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Growth and Content of Flavonoids of Mustard Greens (*Brassica juncea* L.) with Different Humic Acid and Water Content

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ABSTRACT

Water deficiency disrupts metabolic pathways and reduces plant growth. The high demand for Brassica juncea (L.) due to its nutritional value necessitates improved cultivation practices to ensure high-quality yields. Although humic acid is known to support plant productivity, studies examining its interaction with soil moisture levels in B. juncea remain limited. This study aimed to determine the optimal soil moisture level and humic acid dosage for enhancing the growth and flavonoid content of B. juncea (L.). The experiment was conducted using a Randomized Block Design (RBD) with two factors: four levels of humic acid dosage (0 g/kg, 4 g/kg, 8 g/kg, and 12 g/kg) and four watering treatments based on field capacity (FC), namely 20%, 40%, 60%, and 80%, each with four replications. Observations were made 42 days after planting, covering plant height, number of leaves, leaf area, dry weight, and total flavonoid content. Data were analyzed using ANOVA at a 95% confidence level, and significant differences among treatments were further tested using Duncan's Multiple Range Test (DMRT). The results showed that the treatment combination of 20% FC and 12 g/kg humic acid produced the highest values for growth and flavonoid content of B. juncea L. under drought stress conditions, with plant height (23.67 cm), number of leaves (8 leaves), leaf area (1,022 cm²), dry weight (0.55 g), and total flavonoid content (0.108 mg QE/g sample).

INTRODUCTION

Mustard greens—also known as mustard meatballs, caisim, or caisin—are easy to cultivate and can be consumed fresh or processed into pickles or candies. Besides serving as a food source, they provide several health benefits, such as treating urinary tract infections, coughs, and headaches, cleansing the blood, and helping prevent cancer (Pary, 2015). The advantage of mustard greens is their high nutritional content, which includes many antioxidants and vitamins important for health (Damayanti et al., 2013). High antioxidant levels are supported by the mustard greens' content of biologically active phenolic compounds and flavonoids (Wahjuni et al., 2019). Flavonoids are a type of secondary metabolite of polyphenols that are widely found in plants that have a wide variety of benefits, so that higher accumulation of flavonoids in vegetables can increase their economic value (Kumar et al., 2018).

The need for vegetables continues to increase each year, but demand is not matched by production. Mustard production can be increased through plant maintenance, primarily by ensuring water and nutrient sufficiency (Mahfudiawati et al., 2016). Water pressure is considered one of the most influential environmental factors in limiting plant growth and productivity. Limited water availability reduces water potential gradients, thereby decreasing the rates of water and nutrient transport and affecting the yield of fresh weight of mustard plants (Taiz & Zeiger, 2002).

In addition, water deficit leads to cell shrinkage, increased cytoplasmic visibility, cell wall folding, membrane damage, and protein denaturation (Lotfi et al., 2018), as well as physiological, metabolic, and biochemical changes that ultimately alter plant productivity (Kapoor et al., 2020). In addition, drought conditions can limit photosynthesis in plants and alter nutrient uptake, thereby lowering the production of secondary metabolites (Juarez et al., 2011).

Plants under stress require additional nutrients to sustain growth, including humic acid. This compound is widely used in commercial formulations because it contains phytohormones that help protect plants from oxidative damage caused by environmental stressors (Vasconcelos, 2020). Humate enhances nutrient absorption (Halpern et al., 2015), thereby increasing plant growth, as evidenced by increased levels of active compounds (Tripatmasari et al., 2014; Proklamasiningsih et al., 2019). The administration of humic acid has been shown to increase flavonoid compounds and antioxidant content, such as in the god leaf plant (*Gynura pseudochina* (Proklamasiningsih et al., 2020) and red chili (*Capsicum annuum* L.) (Aminifard et al., 2012).

Previous research on humic acid has largely emphasized its contribution to plant growth through nutrient supply and its ability to improve soil physical properties for better nutrient absorption. This study further explores its influence on the biosynthesis of flavonoid compounds when plants experience limited water availability. Therefore, this study was conducted to determine the effect of different water and humic acid content on the growth and flavonoid content of mustard greens. Besides that, to provide information about the proper levels in the application of water and humic acid to increase the growth and content of flavonoids in mustard greens.

