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Seed germination and growth of Joseph's coat (*Amaranthus tricolor* L.) following exposure with Naphthalene-1-Acetic Acid (NAA) and 2,4-Dichlorophenoxyacetic Acid (2,4-D)

Hadyan Pratama Lutfi Ilmam*, Kumala Dewi

Faculty of Biology, Universitas Gadjah Mada
Jl. Teknika Selatan, Sekip Utara, Yogyakarta 55281, Indonesia
*Corresponding author: hadyan.ilmam@gmail.com

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Abstract

Amaranthaceae is a family of plants that can be used as vegetables and medicinal herbs. *Amaranthus tricolor* L. is commonly cultivated because it has fast growth rate and short life cycle that can be boosted by growth regulators such as auxins. *A. tricolor* L. is commonly cultivated because it has a fast growth rate and short life cycle. Growth regulators, such as auxins, can boost the growing process. This research aimed to study the effects of Naphthalene-1-Acetic Acid (NAA) and 2,4-Dichlorophenoxyacetic Acid (2,4-D) on the seed germination and growth of *A. tricolor* L. and to determine effective concentration of NAA or 2,4-D application to *A. tricolor* L. This research was arranged in a completely randomized design with exogenous hormones application as treatments. The treatments consisted of various concentrations of NAA and 2,4 D (0 ppm, 10 ppm, 20 ppm, 40 ppm, and 80 ppm) applied to *A. tricolor* L. plants every two weeks. Germination test of *A. tricolor* L. was carried out for 14 days, and the application of NAA and 2,4-D on *A. tricolor* L. plant was given for 56 days. Observations were made on the plant height, fresh and dry weight, stomatal density, and the content of chlorophyll and carotenoid. Data analysis was conducted using one-way analysis of variance and Duncan Multiple Range Test (DMRT) with significance level of 5%. NAA treatment delayed seed germination by one day compared to control, while 2,4-D treatment inhibited germination for several days with the higher concentration of 2,4-D applied, the greater inhibition of seed germination. NAA of 10 ppm increased plant height, fresh and dry weight, chlorophyll content, and leaf area of *A. tricolor* L. The application of NAA and 2,4-D reduced stomatal density and carotenoid content of *A. tricolor* L., with greater effects at higher concentrations of synthetic auxins. This research concluded that NAA or 2,4-D inhibited germination of *A. tricolor* L. seeds, NAA of 10 ppm effectively increased plant growth and chlorophyll content, but higher NAA concentrations inhibited growth. Application of 2,4-D with concentrations above 40 ppm could be lethal for *A. tricolor* L.

INTRODUCTION

Amaranthaceae is a family of plants that consist of more than 400 species, and some of its species are used as cultivated plants. Amaranths can grow rapidly even during the dry and hot season. One of Amaranths genus, *Amaranthus*, is commonly used as vegetable amaranth, ornamental plants, and weeds. Amaranthus considered as weeds are *Amaranthus hybridus*, *Amaranthus powelii*, and *Amaranthus quitensis*. *Amaranthus lividis* and *Amaranthus tricolor* are used as vegetable amaranths (Rastogi and Shukla, 2013).

Amaranthus tricolor L. or Joseph's coat is a kind

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of common vegetable that has a unique color, rich in nutrients, and delicious taste. *A. tricolor* L. has greenish, reddish, or purplish leaves and can grow in different conditions, especially in hot and dry weather. *A. tricolor* L. is commonly consumed as soup ingredients and noodles. Leaves of *A. tricolor* L. contain proteins (66.26 g/kg–11.38 g/kg), fiber (91.94 µg/g–59.96 µg/g), fat (4.35 g/kg–1.42 g/kg), carbohydrates (98.54 g/kg–15.48 g/kg), iron (1089.19 µg/g), calcium (10.13 mg/g), magnesium (30.01 mg/g), potassium (24.96 mg/g), and zinc (986.61 µg/g). Leaves of *A. tricolor* L. also contain vitamin C (955.19 µg/g), beta-carotene (1043.18 µg/g), betalains (66.40 µg/100g), betaxanthins (33.09 µg/100g), and betacyanin (33.30 µg/100g), so *A. tricolor* L. can be used as highly nutritious tropical vegetable (Sarker and Oba, 2019). Leaves of *A. tricolor* L. are rich in alkaloids, glycosides, phenolics, flavonoids, amaranthine, tannins, and other pigments; therefore, this plant can be potentially useful in agriculture, pharmaceuticals, and food industries (Jahan et al., 2022).

