ISSN: 1412-4718, eISSN: 2581-138x

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Differential effects of thiamine, BAP, and thidiazuron combinations on shoot multiplication and phenolic-flavonoid accumulation in pineapple in vitro cultures

Abstract. The demand for high-quality pineapple (*Ananas comosus* (L.) Merr.) planting materials continues to increase, while conventional propagation methods are limited in multiplication rate, uniformity, and production efficiency. In vitro culture offers an alternative approach for rapid and large-scale seedling production. However, information on the effects of thiamine and cytokinin combinations on both shoot multiplication and phenolic-flavonoid accumulation in pineapple in vitro remains limited. This study evaluated ten defined combinations of thiamine and cytokinin for shoot multiplication and total phenolic-flavonoid contents of pineapple explants. The experiment employed a completely randomized design with 10 treatment combinations of thiamine (0.1 and 0.4 mg/L), cytokinin TDZ (0.01 and 0.1 mg/L) and BAP (1 and 2 mg/L) with three replications. The combination of 0.1 mg/L thiamine and 1 mg/L BAP produced the highest number of shoots (3.33 shoots) and leaves (17.56 leaves). In contrast, 0.4 mg/L thiamine combined with 0.1 mg/L resulted in the earliest shoot emergence (54.67 day after subculture) and the highest total phenolic content (13.62 mg GAE/g DW) and total flavonoid content (15.41 mg QE/g DW). No visible browning symptoms were observed in this treatment. These findings demonstrate that the optimal culture conditions differed according to the intended objective, 0.1 mg/L thiamine with 1 mg/L BAP favored shoot proliferation and leaf development, whereas 0.4 mg/L thiamine with 0.1 mg/L TDZ favored phenolic and flavonoid accumulation.

Keywords: *Ananas comosus* (L.) Merr · Browning symptoms · Cytokinin · Secondary metabolite · Shoot proliferation

Submitted: 4 June 2026, Accepted: 11 August 2026, Published: 14 August 2026
DOI: https://doi.org/10.24198/kultivasi.v25i2.70924

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Rachman DS, Suminar E, Nuraini A, Wicaksono FY, Amien S, Mubarok S, Hermanto. 2026. Differential effects of thiamine, BAP, and thidiazuron combinations on shoot multiplication and phenolic-flavonoid accumulation in pineapple in vitro cultures. Jurnal Kultivasi, 25 (2): 141-152.

Jurnal Kultivasi Vol. 25 (2) August 2026
ISSN: 1412-4718, eISSN: 2581-138x

Introduction

Pineapple (*Ananas comosus* (L.) Merr.) is an economically important tropical fruit in the Bromeliaceae family, valued for its nutritional and functional properties. The fruit contains carbohydrates, dietary fiber, organic acids, minerals, vitamins, and bioactive compounds, particularly phenolics and flavonoids, which contribute to its antioxidant activity (de Ancos et al., 2017; Mohd Ali et al., 2020; Bruce & Boateng, 2025). In Indonesia, pineapple is an important horticultural commodity, with production showing an average annual growth of 13.42% during 2000-2022 (Pusat Data & Sistem Informasi Pertanian, 2022). However, national pineapple production has fluctuated in recent years, as reflected in production data for 2020-2024 Badan Pusat Statistik (2024). The growing demand for pineapple products has consequently increased the need for high-quality and uniform planting materials to support sustainable production.

Pineapple is conventionally propagated vegetatively using suckers, slips, and crowns (Megersa, 2017). However, these methods have relatively low multiplication rates (1:10-1:15 per years), require considerable time for large-scale propagation, and may be associated with pathogen transmission and limited uniformity of planting materials (Patil et al., 2021). Plant tissue culture provides an alternative approach for the rapid multiplication of uniform and relatively pathogen-free planting materials while reducing seasonal limitations (Chandran et al., 2020; Fitramala et al., 2017; Ziraluo, 2021). The success of in vitro propagation is strongly influenced by the composition of the culture medium, particularly vitamins and plant growth regulators. Thiamine (vitamin B1) is known to play a role in DNA and RNA biosynthesis and carbohydrate metabolism, which supports cell division and tissue viability (Fitzpatrick & Chapman, 2020). Increasing thiamine concentrations has also been reported to suppress browning symptoms caused by phenolic oxidation in plant cultures susceptible to oxidative stress, including pineapple (Taha et al., 2022). On the other side, cytokinin PGRs such as 6-benzylaminopurine (BAP) and thidiazuron (TDZ) play an important role in the induction and proliferation of shoots through the stimulation of cell division (Dinani et al., 2018; Aulia et al., 2020).

