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MORPHOLOGICAL AND BIOCHEMICAL RESPONSES OF *Amaranthus viridis* TO CHEMICAL FERTILIZER AND BLACK SOLDIER FLY FRASS

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ABSTRACT

The prolonged use of chemical fertilizer is associated with adverse environmental effects. To date, insect-derived fertilizer from Black Soldier Fly (BSF), has emerged as a promising organic alternative. However, the impact of BSF frass on the morphology and biochemical compositions of leafy vegetables necessitates further investigation. Therefore, the objective of this study was to evaluate the effects of chemical NPK fertilizer and BSF frass fertilizer, applied individually or in combination, on the morphology and biochemical composition of *Amaranthus viridis (A. viridis)*. The experiment consisted of four treatments; untreated control (T1), BSF frass (T2), NPK fertilizer (T3), and BSF frass + NPK fertilizer (T4). The results indicated that *A. viridis* plants in T4 exhibited the highest stem length, root length, leaf area, as well as weight of the stems, leaves, and roots. However, no significant differences were observed in terms of plant morphology and total chlorophyll content among the fertilizer treatments. Additionally, T4 significantly increased the total phenolic content in *A.viridis* (0.481 ± 0.012 mg GAE/g DW) compared to plants treated solely with NPK fertilizer. These results indicate that the combined application of BSF frass and NPK fertilizer significantly enhances plant growth of leafy vegetables, particularly by influencing specific biochemical compositions.

Keywords: Organic fertilizer, plant morphology, metabolites, vegetables, sustainable agriculture.

INTRODUCTION

Amaranthus, an herbaceous plant belonging to the Amaranthaceae family, is recognized for its rich nutritional profile, including high levels of vitamins, minerals, and antioxidants (Sarker et al., 2020). In Malaysia, *A. viridis*, commonly known as *Bayam panjang*, is widely available in grocery stores, being regularly consumed in both urban and rural areas (Amin et al., 2006). According to the latest report from the Department of Statistics Malaysia (2022) the country recorded a self-sufficiency ratio of 112% for *Amaranthus* sp. in 2021, indicating a surplus for export or

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alternatives uses. This statistic highlights the high consumption and demand of *A. viridis* in Malaysia, underscoring the importance of identifying strategies to enhance its growth and yield. Due to its high nutritional content, *A. viridis* has the potential to meet the dietary needs of the population (Khan et al., 2022), making it a promising crop for food security and nutrition.

Chemical fertilizers are still widely used in agriculture to enhance crop yields and accelerate harvest cycles (Finez and Talimbe, 2023). Among them, NPK fertilizer is particularly notable for its broad applicability (Raksun et al., 2022). According to the FAO (2022), Asia is the world's largest user of chemical fertilizers in agriculture, accounting for 55% of the global use in 2018, with Malaysia alone using 1,739.9 tons that year. However, the prolonged use of chemical fertilizer is associated with adverse environmental effects, including pollution, soil structure degradation, and decreased soil fertility (Raksun et al., 2022). As a result, the use of organic fertilizer has gained increasing attention. Moreover, recent studies suggest that the combined application of inorganic and organic fertilizers can mitigate the detrimental effects of excessive chemical fertilizer, while improving crop growth and yield (Jin et al., 2022; Qaswar et al., 2020; Yang et al., 2020).

One of the emerging organic fertilizers nowadays is Black Soldier Fly (BSF) frass, which is produced through the bioconversion of organic waste by BSF larvae, resulting in highly nutritious and hygienic organic fertilizer (Anyega et al., 2021). Frass contains substantial amounts of nitrogen, phosphorus, and potassium, as well as organic matter and other components such as chitin, derived from the larvae's exoskeleton (Elissen et al., 2023). Researchers have identified chitin, and particularly chitosan, which is a linear polysaccharide formed thorough the de-acetylation of chitin, as potent plant defence compounds that can make plants withstand or tolerate a wide range of diseases and pests (Orzali et al., 2017).