A. tricolor L. is widespread globally with the highest abundance in tropical region, especially in South and Southeast Asia. *A. tricolor* L. is originally from India and cultivated in Indonesia or other countries (Rastogi and Shukla, 2013). *A. tricolor* L. is commonly cultivated in tropical-subtropical region with adequate groundwater and loose soil. *A. tricolor* L. can grow quickly with short life cycle, but its growth can be accelerated by plant growth regulators (Bala et al., 2019).

One of the plant growth regulators that is commonly used in agriculture is auxin. Auxin is a group of compounds that have a big role in plant physiology, such as initiating cell elongation with acid-growth theory, improving lateral root growth and inducing adventitious root, inducing vascular differentiation, leaf budding, regulating apical growth in geotropism and phototropism, inhibiting leaf abscission, and regulating fruit growth. Auxin is naturally synthesized using Indole-3-pyruvic acid pathway with tryptophane as a precursor. Auxin is synthesized in apical buds and young leaves and polarly transported as Indole acetic acid (IAA) and Indole butyric acid (IBA) through plant (Urry et al., 2016).

Synthetic auxins that are commonly used are Naphthalene-1-acetic acid (NAA) and 2,4-Dichlorophenoxy acetic acid (2,4-D). These synthetic auxins have a similar chemical structure to natural auxins but with specific uses for each synthetic auxin types, for example, NAA is commonly used to induce rooting in plants, and 2,4-D is commonly used as an herbicide for weed control. As a plant growth regulator, 2,4-D is not easily degradable; therefore, it can accumulate in plants and causes auxin overdose, thus can be lethal in plants (Todd et al., 2020). Chemical stability enables 2,4-D to be used as herbicide to control weeds in agriculture, but 2,4-D lethality may affect horticultural plant's survivability (Zuanazzi, Ghisi and Oliveira, 2020). The effects of synthetic auxins, such as NAA and 2,4-D, on germination and plant growth is not well understood on some horticultural plants such as amaranths; therefore, this research was conducted to study the effects of NAA and 2,4-D on the seed germination and growth of *A. tricolor* L. and to determine the effective concentration of NAA or 2,4-D application to *A. tricolor* L.

MATERIALS AND METHODS

Location, time, and plant materials

This study was conducted in Laboratory of Plant Physiology, Universitas Gadjah Mada for germination test in March 2023 for 14 days, while the growth test of *A. tricolor* L. was conducted in the Experimental Greenhouse at Faculty of Biology, Universitas Gadjah Mada from May 2023 to July 2023 for two months. *A. tricolor* L. seeds were purchased from the local agricultural shop located at Daerah Istimewa Yogyakarta.

Germination test with synthetic auxins

The planting media were prepared using cotton mediums inside petri dishes. Cotton media were given with 20 mL of NAA or 2,4-D with concentrations of 0 ppm; 10 ppm; 20 ppm; 40 ppm; and 80 ppm (n = 5). *A. tricolor* L. seeds were selected with similar sizes, then the seeds were put inside petri dishes with each petri dishes containing 20 seeds. Petri dishes were sealed and put in bright conditions for two weeks. The seeds were observed every 24 hours to record the number of germinated seeds of every treatment. After two weeks, five sprouts were selected from each petri dishes of every treatment, and their length was measured using millimeter block paper.

Growing conditions and synthetic auxin treatments

A. tricolor L. seeds were grown on a mixed soil media that consisted of soil and dried bran (3:1) with adequate light conditions. *A. tricolor* L. seeds were planted in 27 pots with 10 seeds in every pot. Plants were given 10 mL water daily for eight weeks. NAA or 2,4-D were sprayed on plants at concentrations of 0 ppm, 10 ppm, 20 ppm, 40 ppm, and 80 ppm every two weeks. Plant height measurement was conducted weekly for each plant and treatment.