In addition to morphological responses, in vitro culture conditions can influence the accumulation of secondary metabolites, particularly phenolic and flavonoid compounds, which are associated with antioxidant activity and plant defense responses (D'Amelia et al., 2018; Li et al., 2020). The combined effects of thiamine availability and cytokinin supplementation may therefore be reflected in both propagation performance and biochemical characteristics. Previous studies on pineapple and other plant species have predominantly focused on optimizing cytokinin type and concentration to improve shoot induction and proliferation. However, information on the combined application of thiamine with BAP or TDZ and its simultaneous effects on propagation efficiency and phenolic and flavonoid accumulation in pineapple explants remains limited. Therefore, evaluating morphological and biochemical parameters concurrently may provide a more comprehensive characterization of pineapple explants exposed to different thiamine-cytokinin combinations.

This study evaluated ten defined combinations of thiamine with BAP or TDZ in pineapple explants cultured in vitro. The variables measured included shoot height, shoot emergence time, number of shoots, number of leaves, fresh weight, dry weight, total phenolic content, and total flavonoid content. The study aimed to simultaneously characterize the morphological and biochemical responses of pineapple explants to different thiamine-cytokinin combinations and to identify treatment combinations associated with differences in shoot multiplications and phenolic-flavonoid accumulation.

Materials and Methods

Research Time and Location. The experiment was conducted during the period of November 2025 to February 2026 at the Plant Tissue Culture Laboratory, Seed Technology, Faculty of Agriculture, Universitas Padjadjaran, Jatinangor.

Tools and Materials. The materials used in this experiment included pineapple shoots derived from plantlets of cv. Smooth Cayenne originating from Subang, West Java, Indonesia, which had been previously established through in vitro culture and maintained as a collection at the Plant Tissue Culture Laboratory. Other materials included Murashige & Skoog (MS) medium, thidiazuron (TDZ), thiamine, 6-

benzylaminopurine (BAP), sugar, agar, distilled water, HCl, NaOH, 70% alcohol, 80% methanol, Folin-Ciocalteu reagent, gallic acid and quercetin standards, 7.5% Na_2CO_3, 10% $AlCl_3$, and 1M CH_3COOK.

The equipment used in this experiment include culture bottles, beaker glass, hot plate magnetic stirrer, analytical balance, pH meter, volume pipette, autoclave, Laminar Air Flow (LAF); culture rack equipped with LED lights, a spirit lamp, tweezers, scalpel, petri dish, milimeter block paper, Whatman filter paper, test tubes, mortar, vortex, cuvette, and UV-spectrophotometer.

Experimental Design. The experiment was conducted using a Completely Randomized Design (CRD) consisting of 10 define treatment combinations. Each treatment combination consisted of three replications, with three culture bottles containing one explant per replicate, resulting in nine experimental units per treatment and a total of 90 culture bottles. The treatment combinations consisted of A (control: 0,1 mg/L Thiamine without additional plant growth regulators), B (0.1 mg/L Thiamine+0.01 mg/L TDZ), C (0.1 mg/L Thiamine+0.1 mg/L TDZ), D (0.1 mg/L Thiamine+1 mg/L BAP), E (0.1 mg/L Thiamine+2 mg/L BAP), F (0.4 mg/L Thiamine), G (0.4 mg/L Thiamine+0.01 mg/L TDZ), H (0.4 mg/L Thiamine+0.1 mg/L TDZ), I (0.4 mg/L Thiamine+1 mg/L BAP), and J (0.4 mg/L Thiamine+2 mg/L BAP). The experiment consisted of two stages: (1) shoot multiplication and observation for 12 weeks after subculture (WAS), and (2) analysis of secondary metabolites, specifically phenolics and flavonoids).

First Phase Experiment. Pineapple shoots in vitro originating from Subang obtained from the Plant Tissue Culture Laboratory collection were used as explants. Shoots were removed from the cultured bottles, cleaned of any remaining agar, cut using a scalpel, their initial length measured using milimeter block paper, and planted into the treatment medium with one explant per bottle under aseptic condition in LAF. The explants were incubated for 12 weeks at room temperature with a lighting period of 16 hours per day. Observation variables consisted of shoot height increase (cm), time of new shoot emergence (day after subculture), number of shoots, number of leaves, and fresh and dry weight of shoots (g).

Second Phase Experiment. Secondary metabolite analysis began by drying pineapple shoots that had been incubated in vitro for 12 weeks in an oven at 40° C for 72 hours. The dried pineapple shoots were then ground using a mortar to obtain a powder. A total of 0.05 g of dried sample powder was macerated in 10 mL of 80% methanol for 48 hours. The macerated extract was subsequently filtered using Whatman filter paper to obtain the filtrate for TPC and TFC analyses

Analysis of Total Phenolic Content (TPC) was carried out following the Folin-Ciocalteu methods according to Muhammad et al. (2020) with modifications. A total of 100 µL extract, 2.7 mL of distilled water, and 200 µL Folin-Ciocalteu reagent were mixed into a test tube, incubated in the dark at room temperature for 5 minutes, then homogenized using a vortex for 10 seconds. 2 mL of NA_2CO_3 was added and incubated again for 30 minutes. Absorbance was measured at 760 nm using a UV-Vis spectrophotometer with three replications. Gallic acid at concentrations of 25, 50, 100, 200, and 400 ppm was used to construct the calibration curve, resulting in the equation y = 0.0016x + 0.0236 with a coefficient of determination (R^2) of 0.992. The TPC was calculated based on the calibration equation and expressed as mg of gallic acid equivalents per g of dry weight (mg GAE/g DW).