Several studies have described the positive effects of BSF frass fertilizer applications on crop production, nutrient use efficiency, plant growth, and soil biochemical properties (Agustiyani et al., 2021; Beesigamukama et al., 2020). In addition, BSF frass fertilizer has been used in research involving crops such as maize, spring onion, chili pepper, and shallot (Quilliam et al., 2020; Tanga et al., 2021; Zahn, 2017). Notably, a study on Swiss chard found that yields obtained with BSF frass were comparable to those achieved with inorganic fertilizers and significantly higher yields than those from non-fertilized plants (Chirere et al., 2021). Abd Manan et al. (2024) recently compiled various studies on the application of BSF frass to different plant species. Although several studies have examined the effects of BSF frass fertilizer on leafy vegetables such as lettuce and kale (Anyega et al., 2021; Chiam et al., 2021), limited information is available regarding its impact on the biochemical composition or nutritional value of these plants. Given its emerging role in agriculture, the use of BSF frass as an organic fertilizer requires comprehensive evaluation to determine its effectiveness in enhancing crop growth, and biochemical compositions. Hence, this study aimed to evaluate the effects of BSF frass fertilizer and NPK fertilizer, applied individually or in combination, on the morphology and biochemical compositions of *A. viridis*.

MATERIALS AND METHOD

Plant materials, black soldier fly (BSF) frass and NPK fertilizer

A. viridis seeds and NPK 15:15:15 chemical fertilizer were purchased from a local supplier in Johor, while the BSF frass was obtained from a local manufacturer in Perak, Malaysia. Initially, *A. viridis* seeds were soaked overnight in distilled water at room temperature. Subsequently, the seeds were sown at a depth of 0.5 cm to 1.0 cm in a germination tray. The trial was conducted at the greenhouse of the Universiti Teknologi Malaysia in January 2024. Seedlings were transplanted to polybags two weeks after sowing or once they developed two to three true leaves (Sabri et al., 2020). The polybags (14 cm × 11.5 cm), were filled with ±1.5 kg of organic soil and spaced 15 cm apart (Nurseha et al., 2023). In the shade house, the polybags were arranged following a randomized complete block design (RCBD) with five replicates per treatment (Fig. 1). The experiment consisted of four different treatments: no fertilizer application as control (T1), 6.36 g of BSF frass (T2), 0.64 g of NPK fertilizer (T3), and a combination of 50% BSF Frass (3.18g) + 50% NPK 15:15:15 fertilizer (0.32g) (T4). Plants were watered twice daily, once in the morning and once in the evening. Fertilizers were applied every two weeks, and plants were harvested on week 5.

Determination of stem length, root length, and fresh weight

Stem length was measured from the soil surface to the apical meristem (Achten et al., 2010), while root length was determined using ruler after harvesting. Fresh weights of stems, leaves, and roots were measured using an electronic balance (Shimadzu Corporation, Model ATX224, Japan) and stored in separate bags. A fully expanded,

Fig. 1. Seedlings of *Amaranthus viridis* transplanted into polybags for the experiment.

healthy leaf from each treatment was selected, and total leaf area was estimated using ImageJ software after harvesting (Pride et al., 2021). Fresh leaves, stems, and roots were stored at −20 °C for further analysis.

Chlorophyll and carotenoid pigment extraction

Healthy leaves of *A. viridis* were collected and immediately wrapped in aluminum foil. A 0.1 g portion of each fresh leaf sample was cut into small pieces and finely ground with 5 mL of 80% aqueous acetone solution (HmbG® Chemicals) using a mortar and pestle. The mortar and pestle were then rinsed with 80% acetone to ensure complete transfer of the extract. The extract was transferred to a centrifuge tube and centrifuged at 3,000 rpm for 10 min. The supernatant was collected, and centrifugation was repeated until the residue became nearly colorless. The final volume was adjusted to 25 mL with 80% acetone. The absorbance of the extract was measured at 645, 663, 480 and 510 nm using a Ultraviolet-visible (UV-vis) spectrophotometer. A blank sample was prepared using only 80% acetone (Arnon, 1949; Shinde & Khade, 2019; Tongo et al., 2014).

Determination of chlorophyll a, b and total (a+b), and carotenoid content

The chlorophyll a, chlorophyll b and total chlorophyll contents in the 80% acetone extract were quantified using the equations described by Arnon (1949), while the total carotenoid content was calculated according to Shinde and Khade (2019).

$$\text{Total chlorophyll } (\mu g\ mL^{-1}) = 20.2\ (A_{645}) + 8.02\ (A_{663})$$

$$\text{Chlorophyll a } (\mu g\ mL^{-1}) = 12.7\ (A_{663}) - 2.69\ (A_{645})$$

$$\text{Chlorophyll b } (\mu g\ mL^{-1}) = 22.9\ (A_{645}) - 4.68\ (A_{663})$$

$$\text{Total carotenoids } (\mu g\ mL^{-1}) = 7.6\ (A_{480}) - 1.49\ (A_{510})$$

where A_{645}, A_{663}, A_{480} and A_{510} represent absorbance at wavelengths of 645, 663, 480 and 510 nm, respectively. The chlorophyll and carotenoid content in the leaf tissue of the sample was measured in mg/g using the formula V/(1000×W) = 25/ (1000×0.1) = 0.25, where V is the final volume of chlorophyll extract in 80% acetone (25 ml), and W denotes the fresh weight of the tissue extracted (0.1 g). Chlorophyll and carotenoid content (mg g^{-1}) were calculated by substituting 0.25 into the respective equations.