Fresh and dry weight measurement

A. tricolor L. was harvested at 56 days after planting (DAP), and 18 uniform plants from each treatment were selected. The roots were rinsed from soil residues, and then plant specimens from each treatment were measured for fresh weight. Specimens from each treatment were dried using an oven at 58°C for seven days, and dry weight measurement was conducted daily.

Leaf area measurement

Leaves of *A. tricolor* L. were selected from three individuals for each treatment, with third, fourth, and fifth leaves were selected from each plant. Nine leaves from each treatment were harvested and then scanned using EPSON L360 scanner with 300 depths per inch (dpi) resolution. Scanned leaves images were measured using ImageJ software that has been calibrated for leaf area measurement (Agehara et al., 2020).

Stomatal density calculation

A. tricolor L. leaves were cleaned on the abaxial and adaxial sides. Transparent nail polish was applied on the abaxial and adaxial sides of *A. tricolor* L. leaves from each treatment. After 15 minutes, transparent tape was applied onto polished leaves, then the tape was removed carefully to separate the leaf epidermis from mesophyll. Leaf epidermis was put on glass slides, then samples were observed using a microscope with 10×40 magnification. Observed stomata were counted, and stomatal density was calculated using the following equation (Poole and Kürschner, 1999):

$$\text{Stomata density} = \frac{\text{Total number of stomata}}{\text{Area}} \quad .....(1)$$

Chlorophyll and carotenoid content measurement

Leaf tissues from each treatment were collected, then 0.1 grams of leaf tissue were extracted using 10 mL acetone 80%. Leaf extracts were filtered using Whatman paper No. 3, and then 1 mL filtered leaf extracts were measured using spectrophotometry at three wavelengths (480 nm, 645 nm, and 663 nm). Absorbance values of leaf samples from each treatment were recorded three times, then the absorbance values obtained from spectrophotometry were used for calculation of chlorophyll (Arnon, 1949) and carotenoid (Hendry and Grime, 1993) content with the following equation:

$$\text{Chlorophyll (mg/g)} = \frac{[(20.2\times A_{645}) + (8.02\times A_{663})] \times V}{1{,}000 \times W} \quad .....(2)$$

$$\text{Carotenoid (mg/g)} = \frac{[A_{480}+(0.114\times A_{663})-(0.638\times A_{645})]\times V}{1{,}000 \times W} \quad ...(3)$$

Remarks: A_x = absorbance value at x nm, V = extract volume (mL), W = sample weight (g).

RESULTS AND DISCUSSION

Effects of synthetic auxin on the germination of *Amaranthus tricolor* L.

A. tricolor L. germination negatively was affected by synthetic auxin treatment, especially 2,4-D treatment (Figure 1). NAA treatment at concentrations of 10 ppm, 20, ppm and 40 ppm decelerated germination for one day, while NAA at a concentration of 80 ppm decelerated germination for two days. Seed germination was inhibited by 2,4-D treatment, with increased inhibition duration as 2,4-D concentration was raised. The germination of *A. tricolor* L. with 2,4-D 10 ppm treatment nearly reached 100% after seven days, while 2,4-D treatment above 20 ppm decelerated and inhibited germination until 14 days. Seeds treated with 2,4-D at a concentration of 20 ppm germinated after two days, while those treated with 2,4-D at concentrations pf 40 and 80 ppm germinated after seven days.

There was significant different effect between NAA treatment with 2,4-D treatment and control on the germination of *A. tricolor* L. seeds on the first day. NAA treatments slightly decelerated seed germination, with increasing effects as NAA concentration increased. The NAA effects only lasted for one day; therefore, the germination rates of *A. tricolor* L. seeds between NAA concentrations were not significantly different after three days. The 2,4-D treatments strongly inhibited seed germination, with increased inhibition