Analysis of Total Flavonoid Content (TFC) was carried out following the López-Hidalgo *et al*. (2021) with modifications. A total of 100 µL extract, 1.5 mL of 80% methanol, 100 µL $AlCl_3$, 100 µL CH_3COOK 1 M, and 3.2 mL of distilled water were mixed into a test tube, then incubated in the dark at room temperature for 40 minutes. Absorbance was measured at 415 nm using a UV-Vis spectrophotometer with three replications. Quercetin at concentrations of 25, 50, 100, 200, and 400 ppm was used to construct the calibration curve, resulting in the equation y = 0.0014x – 0.0239 with a coefficient of determination (R^2) of 0.9994. The TFC was calculated based on the calibration equation and expressed as mg of quercetin equivalents per g of dry weight (mg QE/g DW).

Statistical Analysis. Data were analyzed using analysis of variance (ANOVA) with software SmartStat XL version 3.6.5.4. Prior to ANOVA, the assumptions of normality and homogeneity of variance were assessed using the Shapiro-Wilk and Levene's tests, respectively. When ANOVA indicated a significant treatment effect, treatment means were further compared using the Scott-Knott test at the 5% significance level. The Scott-Knott test was used to classify treatment means into statistically distinct groups,

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ISSN: 1412-4718, eISSN: 2581-138x

facilitating the identification of groups with similar responses.

For variables assessed at 4, 8, 12 weeks after subculture (WAS), data were analyzed separately for each observation time. This approach was used to evaluate treatment effects at each stage of culture development and determine when differences among treatment became evident. Statistical comparisons were therefore performed independently at each observation time rather than modelling the temporal dependence among repeated observations.

Results and Discussion

Shoot Height Increase. Shoot height reflects the potential for morphogenesis and the explants ability to adapt to environmental conditions and development limitations. According to Mawaddah et al. (2021), shoot elongation is the result of cell division and elongation activity that occurs in stem segment and apical meristems, resulting in increased plant height. Table 1 shows that the combination of thiamine and cytokinin at various concentrations significantly affected pineapple shoot height increase. In the initial phase (4 WAS), the response to shoot height increase was still low and tented to be unstable between treatments. Differences began to appear at 8 and 12 weeks after subculture, where treatment G (0.4 mg/L Thiamine+0.01 mg/L TDZ) produced the highest shoot increase and indicated that the explants had entered the active growth phase.

Table 1. Average increase in pineapple shoot height in response to different combinations of thiamine and cytokinin at 4, 8, and 12 WAS

Combination Treatment	Shoot Height Increase		
	4 WAS	8 WAS	12 WAS
A = Control	0.51 a	0.67 b	0.91 b
B = 0.1 mg/L Thiamine + 0.01 mg/L TDZ	0.27 c	0.44 c	0.73 c
C = 0.1 mg/L Thiamine + 0.1 mg/L TDZ	0.32 c	0.67 b	1.01 b
D = 0.1 mg/L Thiamine + 1 mg/L BAP	0.52 a	0.79 a	0.98 b
E = 0.1 mg/L Thiamine + 2 mg/L BAP	0.43 b	0.66 b	0.87 c
F = 0.4 mg/L Thiamine	0.50 a	0.72 b	0.92 b
G = 0.4 mg/L Thiamine + 0.01 mg/L TDZ	0.51 a	0.83 a	1.13 a
H = 0.4 mg/L Thiamine + 0.1 mg/L TDZ	0.56 a	0.70 b	0.84 c
I = 0.4 mg/L Thiamine + 1 mg/L BAP	0.49 a	0.78 a	0.98 b
J = 0.4 mg/L Thiamine + 2 mg/L BAP	0.43 b	0.61 b	0.76 c

Note: Mean values in a column followed by the same letter indicate no significant difference based on the Scott-Knott test at the 5% level. WAS: Week After Subculture.