RESEARCH METHODS

Method

The research was a laboratory experiment conducted using a Group Random Design (RAK) with two factors. The first factor is the administration of humic acid at four dose levels: 0 g/kg, 4 g/kg, 8 g/kg, and 12 g/kg. In comparison, the second factor is the administration of water content based on field capacity (KL), with four levels: 20%, 40%, 60%, and 80%. Each treatment was repeated four times.

Materials and Equipment

The materials used in this study include water, aquades, $AlCl_3$ 10%, humic acid [Humivit], mustard green seeds (*Brassica juncea* var. Dora), CH_3COOK 1 M, 80% ethanol, block millimeter paper, filter paper [Whatman no. 1], labels, methanol 80%, *polybag* UK. 25 x 25 cm, manure, quercetin standard, Andosol soil.

Procedure

Seed Sowing and Seedling Planting

The seeds are soaked in water, then drained and sown. Seedling media uses a mixture of soil and manure (50:50). 21-day-old seedlings that already have 3-4 leaf blades ready to be transplanted to A Lot of Media. Appearance of Andosol Manure Soil Mixture (50:50) as much as 2 kg/polybag (Istiqomah & Serdani, 2018).

Field Capacity Measurement

The field capacity is determined by the gravimetric method. The soil that the wind has dried is weighed at up to 50 g, then saturated with water until no more water drips. The soil is weighed, then dried in an oven at 105-110 ℃ for 24 hours (Abdurachman et al., 2006). The soil is weighed back as dry weight. The moisture content is determined by the formula (Siregar et al., 2017):

$$\text{Dry matter content (\%)} = \frac{W - w}{T} \text{ x } 100\%$$

$$\text{Moisture content (\%)} = 100\% - \%\ \text{Dry matter}$$

Information:
W = air dry soil weight + weigh bottle (g);
w = weight of weighing bottle (g);
T = weight of moist soil taken for dioven (g)

Treatment Delivery

Humic acid is added to the planting medium twice, on the 7th and 21st days after planting (Fauziah et al., 2019), at the predetermined dosage. Moisture content treatment is carried out from the beginning of seedling planting until harvest, with watering every 3 days at 07.00-09.00 WIB (Nurjanaty et al., 2019), according to the predetermined field capacity. Mustard greens are harvested at the age of 42 days after planting (hst).

Data Collection

Observations were made at 42 days after planting on the following parameters.

Leaf Area (cm^2) and Number of Leaf

Leaf area measurement is carried out based on the gravimetric method, which involves comparing the total leaf weight with the weight of a leaf sample that has a known area. All leaf patterns (leaf replicas) drawn on the millimeter paper were weighed using an analytical scale. A 10 cm × 10 cm piece of paper is cut and weighed. The area of the leaves is calculated using the following formula:.

$$\text{Leaf Area (cm}^2) = \frac{\text{weight of leaf replica (g)}}{\text{weight of 10 cm} \times \text{10 cm paper (g)}} \times 100$$

(Irwan and Wicaksono, 2017)

The number of leaves counted is the leaves that have opened perfectly (Munthe et al., 2018).

Plant Height (cm) and Dry Plant Weight (g)

The height of the plant is measured from the base of the stem to the end of the plant's growth point (Munthe et al., 2018). Dry weight is measured by drying the sample in an oven at 80 ℃ until it reaches a constant dry weight.

Total Content of Flavonoids

Extraction and preparation of treatment samples

Mustard leaves are dried, then mashed and sifted. A total of 0.4 g of sample powder was macerated with 80% methanol, 10 mL 2 twice, then homogenized using *a shaker* for 12 hours at room temperature. The extract was filtered using Whatman Number 1 filter paper, and 10 mL of filtrate was stored in a vial bottle (Chang et al., 2002).