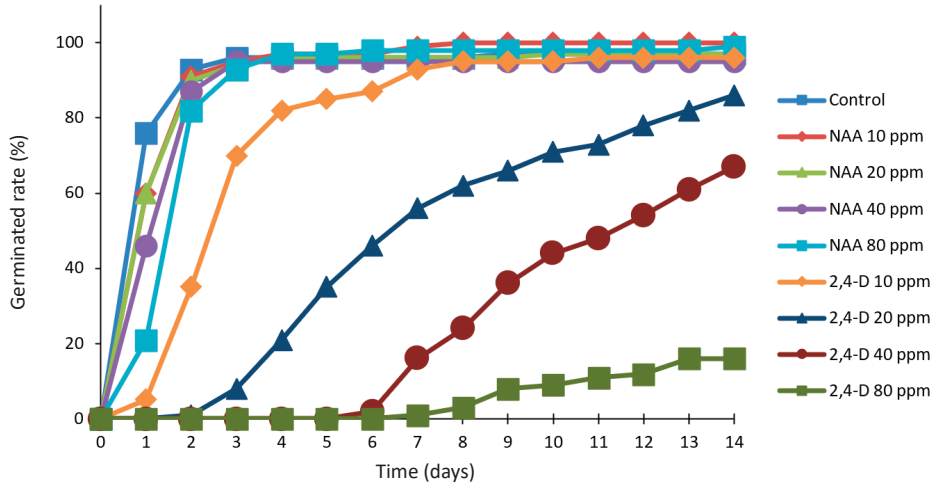

Figure 1. Germination rate of *Amaranthus tricolor* L. seeds as affected by NAA and 2,4-D treatment at concentrations of 10, 20, 40, and 80 ppm for 14 days

duration as 2,4-D concentrations increased. After 14 days, the germination rate of the seed treated with 2,4-D at concentrations of 20, 40, and 80 ppm were 86%, 67%, and 16%, respectively.

Synthetic auxins treatment with different concentrations decelerated and inhibited seed germination at different rates for each treatment. This occurred because auxins indirectly affects gibberellic acid (GA) and abscisic acid (ABA) signals in germination (Wu, Wu and Gan, 2020). Excess exogenous auxins increase ABA biosynthesis and inhibit GA biosynthesis; thereby interrupting germination process (Shuai et al., 2017). Increased concentration of NAA treatment further reduced the germination rate of *A. tricolor* L. on the first day one, but the germination rate returned to normal after the third day with germination rate between NAA treatments and control no longer significant. NAA have similar structure and properties to IAA; therefore, plants can regulate excess NAA into other substances, such as naphthol or salicylic acid (Proctor, 1963), so that excess auxin will not inhibit seed germination (Butova et al., 2023).

Treatment of 2,4-D at different concentrations significantly inhibited seed germination of *A. tricolor* L. compared to NAA treatments and control. Synthetic auxin like 2,4-D is easily dissolved and absorbed into young plant tissue; therefore 2,4-D can accumulate inside a plant cell and inhibit germination with greater effects if compared to NAA (Islam et al., 2018). The effects of 2,4-D on inhibition of germination last longer than NAA treatments, because 2,4-D is relatively stable structurally and can only be conjugated in a plant's system; thus, 2,4-D persists longer and harder to be degraded by plant, especially on sensitive broadleaf plants (Peterson et al., 2016). Germinated seeds after 14 days of treatment are shown in Figure 2.

A. tricolor L. sprouts treated with synthetic auxin treatment for 14 days were shorter when compared to those with control treatment. Sprouts with control treatment had purplish-red stems, yellowish-white main root, and very thin lateral roots. Sprouts with NAA and 2,4-D treatments had thicker, shorter stems and roots without lateral roots, with distinct pale stems on sprouts treated with 2,4-D at concentrations of 40 and 80 ppm (Figure 2).

The differences in sprout morphology between the control and NAA and 2,4-D treatment are caused by excess exogenous auxins for each treatment. This excess auxin affects plant growth by focusing growth on the main root, so the main root becomes thicker, and no lateral roots are formed. In addition, higher concentrations of auxins can stimulate ethylene biosynthesis that causes a triple response effect, including thickened stem, stunted stem growth, and curved stem shape (Ahammed, 2020). The presence of ethylene inhibits cell expansion, so stem and roots become shorter (Duboiscet al., 2018). The measurement of sprouts is shown in Figure 3.