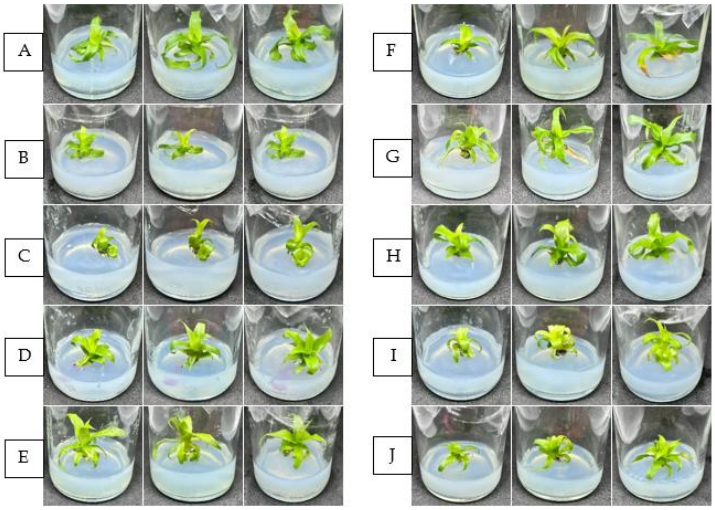

Figure 1. Visualization of shoot height in response to different combinations of thiamine and cytokinin

Note: *A = Control, B = 0.1 mg/L Thiamine + 0.01 mg/L TDZ, C = 0.1 mg/L Thiamine + 0.1 mg/L TDZ, D = 0.1 mg/L Thiamine + 1 mg/L BAP, E = 0.1 mg/L Thiamine + 2 mg/L BAP, F = 0.4 mg/L Thiamine, G = 0.4 mg/L Thiamine + 0.01 mg/L TDZ, H = 0.4 mg/L Thiamine + 0.1 mg/L TDZ, I = 0.4 mg/L Thiamine + 1 mg/L BAP, J = 0.4 mg/L Thiamine + 2 mg/L BAP. In every row of treatment, left picture, middle picture, and right picture are taken at 4, 8, and 12 WAS, respectively.*

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Based on Table 1, the combination of 0.4 mg/L Thiamine and 0.01 mg/L TDZ showed an average the highest shoot was 1.13 cm and was significantly different compared to the other treatments. Meanwhile, the other treatments showed relatively lower growth as shown in Figure 1. The visualization of the image shows a tendency for increasing shoot height in each treatment given. Thidiazuron acts as an exogenous cytokinin while also inducing endogenous cytokinin biosynthesis in plant cell (Setiadi et al., 2025). At low concentration, TDZ can stimulate the biosynthesis or inhibit the degradation of endogenous of cytokinin, due to exogenous cytokinin at high concentrations can cause explant saturation and reduce the growth response (Wang et al., 2025). The cell elongation process requires high energy as indicated by an increase in the ATP ratio. Thiamine concentrations up to 0.4 mg/L can increase metabolic activity intensively, so that the availability of energy in the form of ATP can encourage cell expansion (Lukman et al., 2023).

Time of New Shoot Emergence. The time of emergence of shoots in each treatment showed a fairly varied range, which ranges from 54 to 83 day after subcultures, but there was no difference significantly with treatment C, J, G, E, and I (Table 2). The addition of TDZ at low concentration (0.1-0.5 mg/L) shows high effectiveness in stimulating physiological activity, so it is able to triggers the formation of shoots (Rani et al., 2023). Increased thiamine concentration also known to accelerate the explant response to condition in vitro, especially in shortening the lag phase adaptation. This is related to energy produced by thiamine through carbohydrate metabolism, so that they can activate meristematic cells to enter the active division phase (Srilestari & Suwardi, 2019).

The combination of thiamine and TDZ in optimal concentrations proves the existence of synergy between metabolic and hormonal factors. Thiamine contributes to supporting energy availability for the metabolic activity of cells, while TDZ acts as a signal that directs the phase differentiation towards shoot formation (Liu et al., 2022; Putri et al., 2024). The combination of the two allows the explant to accelerate the transition from the adaptation phase to the active growth phase, so that the time of emergence of shoots new become shorter. This aligns with the concept that plant tissue culture performance depends on the interaction between nutrient availability and growth regulator concentrations in the culture medium. In contrast, media without added growth regulators (control) produced fewer shoots with slower growth due to the absence of exogenous cytokinins, which play an important role in stimulating cell division and promoting differentiation toward bud formation.