Preparation of flavonoid standard solutions

A standard solution of quercetin is used to create calibration curves. A total of 0.15 mg of standard quercetin was dissolved in 15 ml of 80% ethanol and then diluted to 20, 40, 60, 80, and 100 ppm. The diluted quercetin solution was then diluted to 0.5 ml and mixed with 1.5 ml of 80% ethanol, 0.1 ml of 10% $AlCl_3$, 0.1 ml of CH3COOK 1 M, and 2.8 ml of aqueducts separately. The mixed solution was then incubated for 30 minutes (Chang et al., 2002).

Spectrophotometric Analysis

The examination of the total absorbance value of flavonoids was measured at a wavelength of 415 nm using a UV-Vis spectrophotometer. The blank is prepared by pipetting up to 1.5 ml of 80% ethanol, 0.1 ml of $AlCl_3$, 0.1 ml of CH_3COOK 1 M, and 2.8 ml of aqua. The total flavonoid content is expressed by the equivalent mass of quercetin (Chang et al., 2002).

Data Analysis

The observation data were analyzed descriptively and statistically using Variance Analysis (ANOVA); if the results indicated a significant effect, the Duncan Multiple Distance Test at the 95% level (α = 0.05) was used to determine the differences.

RESULTS AND DISCUSSION

Effect of Humic Acid and Water Content on the Growth of Mustard Mustard

Plant Height

Plant height is the most frequently used and easily observable growth indicator for assessing the influence of the environment or the applied treatment, and can describe growth rate (Dong et al., 2019).

The ANOVA results showed that the application of humic acid, moisture content, and their interaction had a significant effect (P-value < 0.05) on mustard green height. To assess the difference between treatments, a Multiple Distance Test was conducted; the results are presented in **Table 1.**

Based on Table 1, humic acid applied at 1–12 g/kg increased plant height compared to the control at 20–60% field capacity, mainly because it enhances nutrient uptake under stress conditions. In contrast, at 80% field capacity, the addition of humic acid reduced plant height. This decrease occurs because plants with sufficient water may absorb excess moisture, causing growth suppression as noted by Zlatev and Lidon (2005).

The average height of the tallest plant, 23.67 cm, was observed in the 20% KL treatment with 12 g/kg humic acid. It is suspected because, during treatment with 20% KL, the soil water content is very low. The addition of humic acid contributes to the availability and uptake of water and N by plants. Nitrogen plays a role in spurring vegetative growth and photosynthesis (Ginandjar et al., 2019). Photosynthate produced by photosynthesis will be dispersed and accumulated in plant organs, including stems, thereby increasing plant height (Herman & Dibyo, 2013).

In **Table 1,** the lowest average plant height of 12.875 cm was observed in the 20% KL treatment without humic acid. Hussain and Ali (2015) reported that stem elongation and leaf area can be hampered when plants experience water shortages. Dong et al. (2019) also reported that water stress can inhibit plant growth, especially plant height and leaf area. During cell elongation, plants require an appropriate water balance because the force driving elongation is turgor pressure (Latuharhary & Saputro, 2017). Drought conditions can limit nutrient uptake by plants due to reduced soil moisture, resulting in shorter stems (Kapoor et al., 2020).

Table 1. Average Increase in Height of Mustard Plants (cm) in Humic Acid Treatment and Different Water Content

Moisture Content (Spacious)	**Humic Acid**			
	0 g/kg	4 g/kg	8 g/kg	12 g/kg
20%	12,875a A	20ab B	23,25b B	23.67b C
40%	14,75a A	17,625a A	20.875b B	17,975a B
60%	15a B	17,25ab A	17,475ab AB	17,925b B
80%	18.65b B	16.5ab A	15,175ab A	14,625a A

Remarks: The average values with the same lowercase letter on the same row and the same uppercase letter in the same column show no significant difference according to the Duncan Multiple Spacing test at the 5% significance level.