A. tricolor L. sprouts in the control treatment were longer compared to those treated with NAA and 2,4-D, with an average sprout length in the control treatment was 2.173±0.162 cm (Figure 3). Sprouts in NAA treatments were significantly shorter than those in control treatment (about 25–35% of control treatment), with increased NAA concentrations further reducing sprout length, although the differences were

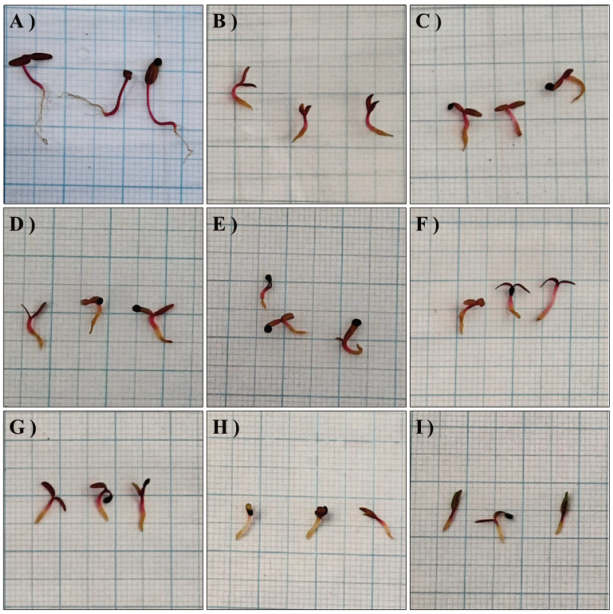

Figure 2. *Amaranthus tricolor* L. sprouts after 14 days with synthetic auxins treatments; A) Control; B) NAA 10 ppm; C) NAA 20 ppm; D) NAA 40 ppm; E) NAA 80 ppm; F) 2,4-D 10 ppm; G) 2,4-D 20 ppm; H) 2,4-D 40 ppm; I) 2,4-D 80 ppm

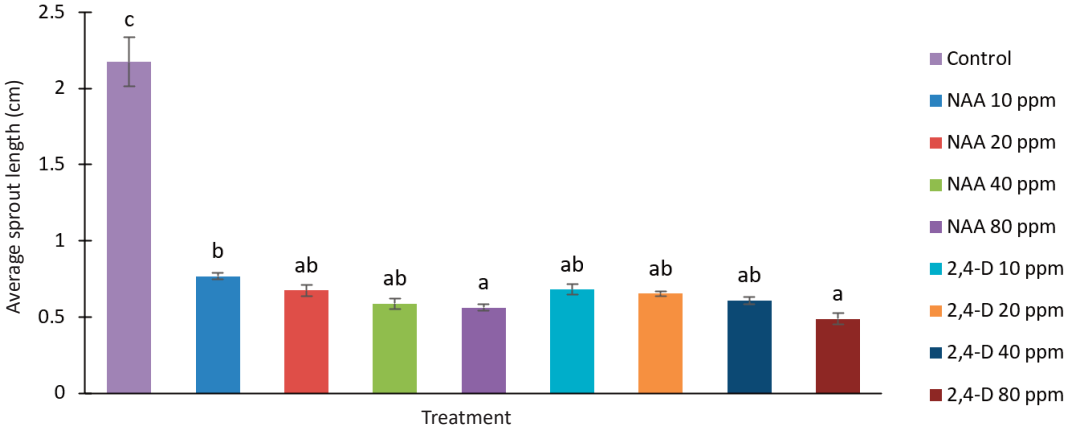

Figure 3. Average sprout length of *Amaranthus tricolor* L. as affected by NAA and 2,4-D treatment at concentrations of 10, 20, 40, and 80 ppm after 8 weeks; Bars associated with different letters are statistically different as assessed by the Duncan Multiple Range Test (DMRT) at 5% level

not significant, except sprouts with NAA treatment at concentrations of 10 and 80 ppm. Sprouts treated with 2,4-D were also shorter in size compared to those in control treatment (22–31% of control treatment), with increased 2,4-D concentrations further reducing sprout length, although the differences were not significant. Sprouts in the 2,4-D treatments were shorter when compared to sprouts with NAA treatments although the difference in sprout length was not significant.