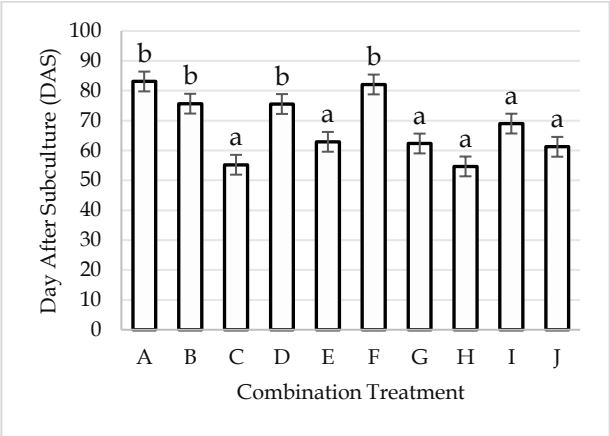

Figure 2. New shoots emergence time in response to different combinations of thiamine and cytokinin

Note: A = Control, B = 0.1 mg/L Thiamine + 0.01 mg/L TDZ, C = 0.1 mg/L Thiamine + 0.1 mg/L TDZ, D = 0.1 mg/L Thiamine + 1 mg/L BAP, E = 0.1 mg/L Thiamine + 2 mg/L BAP, F = 0.4 mg/L Thiamine, G = 0.4 mg/L Thiamine + 0.01 mg/L TDZ, H = 0.4 mg/L Thiamine + 0.1 mg/L TDZ, I = 0.4 mg/L Thiamine + 1 mg/L BAP, J = 0.4 mg/L Thiamine + 2 mg/L BAP. The same letter above the bar indicate no significant difference based on the Scott-Knott test at the 5% level.

Number of Shoots. Shoots are formed from the presence of bud on the expansion, which then develop when grown on culture media so that it triggers the growth of new shoots (Lutfiani et al., 2022). The difference in the number of shoots produced by each treatment is influenced by the ability of the explant in absorbing nutrients in the media and the given PGR. Table 3 shows various the concentration of thiamine and cytokinin had a significant effect on the number of shoots at 4, 8, and 12 WAS. Media with the addition of 0.1 mg/L Thiamine and 1 mg/L BAP became a marked superior treatment by a consistent increase in the number of shoots up to 12 weeks, with an average of 3.33 shoots and significantly different compared to other treatments. This result is in line with (Dahniar & Elvavina, 2022) who reported that the addition of 1 mg/L BAP was able to produce 10 pineapple shoots in 12 weeks.

The application of BAP at optimal concentrations can promote shoot multiplication

ISSN: 1412-4718, eISSN: 2581-138x

and support direct shoot regeneration without an intervening callus phase. BAP plays a role in stimulating cell division in tissues, which then triggers the formation of buds through the development of unipolar cells or organogenesis (Ajadi et al., 2018). As a type of cytokinin, BAP can suppress elongation cells, so that the formation of shoots becomes more dominant (Saepudin et al., 2023). Addition of vitamins B1 also plays a dual role in plant metabolism, namely as a catalyst and as a co-enzyme involved in various biochemical reactions of cells (Munir et al., 2016). In this study, the standard thiamine concentration of MS media, namely 0.1 mg/L, was in the acceptable range enough to increase the number of shoots on pineapple plants.

Number of Leaves. Leaves are the main organ of plants that function as a place where this happens the photosynthesis process is supported by high chloroplast content, especially in the mature leaf (Andrian et al., 2022). Increasing the number of leaves will encourage photosynthesis activity and photosynthates production to support growth. According to Lutfiani et al. (2022), shoot proliferation contributes to leaf formation, resulting in a greater number of leaves as shoot production increases. Table 4 shows that all treatments at the age of 4 week after subculture has not had a real effect, whereas at 8 and 12 weeks a real effect began to be seen on the number of leaves. The response was not significantly different between treatments at the age of 4 weeks after planting, indicating that all the combinations of treatments given were not able to trigger differences in the number of leaves in the early growth phase. However, treatment with the addition of 1 mg/L BAP combined with 0.1 mg/L thiamine or 0.4 mg/L produced the same number of leaves highest, namely 10.78 and 10.83 strands. This condition indicates that the explant is still in the growth phase. adaptation to the culture environment, so that cell proliferation and differentiation activities have not yet takes place optimally.

Entering the age of 8 and 12 WAS, the differences between treatments began to be significant. Treatment D (0.1 mg/L Thiamine + 1 mg/L BAP) produced the highest number of leaves, namely 17.56 leaves, but not significantly different from treatments E and I. In contrast, the control treatment (without ZPT) and the combination with TDZ tends to produce a lower number of leaves and is significantly different from the treatment combined with BAP. A concentration of 1 mg/l BAP is thought to be the optimal concentration optimal in supporting leaf formation. This is in line with Purita et al. (2017) which reported that increasing BAP concentration correlated with decreasing leaf number. The activity of BAP as a cytokinin not only stimulates shoot growth, but also influences the formation of the number of leaves through the process of proliferation and differentiation of tissue determine the formation of leaf primordia.