Sample extraction for total phenolic content analysis

Leaves and stems of *A. viridis* were oven-dried (Memmert, Model UFE800, Germany) at 55 °C until constant weight was achieved. The dried samples were ground into a fine powder using a mortar and pestle, and then stored in zip-lock bags and stored at −20 °C until analysis (Obeng et al., 2020). Following the extraction method described by Panawala et al. (2016), with slight modifications, 0.1 g of powdered leaf and stem sample from each fertilizer treatment was extracted with 10 mL of 80% (v/v) aqueous methanol at 40 °C for 24 h. Subsequently, the mixtures were cooled to room temperature and centrifuged (Eppendorf, Model 5810, Germany) for 15 min at 4,500 rpm. The resulting supernatant was collected and used for the determination of total phenolic content (TPC) and flavonoid content.

Determination of total phenolic content

The preparation of the standard solution and calibration curve for gallic acid followed the method outlined by Shirazi et al. (2014). A standard solution was prepared by dissolving 1g of gallic acid (Sigma Aldrich) in 100 mL of methanol (Merck), yielding a 1% (w/v) or 10 mg mL^{-1} solution. The standard solution was then serially diluted in methanol to achieve concentrations of 0, 0.1, 0.2, 0.3, and 0.4 mg mL^{-1}. These concentrations were used to generate a standard calibration curve for gallic acid. TPC was estimated using the Folin-Ciocalteu reagent (Merck), with gallic acid as the reference standard, following the procedure described by Hossain et al. (2022), with slight modifications. Briefly, 1 mL of each diluted gallic acid solution (0, 0.1, 0.2, 0.3, and 0.4 mg mL^{-1}) and 1 mL of Folin–Ciocalteu reagent were added to the test tube containing 9 mL of distilled water and vortexed. After 5 min, 10 mL of 7% (w/v) Na_2CO_3 was added to each tube, followed by incubation in the dark at room temperature for 90 min. Absorbance was measured at 760 nm using a UV-Vis spectrophotometer (Thermo Fisher Scientific, GENESYS 10S UV-Vis, United States). A reagent blank was prepared by substituting distilled water for the extract solution. This procedure was repeated using the extract solutions obtained from both leaf and stem samples of all treatments. A standard calibration curve was constructed by plotting gallic acid concentrations on the X-axis and their corresponding absorbance values on the Y-axis. The resulting linear regression equation was used to calculate the TPC in each extract. Result were expressed as mg gallic acid equivalent (GAE) g^{-1} of dry weight (DW), using the formula:

$$C = (c \times v)\, m^{-1}$$

where C represents total phenolic content, c represents gallic acid concentration in mg mL^{-1}, v represents extract volume in mL, and m represents crude extract weight in g.

Statistical analysis

Data from all five replicates were used to calculate the mean ± standard error (SE). ANOVA was carried out by using IBM SPSS. Means were compared using Tukey's post hoc test at a 95% confidence level ($p \leq 0.05$).

RESULTS AND DISCUSSION

Effects of different fertilizer treatments on the morphological properties of *Amaranthus viridis*

Fig. 2 illustrates the effect of different fertilizer treatments on the stem length of *A. viridis* after harvesting. Stem length increased across all treatments throughout the experiment. T4 recorded the greatest value of 7.3 ± 0.2 cm, followed by T2 and T3, with values of 6.7 ± 0.4 cm and 6.4 ± 0.4 cm, respectively. Conversely, the non-fertilized plants (T1) exhibited the shortest value of 4.0 cm. Therefore, stem length in fertilizer-treated plants of *A. viridis* was significantly higher compared to control ($p<0.05$). However,