NAA and 2,4-D treatments decreased sprout growth with sprouts length only one-fifth to one-third of the sprout length in control treatment. Different synthetic auxin treatments, in this context NAA and 2,4-D, did not show significantly different results, so auxin is not directly involved in the inhibition of sprout growth. Auxins, especially synthetic auxins, at high concentrations can induce biosynthesis of ethylene and induce ABA signals, thereby causing stress response in plants (Sybilska and Daszkowska-Golec, 2023). The sprout's length in 2,4-D treatments was shorter compared to that in NAA treatments, because

the 2,4-D compound tends to be more stable and difficult to degrade compared to NAA, so the auxin effects in 2,4-D treatments are stronger than in NAA treatments.

Effects of synthetic auxin on the growth of *Amaranthus tricolor* L.

Plant height as affected by NAA and 2,4-D treatments at various concentrations showed various trends, with NAA and 2,4-D treatments increasing plant height until the second week, but the growth rate of plants was decelerated after the third week (Figure 4). In general, NAA at a concentration of 10 ppm and 2,4-D at a concentration of 10 ppm increased plant height by up twice that in control treatment, while NAA at a concentration of 20 ppm increased plant height by 35% compared to that in control treatment. The highest plant after 8 weeks of treatment was found in NAA treatment at a concentration of 10 ppm with an average plant height of 8.48±0.63 cm, and the lowest was found in the 2,4-D treatment at a concentration of 80 ppm with all plants in the treatment died after the sixth week. The 2,4-D treatment at a concentration of 40 ppm was lethal on treatment plants, while the plant height as affected by NAA and 2,4-D treatments at a concentration of 20 ppm were similar when compared to control treatment.

NAA treatment increased plant height quicker than the control treatment after the first application of NAA (second to fourth week). This shows that NAA treatment stimulates plant growth, especially in the shoot apex, by stimulating cell division at the shoot apex, so the stem grows faster with NAA treatment. Increased NAA concentrations were inversely related to the growth acceleration of stems, with the plant height as affected by NAA treatment at a concentration of 80 ppm was similar to that in control treatment. Auxin applied at high concentrations induces stress hormones, such as ABA and ethylene, thereby inhibiting shoot growth (Sybilska and Daszkowska-Golec, 2023).

The 2,4-D treatment increased plant height when compared to control after 2,4-D application (second to third weeks). This shows that 2,4-D stimulates plant growth, especially in stems, by accelerating cell division in shoot apex; therefore stem growth is faster with 2,4-D treatment than with the control treatment. Changes in the effect of 2,4-D on the plant height occurred after the fourth week, where 2,4-D treatment at concentrations of 10 and 20 ppm reduced stem growth, and 2,4-D treatment at concentrations 40 and 80 ppm caused mortality of plants. The 2,4-D compounds are difficult to degrade, thereby inducing auxin accumulation and increasing ethylene and ABA levels in plants. Increased auxin, ethylene, and ABA levels that exceeds the limit causes uncontrolled growth that is indicated by physical symptoms such as tissue swelling, stem bending, and decreased plant productivity (Peterson et al., 2016).

NAA treatments have better effects on the plant height compared to 2,4-D treatment, with NAA treatment increasing plant growth at concentrations of 10 to 80 ppm, while 2,4-D treatments are lethal to plants with a higher risk of lethality at higher