Table 2. Average number of pineapple shoots in response to different combinations of thiamine and cytokinin at 4, 8, and 12 WAS

Combination Treatment	Number of Shoots		
	4 WAS	8 WAS	12 WAS
A = Control	1.33 b	1.33 b	1.67 c
B = 0.1 mg/L Thiamine + 0.01 mg/L TDZ	1.33 b	1.67 b	2.00 c
C = 0.1 mg/L Thiamine + 0.1 mg/L TDZ	1.67 a	2.00 a	2.33 b
D = 0.1 mg/L Thiamine + 1 mg/L BAP	2.00 a	2.67 a	3.33 a
E = 0.1 mg/L Thiamine + 2 mg/L BAP	1.67 a	2.00 a	2.33 b
F = 0.4 mg/L Thiamine	1.11 b	1.33 b	1.67 c
G = 0.4 mg/L Thiamine + 0.01 mg/L TDZ	1.67 a	2.33 a	2.67 b
H = 0.4 mg/L Thiamine + 0.1 mg/L TDZ	1.33 b	2.00 a	2.33 b
I = 0.4 mg/L Thiamine + 1 mg/L BAP	1.33 b	1.67 b	2.00 c
J = 0.4 mg/L Thiamine + 2 mg/L BAP	1.11 b	1.44 b	1.56 c

Note: Mean values in a column followed by the same letter indicate no significant difference based on the Scott-Knott test at the 5% level. WAS: Week After Subculture.

Table 3. Average number of pineapple leaves in response to different combinations of thiamine and cytokinin at 4, 8, and 12 WAS

Combination Treatment	Number of Leaves		
	4 WAS	8 WAS	12 WAS
A = Control	8.67 a	9.56 b	10.22 b
B = 0.1 mg/L Thiamine + 0.01 mg/L TDZ	8.78 a	10.22 b	11.22 b
C = 0.1 mg/L Thiamine + 0.1 mg/L TDZ	6.00 a	6.56 b	7.00 b
D = 0.1 mg/L Thiamine + 1 mg/L BAP	10.78 a	13.67 a	17.56 a
E = 0.1 mg/L Thiamine + 2 mg/L BAP	9.11 a	13.11 a	15.22 a
F = 0.4 mg/L Thiamine	9.44 a	10.11 b	10.67 b
G = 0.4 mg/L Thiamine + 0.01 mg/L TDZ	8.41 a	10.67 b	11.22 b
H = 0.4 mg/L Thiamine + 0.1 mg/L TDZ	8.67 a	9.22 b	11.22 b
I = 0.4 mg/L Thiamine + 1 mg/L BAP	10.83 a	12.11 a	13.17 a
J = 0.4 mg/L Thiamine + 2 mg/L BAP	7.78 a	9.00 b	9.56 b

Note: Mean values in a column followed by the same letter indicate no significant difference based on the Scott-Knott test at the 5% level. WAS: Week After Subculture.

The presence of thiamine as vitamin B1 also influences the increase in the number of leaves in culture media. According to Naheed et al. (2022), thiamine functions as a coenzyme in carbohydrate metabolism and fats, then produce energy through photosynthesis to support growth leaves. Thiamine also plays a role similar to phytohormones in stimulating growth rates and development of plant tissue even though it is needed in very small amounts (Nurreni et al., 2025). This is consistent with Cryssanti et al. (2019), who reported that low concentrations of thiamine can trigger greater leaf growth in pitcher plants than high concentrations. Therefore, the media formulation with the addition of thiamine and cytokinin at various concentrations also provide different responses to number of leaves.

Fresh and Dry Weight of Shoots. The fresh weight of the plantlet is the accumulation of biomass formed as a result of plant activity cell division and enlargement, which is related to the absorption of water and organic materials in the tissue (Baskara et al., 2018). Physiologically, fresh weight is dominated by water, reaching ±80-90% of the total fresh weight of plants and carbohydrates as other main components (Oberhuber, 2017). Different with fresh weight, dry weight reflects the accumulation of organic matter resulting from activity metabolism because the water content in the tissue has been removed, so it is commonly used as an indicator of the quality of plantlet growth. Table 5 shows that the combination of thiamine and cytokinin at various concentrations had a significant effect on the fresh weight and dry weight of shoots.

Treatment C (0.1 mg/L Thiamine + 0.1 mg/L TDZ) tended to produce higher fresh weight than treatments A, D, E, and G, with the weight reaching 0.319 g. Although statistical analysis showed that the difference was not significant, treatment C numerically increased fresh weight by 19.92% compared to the control. The combination of thiamine and cytokinin are able to stimulate optimal explant growth, so that nutrient absorption is from the media can be absorbed optimally. Azmi & Handriatni (2018) argue that the fresh weight reflects the total biomass of all parts of the plant (roots, stems, and leaves), so it is correlated positive with vegetative growth. When viewed from the parameters of the number of shoots and the number of leaves, this treatment showed lower results compared to treatment with addition of BAP. This is thought to occur because the metabolic process in the planlets are disrupted due to the application of TDZ at high concentrations (Raisya et al., 2020). In line with Erland et al. (2020), the addition of TDZ can increase the accumulation of certain minerals and metabolites indicates a physiological stress response.