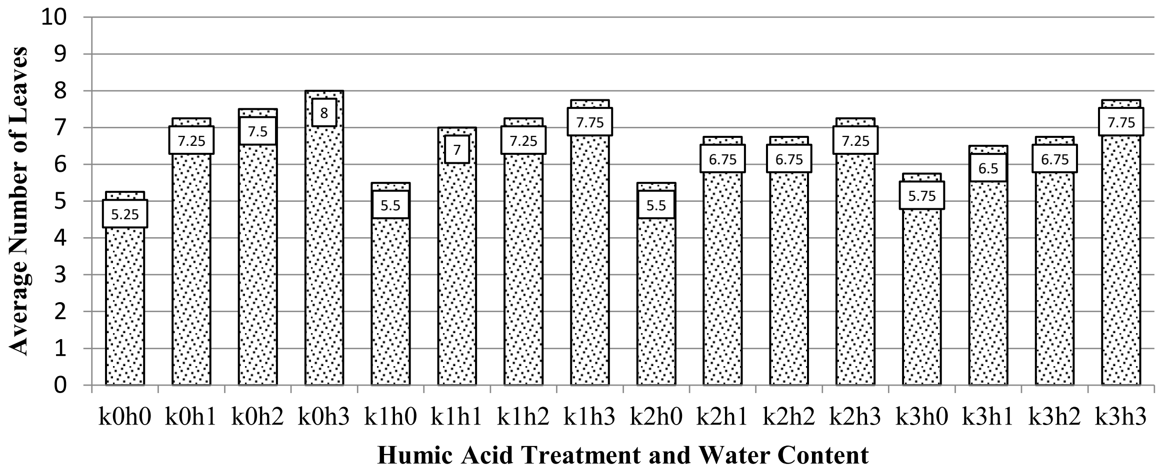

Figure 1. Graph of the Average Number of Mustard Leaves in Humic Acid Treatment and Different Water Content.
Description: k0 = 20% KL, k1 = 40% KL, k2 = 60% KL, k3 = 80% KL, h0 = 0 g/kg, h1 = 4 g/kg, h2 = 8 g/kg, and h3 = 12 g/kg

Number of Leaves

The number of leaves affects a plant's photosynthetic efficiency. Leaves serve as sites of absorption and conversion of sunlight through photosynthesis for plant growth (Mathur et al., 2018). Based on ANOVA, it is known that the administration of humic acid, the moisture content is different, and the interaction has no real effect (P-value < 0.05) on the average number of leaves. The average number of mustard greens with different water and humic acid content is presented in **Figure 1**.

Figure 1 shows that the average number of leaves increased with increasing humic acid dose across all field capacity treatments. Bakry et al. (2014) stated that humic acid plays a role in activating the plant's defense system against environmental stress by stimulating levels of growth regulators and enhancing the protection of photosynthetic pigments. Increased photosynthesis leads to greater carbohydrate availability, which in turn increases the number and area of leaves (Bassi et al., 2018).

Based on **Figure 1**, the average highest number of leaves (8 leaves) was found in the k0h3 treatment (20% KL + 12 g/kg humic acid). The plant's tolerance to the lowest moisture content at the 20% KL treatment is supported by humic acid, which helps the plant survive these unfavorable conditions. According to Sembiring et al. (2015), humic acid can increase nutrient absorption, primarily N and P, which are involved in leaf formation. The application of humic acid can reduce evapotranspiration, increase *water holding capacity,* reduce soil erosion, improve soil structure, and increase soil KTK (Khaled & Fawy, 2011). The higher the KTK value in soil, the greater the soil's potential to provide nutrients for plants (Bhaskoro et al., 2015).

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Table 2. Average Area of Mustard Leaves (cm2) in Humic Acid Treatment and Different Water Content

Moisture Content (Spacious)	Humic Acid			
	0 g/kg	4 g/kg	8 g/kg	12 g/kg
20%	0.295a A	0.815ab C	0.913b B	1.022c C
40%	0.368a A	0.693ab B	0.902b B	0.953b B
60%	0.513a B	0.652a A	0.738ab AB	0.915b B
80%	0.535a B	0.625ab A	0.663b A	0.663b A

Remarks: The average values with the same lowercase letter on the same row and the same uppercase letter in the same column show no significant difference according to the Duncan Multiple Spacing test at the 5% significance level.