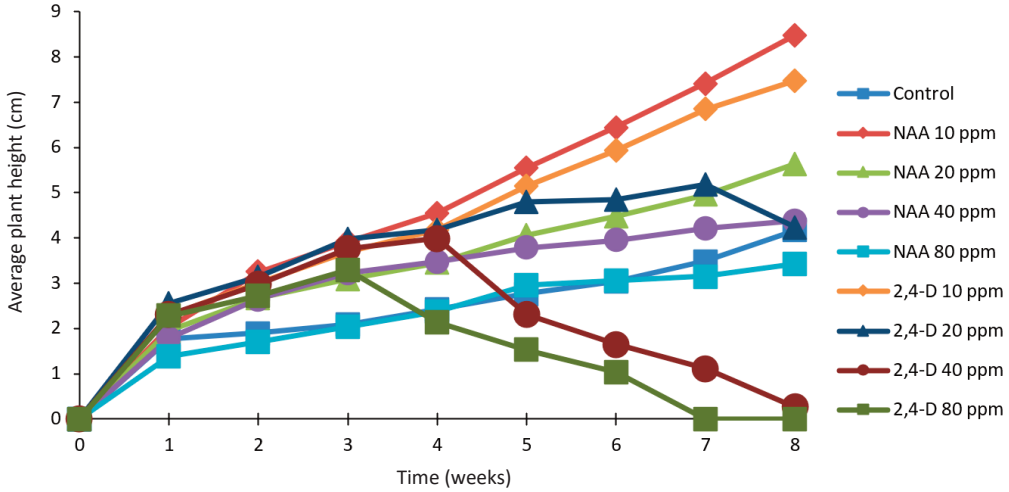

Figure 4. Average height of *Amaranthus tricolor* L. plants as affected by NAA and 2,4-D treatment at concentrations of 10, 20, 40, and 80 ppm after 8 weeks

concentrations, except for 2,4-D treatment at a concentration of 10 ppm that still increased plant growth. This reflects to the structure and function of each synthetic auxin compound, with NAA as a plant growth promoter and 2,4-D as a herbicide (Flasiński and Hąc-Wydro, 2014). Effects of NAA treatment at concentrations above 20 ppm are not effective on the growth of *A. tricolor* L. plants, resulting lower plant height compared to that in NAA treatment at a concentration of 10 ppm. This is because excess auxins in plants can alter signals of other phytohormones, leading to uncontrolled hormone regulation (Sybilska and Daszkowska-Golec, 2023).

Effects of synthetic auxins on the fresh and dry weight

Fresh weights of *A. tricolor* L. plants harvested after 60 days showed that NAA and 2,4-D treatments had significant effects on plant fresh weights (Figure 5). NAA treatment at concentrations of 10 and 20 ppm, as well as 2,4-D treatment at concentrations of 10 ppm significantly increased plant fresh weight compared to control treatments, with the highest value in NAA treatment at a concentration of 10 ppm (15.5361 g). NAA treatment at concentrations of 40 and 80 ppm, as well as 2,4-D treatment at concentrations of 20 and 40 ppm had lower fresh weight compared to control treatments, with the lowest value in 2,4-D treatment at a concentration of 40 ppm (0.2101 g). The 2,4-D treatment at a concentration of 80 ppm inhibited the growth of *A. tricolor* L. plants until the plants dried out and died.

The application of NAA at a concentration of 10 ppm increased the fresh weight of *A. tricolor* L. plants by up to 3.5 times from the control treatment, while the NAA treatment at a concentration of 20 ppm increased the plant fresh weight by 160% from the control treatment. This happens because NAA at concentrations of 10 and 20 ppm can trigger cell division, induce the appearance of leaf buds, and increase cell growth and elongation so that plants grow faster accompanied by an adequate supply of essential macromolecules (Gil et al., 2020). NAA treatment at concentrations of 40 and 80 ppm inhibited plant growth, characterized by plant fresh weight that was only 84% and 71% of plant fresh weight with control treatment. This happens because NAA at high concentration causes auxin accumulation, which induces ABA and ethylene synthesis as plant stress response, resulting in slow plant growth with decreased metabolic rate characterized by lower plant fresh weight. Each NAA treatment at different concentrations shows significant different results from each other, where an increase in NAA concentration can lead to lower plant fresh weight with NAA concentration effective in increasing plant fresh weight was 10 ppm.