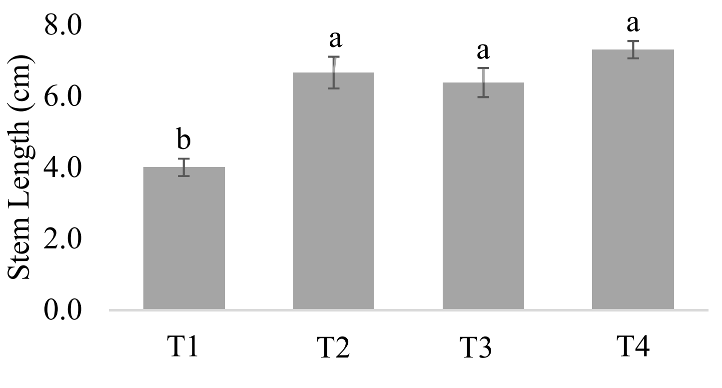

Fig. 2. Stem length of *A. viridis* under different fertilizer treatments. T1: Control, T2: BSF Frass only, T3: NPK only, and T4: Combination of NPK and BSF frass fertilizer. Data are presented as mean ± standard error (n=5). Different letters above the bars indicate statistically significant differences according to Tukey's Test at a significance level of P ≤ 0.05.

no significant differences were observed between T4, T3 and T2 (p>0.05).

The effect of fertilizer application on the root length of *A. viridis* is shown in Fig. 3. T1 recorded the lowest value of 9.3 ± 0.6 cm, contrasting significantly with the fertilizer-treated plants. The longest root length was recorded in T4 (15.2 ± 1.1 cm), followed by T3 and T2, with values of 4.3 ± 1.3 cm and 12.7 ± 1.3 cm, respectively.

Regarding leaf area, the fertilizer treatments inhibited significantly higher values than the control. T4 treatment recorded 26.207 ± 2.137 cm², while T2 and T3 recorded lower values of 23.114 ± 3.596 and 21.876 ± 2.246 cm², respectively (Fig. 4), with no statistically significant differences among these treatments (p>0.05).

Effects of different fertilizer treatments on fresh weights of *Amaranthus viridis*

The results showing the effects of BSF frass and NPK fertilizer treatments on fresh weight of stems, leaves, and roots of *A. viridis* are presented in Table 1.

Based on the results, treatments T2, T3 and T4 improved the growth parameters of *A. viridis*, including stem length, root length, and, leaf area, compared to the untreated control. The combination of chemical and organic fertilizers (T4) demonstrated potential synergistic and complementary effects. This finding is consistent with the results reported by Anyega et al. (2021), and supports the notion that combining organic and inorganic fertilizers can significantly boost crop growth. Additionally, there is evidence that their combined application improves soil properties by increasing soil organic carbon (C), total nitrogen (N), and the amount of macro- and micronutrients (Arif et al., 2014; Brar et al., 2015; Moe et al., 2017). The presence of sufficient nitrogen can stimulate rapid cell elongation and proliferation in plants (Luo et al., 2020), contributing to improved growth. Moreover, the combined use of organic and chemical fertilizer has been shown to increase microbial biomass and enhance overall soil health (Roba, 2018). In the present study, the highest biomass levels for stem, leaves, and roots were observed in *A. viridis* plants under the combined application of NPK fertilizer and BSF frass (T4). In the same treatment, the fresh weight of *A. viridis* roots was significantly higher compared to the control, whereas no significant differences were detected between T4 and the individual fertilizer applications (T2 and T3). Overall, the fresh weight of *A. viridis* plants improved under all fertilizer treatments, whether applied individually or in combination.

Effects of different fertilizer treatments on *Amaranthus viridis* chlorophyll and carotenoid contents

Table 2 presents the levels of total chlorophyll, chlorophyll a, chlorophyll b, and total carotenoids in plants under different fertilizer treatments.

As shown in Table 2, all treatments increased the total chlorophyll content, chlorophyll a, chlorophyll b and total carotenoid content compared to the untreated control (T1). However, no significantly differences were observed among the fertilizer treatments (T2, T3 and T4) for these parameters. Generally, fertilizers supply essential nutrients, including nitrogen, which