Figure 1 also shows that the k0h0 treatment (20% KL without humic acid) resulted in the lowest average number of leaves (5 leaves). Drought stress suppresses cell expansion and cell growth due to low turgor pressure. Plants will adapt by reducing leaf number and leaf area in response to drought, thereby increasing survival but decreasing growth rate and biomass (Abobatta, 2019). According to Samanhudi et al. (2017), the greater the water grip pressure, the greater the value of leaf damage intensity (IKD) that damages chloroplasts, so that the leaves will quickly experience chlorosis and *senescence.* It leads to a decrease in the number of leaves.

Leaf Area

Leaf area is a sensitive indicator of plant response to water stress (Dong et al., 2019), and ANOVA results showed that humic acid, moisture level, and their interaction significantly affected mustard leaf area ($P < 0.05$). Duncan's test results (**Table 2**) indicate that at 0 g/kg humic acid, higher moisture levels reduce the decline in leaf area. Under this condition, the 80% field capacity treatment produced the largest average leaf area, measuring 0.535 cm².

Hendri and Ansar (2014) stated that plants need enough water to maintain turgor and leaf expansion. The application of 12 g/kg humic acid with a moisture content of 20% KL resulted in the highest average leaf area of 1,022 cm^2. Although increased water retention leads to reduced leaf area to avoid excessive transpiration with low stomata density (Riaz et al., 2013), humic acid application can reduce water evaporation by reducing the opening of stomata on the leaves, so that plants and soil can retain more water and increase plant resistance (Li, 2020).

The lowest average leaf area was obtained in the treatment of 20% KL without humic acid, which was 0.295 cm2 **(Table 2).** In drought-stressed conditions, leaf area tends to decrease as plants adapt to reduce transpiration (Hidayati et al., 2017). During the drought, the growth and development of young leaves are stunted. Cell shrinkage and leaf aging are followed by the abortion of old leaves, resulting in a reduction in leaf area.

Dry Weight of Plants

The dry weight of plants indicates the accumulation of inorganic compounds that the plant synthesizes into organic compounds (Suryaningrum et al*.,* 2016), such as proteins, carbohydrates, and lipids (fats), as well as the accumulation of photosynthates located in the stems and leaves (Sehgal et al., 2018). The ANOVA results showed that the application of humic acid, different moisture content, and their interactions had a significant effect (P-value < 0.05) on the dry weight of mustard green plants. To examine differences in these interactions among treatments, the Duncan Multiple Distance Test was performed, with the results presented in **Table 3.**

Based on **Table 3,** the average dry weight increases with increasing humic acid dose across all field capacity treatments. Suhardjadinata et al. (2015) reported that humic acid in the medium can increase soil N levels, stimulating vegetative growth and increasing the number of saplings (Habibullah et al., 2015), thereby significantly affecting plant height, leaf area, and plant dry weight.

Table 3 also shows that in the application of humic acid up to 4 g/kg, dry weight tends to increase in line with the increase in field capacity, but the application of 8 g/kg and 12 g/kg of humic acid leads to a decrease in the dry weight of the plant. The dry weight of the plant is positively related to the level of N in the soil and its absorption by the plant. The higher the N level and absorption, the greater the N requirement during the plant's vegetative phase, thereby increasing plant biomass (Zhao et al., 2014). The highest average dry weight of 0.55 grams was obtained in the treatment with 20% KL and 12 g/kg humic acid in the medium. According to Amanah and Putra (2018), humic acid can maintain plant vigor under drought-stress conditions.

Table 3. Average Dry Weight of Mustard Plants (g) at Humic Acid Treatment and Different Water Content

Moisture Content (Spacious)	Humic Acid			
	0 g/kg	4 g/kg	8 g/kg	12 g/kg
20%	0.04a A	0.043a A	0.198b C	0.55c C
40%	0.049a A	0.053a A	0.183b C	0.41c B
60%	0.062a A	0.11b B	0.16b B	0.293c AB
80%	0.075a B	0.12a B	0.14b A	0.21c A

Description: The average value followed by the same lowercase letter on the same line and the same uppercase letter in the same column shows no significant difference according to the Duncan Double Distance Test at a significant level of 5%.