The highest dry weight was obtained in treatment G (0.4 mg/L Thiamine + 0.01 mg/L TDZ) which is 0.109 g and is significantly different compared to other treatments. The increase in dry weight that occurs in relation to increased photosynthesis results due to the water and nutrient requirements nutrients, so that plant metabolism occurs optimally (Azmi & Handriatni, 2018). In general, increase in dry weight is proportional to the increase in fresh weight (Numba et al., 2024). However, in this study, the treatment that produced the highest dry weight was not followed by the highest numerical fresh weight as

Jurnal Kultivasi Vol. 25 (2) August 2026
ISSN: 1412-4718, eISSN: 2581-138x

well. This condition is common in culture in vitro, because the explanation highly dependent on the media that influences the water content and growth regulating hormones that affects the distribution of photosynthates in plants (Dar et al., 2021; Levinsh, 2023).

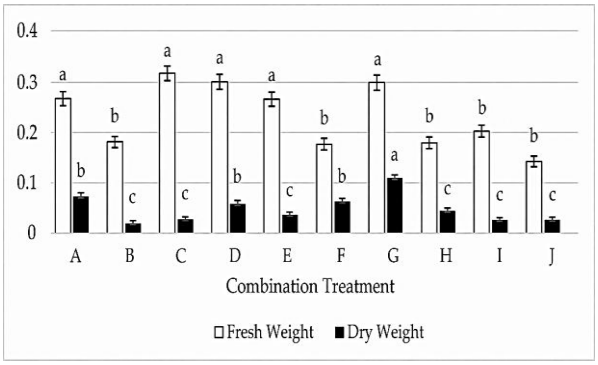

Figure 3. Average fresh and dry weight of pineapple shoots in response to different combinations of thiamine and cytokinin
Note: A = Control, B = 0.1 mg/L Thiamine + 0.01 mg/L TDZ, C = 0.1 mg/L Thiamine + 0.1 mg/L TDZ, D = 0.1 mg/L Thiamine + 1 mg/L BAP, E = 0.1 mg/L Thiamine + 2 mg/L BAP, F = 0.4 mg/L Thiamine, G = 0.4 mg/L Thiamine + 0.01 mg/L TDZ, H = 0.4 mg/L Thiamine + 0.1 mg/L TDZ, I = 0.4 mg/L Thiamine + 1 mg/L BAP, J = 0.4 mg/L Thiamine + 2 mg/L BAP. The same letter above the similar color of bar indicate no significant difference based on the Scott-Knott test at the 5% level.

Secondary Metabolite Analysis (TPC and TFC). Pineapple is a tropical fruit plant that contains various bioactive compounds, especially the phenolic and flavonoid groups (Tuzzahra & Mustakim, 2026). Bioactive compounds in pineapple can be utilized in the health sector due to their antimicrobial, anti-inflammatory, anticancer, and antidepressant activities (Casas-Rodríguez et al., 2025). This potential indicates that pineapple could be developed as a natural raw material for health products and functional foods. If the utilization of pineapple is implemented on a large scale, a high supply of biomass with stable phenolic and flavonoid content will be required. Although pineapple is not classified as a medicinal plant, the evaluation of total phenolic and flavonoid contents in in vitro-derived plantlets is relevant for exploring their potential as sources of bioactive compounds.

Research on phenolic and flavonoid compounds in pineapple is currently still dominated by studies on parts of the fruit, especially the peel, which contains considerable amounts of these compounds (Fauzi et al., 2023). In contrast, studies investigating total phenolic and flavonoid contents at the plantlet stage obtained through in vitro culture remain relatively limited. In vitro culture provides controlled conditions for plant growth and may facilitate the evaluation of phenolic and flavonoid accumulation under define culture conditions. Moreover, tissue culture system can support the production of plant biomass under controlled environmental conditions and reduce dependence on seasonal variation compared with conventional cultivation system (Setiawati et al., 2020).