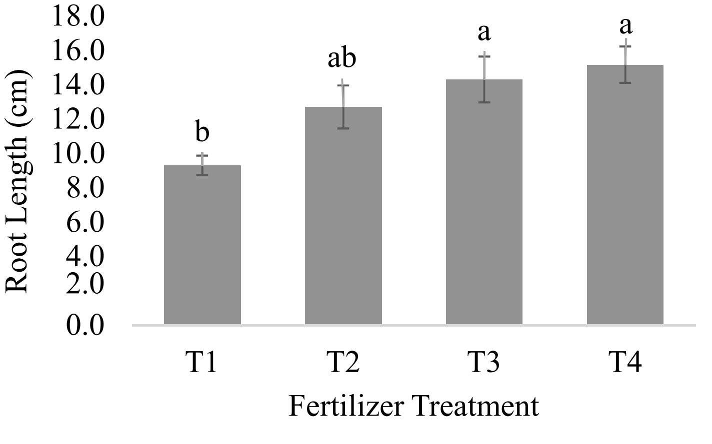

Fig. 3. Root length of *A. viridis* under different fertilizer treatments. T1: Control, T2: BSF Frass only, T3: NPK only and T4: Combination of NPK and BSF frass fertilizer. Data are presented as mean ± standard error (n=5). Different letters above the bars indicate statistically significant differences according to Tukey's Test at a significance level of P ≤ 0.05.

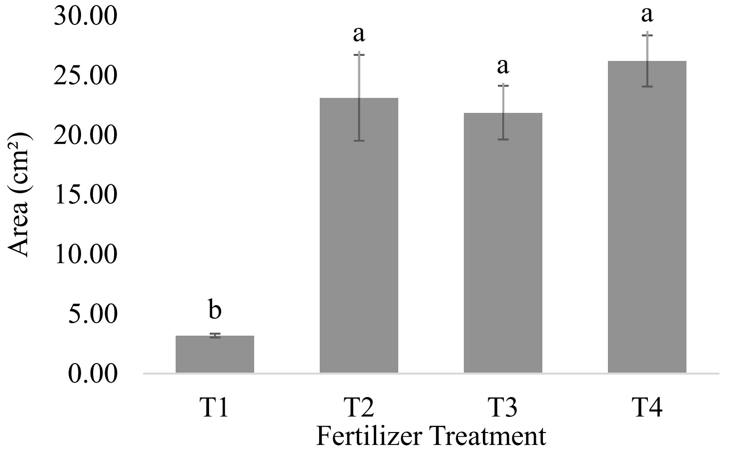

Fig. 4. Leaf area of *A. viridis* under different fertilizer treatments. T1: Control, T2: BSF Frass only, T3: NPK only and T4: Combination of NPK and BSF frass fertilizer. Data are presented as mean ± standard error (n=5). Different letters above the bars indicate statistically significant differences according to Tukey's Test at a significance level of $P \leq 0.05$.

Table 1. Fresh weight of stems, leaves, and roots of *A. viridis* under different fertilizer treatments. T1: Control, T2: BSF Frass only, T3: NPK only, and T4: Combination of NPK and BSF frass fertilizer. Data are presented as mean ± standard error (n=5). Different letters indicate statistically significant differences between treatments according to Tukey's Test at a significance level of $P \leq 0.05$.

	Fresh Weight (g)		
Treatment	**Stems**	**Leaves**	**Roots**
T1	0.16 ± 0.02b	0.09 ± 0.01b	0.08 ± 0.01b
T2	1.14 ± 0.26a	2.22 ± 0.51a	0.39 ± 0.11ab
T3	1.18 ± 0.23a	2.17 ± 0.36a	0.41 ± 0.09ab
T4	1.37 ± 0.10a	2.42 ± 0.14a	0.67 ± 0.10a

Table 2. Total Contents of Chlorophyll ($mg\ g^{-1}$), Chlorophyll a ($mg\ g^{-1}$), Chlorophyll b ($mg\ g^{-1}$) and Carotenoids (mg/g) of *A. viridis* under different fertilizer treatments. T1: Control, T2: BSF Frass only, T3: NPK only and T4: Combination of NPK and BSF frass fertilizer. Data are presented as mean ± standard error (n=5). Different letters indicate statistically significant differences between treatments according to Tukey's Test at a significance level of $P \leq 0.05$.

Treatment	Total Chlorophyll Content ($mg\ g^{-1}$)	Chlorophyll a ($mg\ g^{-1}$)	Chlorophyll b ($mg\ g^{-1}$)	Total Carotenoid Content ($mg\ g^{-1}$)
T1	0.403 ± 0.008b	0.198 ± 0.010b	0.205 ± 0.002b	0.178 ± 0.006b
T2	1.749 ± 0.093a	1.328 ± 0.064a	0.422 ± 0.046a	0.556 ± 0.028a
T3	1.834 ± 0.068a	1.413 ± 0.049a	0.421 ± 0.020a	0.561 ± 0.018a
T4	1.919 ± 0.104a	1.448 ± 0.079a	0.472 ± 0.025a	0.559 ± 0.028a

is a primary component of all amino acids that serve as fundamental structural components of chloroplasts (Ouda and Mahadeen, 2008). Additionally, the magnesium (Mg) present in BSF frass may further enhance chlorophyll content. Mg is the central atom in the chlorophyll molecule responsible for photon capture in both Photosystem I and Photosystem II (Sirohiwal et al., 2021).