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Table 4. Average Total Flavonoid Content of Mustard Mustard (mg QE/g Sample) in Humic Acid Treatment and Different Water Content

Moisture Content (Spacious)	Humic Acid			
	0 g/kg	4 g/kg	8 g/kg	12 g/kg
20%	0.076a B	0.083a B	0.096a C	0.108b C
40%	0.052a A	0.064a AB	0.076ab B	0.103b B
60%	0.05a A	0.054a A	0.072b B	0.088b B
80%	0.049a A	0.053a A	0.057a A	0.085b A

Description: The average value followed by the same lowercase letter on the same line and the same uppercase letter in the same column shows no significant difference according to the Duncan Double Distance Test at a significant level of 5%.

Meanwhile, the 20% KL treatment without humic acid produced the lowest average dry weight of 0.04 g (**Table 3**), likely due to limited water availability for photosynthesis. This inhibition reduces growth rate, causing water stress to lower both fresh and dry weight (Jumawati et al., 2014). Wibowo & Sitawati (2017) stated that water-deficit conditions can reduce turgidity in plant cells, thereby inhibiting cell multiplication and enlargement. Water retention also disrupts nutrient transport and plant biochemical processes, as indicated by the plant's low dry weight (Pour-Aboughadareh et al., 2019).

Effect of Humic Acid and Water Content on Flavonoid Levels

Based on ANOVA, humic acid application, water content levels, and their interaction significantly affected the total flavonoid content of mustard greens ($P < 0.05$). A Duncan Multiple Range Test was then used to compare treatment differences. The results are shown in **Table 4**. **Table 4** shows that the average flavonoid content increased with increased doses of humic acid at all field capacity treatments.Suhardjadinata et al. (2015) reported that humic acid can increase nutrient availability in the planting medium, thereby enhancing the production of active compounds, such as polyphenols.

The addition of humic acid under drought conditions is also one of the efforts to increase secondary metabolite production by boosting plant biomass. Biomass production will increase if plant growth increases (Hardiyati et al., 2020). The administration of humic acid can increase the absorption of nutrients, including Cu, Mg, N, P, and K (Riyandi et al., 2020). Ali et al. (2006) showed that Cu can enhance PAL enzyme activity involved in polyphenol production. Meanwhile, Soedradjad & Sunihar (2017) reported that higher N absorption optimizes enzymes in flavone biosynthesis, thereby increasing flavonoid levels.

Table 4 also shows that the highest flavonoid content of 0.108 mg QE/g was obtained with the 20% KL and 12 g/kg humic acid treatment. Nephali et al. (2020) reported that humic acid can function optimally in plants under environmental stress. One form of plant defense against drought stress is the increased production of secondary metabolites, including phenolics and flavonoids (Garibi et al., 2015), which is driven by the increased production of *Reactive Oxygen Species* (ROS) under drought stress (Gill & Tuteja, 2010). The accumulation of ROS in plant cells due to disruption of osmotic pressure and the toxic effects of drought can lead to the overproduction of phenolic compounds, including flavonoids (Abogadallah, 2010).

In this study, the measurement of secondary metabolite levels is limited to total flavonoid compounds, so it is necessary to develop research on other types of potential secondary metabolites, in addition to being able to use other biostimulants, such as chitosan, to determine the effect of both the growth and content of various secondary metabolite compounds in mustard greens under drought stress.

CONCLUSION

Different water content and humic acid doses affect the growth and flavonoid content of mustard greens (*Brassica juncea* L.). Treatment of 20% field capacity (KL) and humic acid of 12 g/kg provides the best growth and total content of mustard greens flavonoids under drought-stressed conditions with a plant height of 23.67 cm, the number of leaves eight strands, the leaf area of 1.022 cm^2, the wet weight of 5.008 g, the dry weight of 0.55 g, and the total flavonoid content of 0.108 mg QE/g of the sample.

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