)	32.40 ± 0.01	370	371.62 (1.00×)	374.86 (1.01×)	378.10 (1.02×)	386.20 (1.04×)
Manganese (Mn)	1.83 ± 0.01	22.3	22.39 (1.00×)	22.57 (1.01×)	22.76 (1.02×)	23.22 (1.04×)
Zinc (Zn)	1.90 ± 0.00	8.6	8.70 (1.01×)	8.89 (1.03×)	9.08 (1.06×)	9.55 (1.11×)
Molybdenum (Mo)	Not detected	0.25	0.25 (1.00×)	0.25 (1.00×)	0.25 (1.00×)	0.25 (1.00×)
Iron (Fe)	0.80 ± 0.00	27.8	27.84 (1.00×)	27.92 (1.00×)	28.00 (1.01×)	28.20 (1.01×)
Cobalt (Co)	Not detected	0.025	0.03 (1.00×)	0.03 (1.00×)	0.03 (1.00×)	0.03 (1.00×)
Boron (B)	Not detected	6.2	6.20 (1.00×)	6.20 (1.00×)	6.20 (1.00×)	6.20 (1.00×)
Copper (Cu)	0.24 ± 0.02	0.025	0.04 (1.48×)	0.06 (2.44×)	0.09 (3.40×)	0.15 (5.80×)

Note: The numbers inside the bracket show the comparison of the element content in the media with the addition of tempeh extract compared to the MS0 media.

The content of the B3 vitamin in tempeh extract was found to be 4.6 ppm (Table 3), however neither the B1 vitamin nor B6 vitamin which has a crucial role in various metabolic processes within plant cells was not found in the tempeh extract used for this research.

Table 3 Content of B vitamins in tempeh extract

Vitamins	Content in tempeh extract (ppm)
B1 (Thiamine)	Not detected
B6 (Pyridoxin)	Not detected
B3 (Niacin)	4.6

B1 and B6 vitamins are known to be light-sensitive [13, 14]. The absence of B1 and B6 vitamin content in tempeh extract is suspected to be due to photodegradation. This is attributed to the tempeh packaging, which is made from transparent plastic, allowing light to enter.

4. DISCUSSION

The results obtained from this study provide valuable insights into the effect of tempeh extract on banana culture growth. In the initiation stage, some explants experienced browning with a rate of 13.34% and contamination with a rate of 20%. In addition, the browning that occurs at the initiation stage was thought to be due to stress from the addition of tempeh extract, as observed results showed (Figure 1) explants that were inoculated into tempeh extract media did not promote any shoot growth. This was due to excess copper in plants lead to significant changes in nitrogen (N) metabolism, including of the uptake and transportation of nitrate and the regulation of nitrate reductase (NR) and nitrite reductase (NiR) [15]. The NR plays an important role in nitrate assimilation, it converts nitrate into NO2- which then will be converted to NH4-N by NiR, which will be used for another metabolism. NR has two active sites, which at the two active sites have an essential Cys residue each. Cu binding to the Cys residues of these active sites would inhibit the activity of NR [16]. This lead to an impeded N metabolism which eventually lead to the inhibition of shoot growth. On the other hand, at the subculture stage, it was found that plantlets that were inoculated into tempeh extract media have shown poor growth (Figure 3). This was further supported by the result in the subculture stage where the most optimal growth results were obtained in the control treatment which showed a significant difference compared to the treatment with tempeh extract at week 12 (Figure 4,5, and 6). This was due to the MS media used already contains complete nutrients and vitamins, ensuring that the nutritional requirements of the plantlets are adequately met without the need for additional tempe extract [17, 18]. It was also evident that the higher the concentration of tempeh extract applied, the more inhibited the growth of the culture. This suggests that the addition of tempeh extract may inhibit the growth of banana culture. The likely explanation for this decrease in growth can be attributed to the high copper content present in the tempeh extract, which was measured to be 0.24 ± 0.02 $mg.L^{-1}$.

Copper is a crucial trace element for plants, involved in the processes of photosynthetic electron transport, responding to oxidative stress, regulating cell wall metabolism, and mediating hormone signaling [19]. Cu ions also have a role as cofactors in many enzymes such as superoxide dismutase, plastocyanin, cytochrome c oxidase, ascorbate oxidase, and diamine oxidase [20]. However, when copper is present at higher concentrations, it can become toxic to plants causing symptoms such as necrosis, stunting, inhibition of root growth, and chlorosis [21]. The phenomenon of copper toxicity stress has been previously documented in various tissue cultures. For example, in banana tissue culture, increased copper concentrations have been shown to cause a significant decrease in the number and length of shoots [22]. Similarly, studies conducted on maize have indicated that high copper concentrations reduce root length, shoot growth, and leaf size [23]. Additionally, research on *Vitis vinifera* tissue culture exposed to copper has reported a reduction in root and leaf growth [24]. These studies align with the findings of the present study, further supporting the conclusion that the observed browning in the initiation stage and the poor results in the subculture stage can be attributed to copper toxicity stress induced by the tempeh extract treatment.

There were many mechanisms of copper toxicity in plants. However, the effect of copper on photosystem II (PSII) has a major toxic effect on plants. Copper can cause an inhibition of the oxygen-evolving complex (OEC), which catalyses the oxidation of water molecules during light-dependent reactions of photosynthesis, affecting the Mn-cluster and leading to a decrease in oxygen production and overall PSII efficiency [25]. Copper can also disrupt the electron transfer process within PSII, this causes a decrease in the production of ATP, which is essential for synthesis of carbohydrates during photosynthesis [26]. Copper can produce reactive oxygen species (ROS), which causes oxidative stress in plants, by facilitating the formation of hydroxyl radicals (OH^-) through a non-enzymatic chemical reaction involving superoxide (O_2^-) and hydrogen peroxide (H_2O_2) [27].

Although the supplementation of tempeh extract led to poor growth of the banana culture, there was an interesting observation regarding the total leaf count in the 5% ET treatment at week 12 (Figure 6), where an increase in the total leaf count compared to the control treatment was observed. However, this increase was not statistically significant (P: 0.27, Kruskal-Wallis followed by Stepwise Stepdown Multiple Comparison as post-hoc test). The exact influence of this increase in total leaf count was still unknown and requires further investigation.

5. CONCLUSION

This study indicates that the addition of tempeh extract negatively affects the growth of banana tissue cultures. Limited shoot growth was demonstrated at the initiation stage, as well as poor growth was observed at the subculture stage due to the addition of tempeh extract. The suboptimal growth results were suspected to be a stress response to the high copper content in the tempeh extract, which was measured at approximately 0.24 $mg.L^{-1}$. Hence, tempeh extract is not recommended to be

used as a supplement, despite its potential vitamin content to support growth.

AUTHORS' CONTRIBUTIONS

AWD, ATH, and LUK designed the study. AWD carried out the laboratory work. AWD, ATH, and LUK analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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