Effects of different fertilizer treatments on the total phenolic content of *Amaranthus viridis*

The total accumulation of phenolic compounds in *A. viridis* in response to different fertilizer treatments is presented in Fig. 5. In general, fertilizer application significantly increased the TPC of *A. viridis* compared to the control treatment. T4 recorded 0.481 ± 0.012 mg GAE g DW^{-1}, being significantly higher than the sole applications of BSF brass (T2) and NPK (T3). In turn, BSF brass (T2) recorded a slightly higher TPC than T3, with values of 0.435 ± 0.007 mg and 0.398 ± 0.015 mg GAE g^{-1} DW, respectively.

The combined application of BSF frass and NPK fertilizer led to a higher total phenolic content (TPC) in *A. viridis* compared to plants cultivated with NPK fertilizer alone. This indicates that the addition of frass altered the biochemical properties of plants. The rapid nutrient release from chemical fertilizers like NPK may reduce the TPC in plants, as previously observed in leaf mustard (Li et al., 2008). Although chemical fertilizers can promote plant growth, they may simultaneously hinder the synthesis of phytochemical and secondary metabolite due to an increased protein demand associated with growth processes (Shiwakoti et al., 2023). The obtained results confirmed that the integration of organic and chemical fertilizers stimulated TPC production in *A. viridis*, being consistent with previous studies (Koureh et al., 2019; Sarwar et al., 2020; Siddiqui et al., 2020).

CONCLUSIONS

This study investigated the effects of organic Black Soldier Fly (BSF) frass, chemical NPK fertilizer and their combined application on the morphological traits and biochemical composition of *Amaranthus viridis*. The results showed that morphological parameters did not differ significantly among the different fertilizer treatments. However, the combined application of BSF frass and NPK fertilizer led to a notable increase in the total phenolic content (TPC) in *A. viridis*, whereas the sole application of NPK fertilizer resulted in a lower TPC. Overall, the integrated use of organic and inorganic fertilizers promoted plant growth and positively influenced specific biochemical traits, particularly root development. These findings suggest that BSF frass holds promise as a sustainable alternative to reduce dependence on chemical fertilizers in future agricultural practices.

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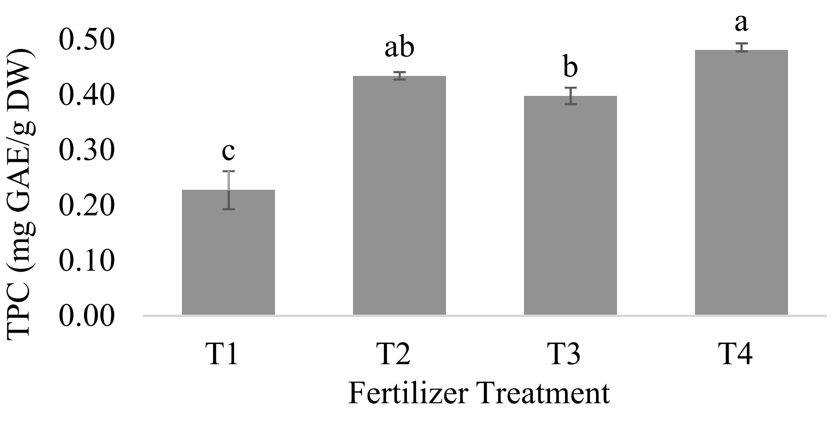

Fig. 5. Total phenolic content of *A.viridis* shoots under different fertilizer treatment. T1: Control, T2: BSF Frass only, T3: NPK only and T4: Combination of NPK and BSF frass fertilizer. Data are presented as mean ± standard error (n=5). Different letters above the bars indicate statistically significant differences according to Tukey's Test at a significance level of P ≤ 0.05.

Author Contributions

Active participation in the bibliographic review: Shao-Zhen; in the development of the methodology: Fazilah and Raihana; in the discussion of the results: Shao-Zhen, Fazilah, and Raihana. All authors reviewed and approved the final version of the article.

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