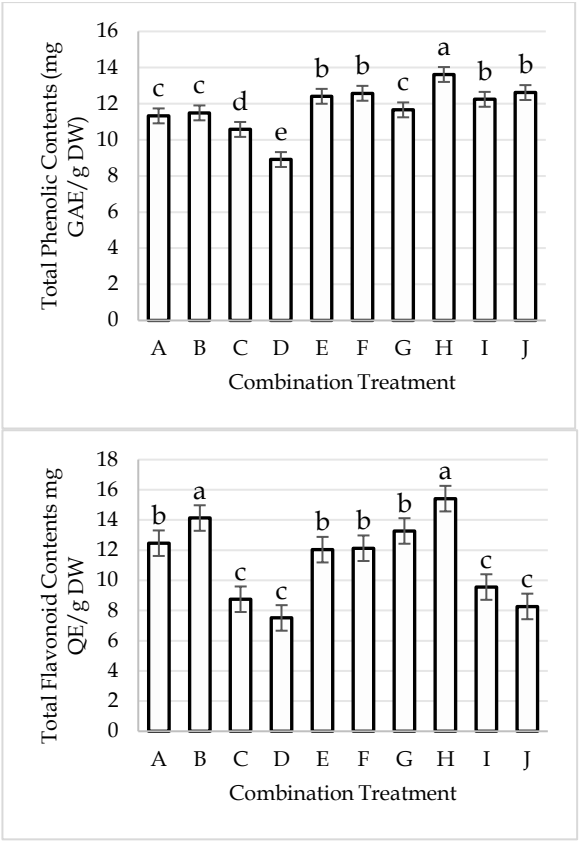

Figure 4. Total phenolic (upper) and flavonoid contents (lower) in response to different combinations of thiamine and cytokinin
Note: A = Control, B = 0.1 mg/L Thiamine + 0.01 mg/L TDZ, C = 0.1 mg/L Thiamine + 0.1 mg/L TDZ, D = 0.1 mg/L Thiamine + 1 mg/L BAP, E = 0.1 mg/L Thiamine + 2 mg/L BAP, F = 0.4 mg/L Thiamine, G = 0.4 mg/L Thiamine + 0.01 mg/L TDZ, H = 0.4 mg/L Thiamine + 0.1 mg/L TDZ, I = 0.4 mg/L Thiamine + 1 mg/L BAP, J = 0.4 mg/L Thiamine + 2 mg/L BAP. The same letter above the bar indicate no significant difference based on the Scott-Knott test at the 5% level.

Table 6 shows that various combinations of thiamine and cytokinin had a significant effect on the total phenolic and flavonoid content of pineapple plantlets in vitro. The highest total phenolic content of pineapple obtained in treatment H (0.4 mg/L Thiamine + 0.1 mg/L TDZ) of 13.62

mg GAE/g DW and different significantly compared to other treatments. A similar response was observed for total flavonoid content, where treatment H (0.4 mg/L Thiamine + 0.1 mg/L TDZ) produced the highest value of 15.41 mg QE/g DW, but not significantly different from treatment B (0.1 mg/L Thiamine + 0.01 mg/L TDZ). These results indicate that the response of pineapple plantlets to thiamine and cytokinin depended on the combination and concentration applied. Importantly, the treatment that promoted the highest shoot multiplication was different from that which promoted the highest accumulation of total phenolic and flavonoid compounds. The combination of 0.1 mg/L Thiamine and 1 mg/L BAP (treatment D) was the most effective treatment for shoot multiplication and leaf development, whereas 0.4 mg/L Thiamine combined with 0.1 mg/L TDZ (treatment H) resulted in the highest total phenolic and flavonoid contents. This distinct response is an important finding of the present study, indicating that culture conditions that promote vegetative development do not necessarily favor the accumulation of phenolic and flavonoid compounds.

The higher total phenolic and flavonoid contents observed in treatment H were associated with the application of 0.4 mg/L Thiamine and 0.1 mg/L TDZ. TDZ is a cytokinin-like plant growth regulator that influences plant growth, metabolism, and has been reported to affect the accumulation of secondary metabolites in several plant species (Erland et al., 2020). TDZ will activate the phenylpropanoid pathway to produce secondary metabolites such as phenolic and flavonoid compounds (Shoja & Shishavani, 2021). Thiamine has also been reported to participate in plant metabolic and physiological processes that may influence plant responses under in vitro conditions (Subki et al., 2018). Although treatment H produced the highest total phenolic and flavonoid contents, no visible browning symptoms were observed in the pineapple plantlets during the observation period. This finding was based solely on visual observation and indicates that the accumulation of total phenolic and flavonoid compounds in treatment H was not accompanied by visible browning symptoms under the conditions of this study.

Conclusion

In vitro shoot multiplication of pineapple (*Ananas comosus* (L.) Merr.) varied depending on the combination of thiamine and cytokinin. The combination of 0.1 mg/L Thiamine and 1 mg/L BAP was the most effective for shoot multiplication and leaf development, as indicated by the highest numbers of shoots and leaves. In contrast, the highest total phenolic and flavonoid contents were obtained with 0.4 mg/L Thiamine combined with 0.1 mg/L TDZ. These findings indicate that culture conditions that favored vegetative development differed from those that promoted phenolic and flavonoid accumulation. Therefore, the choice of culture medium should be tailored to the intended production objective, whether shoot multiplication or the accumulation of phenolic and flavonoid compounds. The treatments identified in this study provide a basis for further optimization of pineapple in vitro culture. However, further validation during rooting, acclimatization, and ex vitro establishment, together with assessments of genetic stability and antioxidant activity, is required before these conditions can be recommended for large-scale propagation or bioactive compound production.

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