The 2,4-D treatment at a concentration of 10 ppm increased the fresh weight of plants to 2.5 times the fresh weight of plants with a control treatment, but 2,4-D treatment at concentrations above 20 ppm decreased the fresh weight of plants significantly

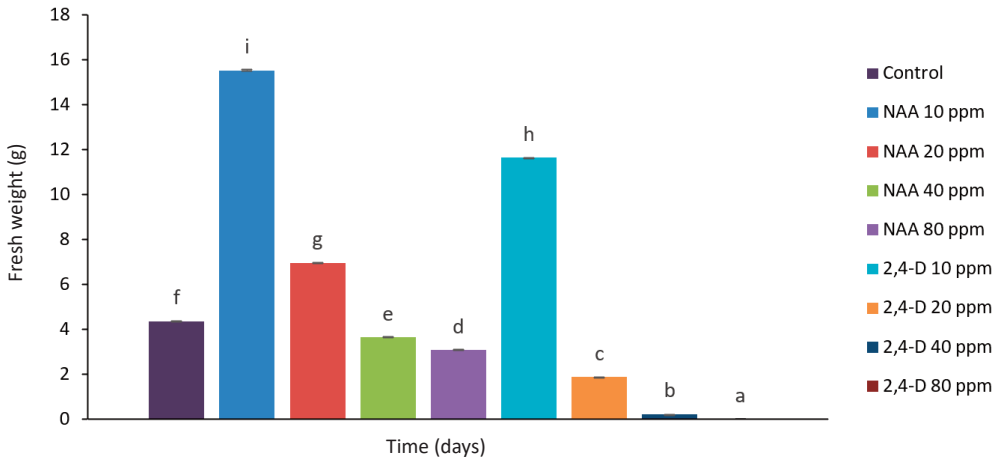

Figure 5. Fresh weight of *Amaranthus tricolor* L. plants as affected by NAA and 2,4-D treatment at concentrations of 10, 20, 40, and 80 ppm after 8 weeks; Bars associated with different letters are statistically different as assessed by the Duncan Multiple Range Test (DMRT) at 5% level

when compared to the control treatment. This happens because 2,4-D at a concentration of 10 ppm accelerates cell growth, cell division, and increases metabolism that is still balanced with the ability of plants to supply nutrients and energy from the environment. The 2,4-D treatment at concentrations of 20 and 40 ppm reduced the fresh weight of plants due to excess accumulation of synthetic auxin in plants so that plants will continue to grow without being balanced with a sufficient supply of nutrients and energy. This translocation causes old plant organs to age and fall off so that the whole plant weight is lower. The dry weight of the plants from each treatment is presented in Figure 6.

The dry weight of *A. tricolor* L. plants as affected by NAA and 2,4-D treatments differed significantly for each concentration (Figure 6). The highest dry weight of plants was in the NAA treatment at a concentration of 10 ppm with a value of 1.2235 g or 3.5 times that of the control treatment. The 2,4-D treatment at a concentration of 10 ppm increased the dry weight of the plants with a value of 0.8864 g or 2.5 times that of the control treatment. NAA treatment at a concentration of 20 ppm increased the dry weight of plants with a value of 0.6206 g or 177% of the control treatment. NAA and 2,4-D treatments at concentrations greater than 40 ppm decreased the dry weight of plants, with 2,4-D treatments having a more significant effect.

The dry weight of *A. tricolor* L. plants demonstrates that NAA treatment at concentrations of 10 and 20 ppm and 2,4-D treatment at a concentration of 10 ppm can increase plant biomass. This increase in biomass is due to synthetic auxins that promote plant growth, especially stem growth, leaf bud induction, leaf development, and root elongation. The increase in growth rate in the treatment is balanced with the ability of plants to supply nutrients and resources so that plant growth is faster and more stable. NAA treatment is better in increasing plant biomass compared to 2,4-D treatment because NAA is structurally easier to enter the system compared to larger molecular 2,4-D compounds. Thus, NAA is easier to regulate and degrade than 2,4-D when there is excess auxin accumulation in plants (Peterson et al., 2016).

The results of measuring plant fresh and dry weight showed that the ratio of fresh weight to dry weight tended to be similar in all treatments. This shows that NAA and 2,4-D treatments have no effect on the osmotic balance of plants, so synthetic auxin treatments do not cause water stress or drought in plants. The effect of these two synthetic auxins is more to change the balance of hormones, especially ABA and ethylene, in inducing the aging of plant organs and triggering the translocation of nutrients from old organs to older organs, thereby stimulating the death of older plant organs. The effect of this auxin is higher when the 2,4-D used in the form that is relatively more difficult-to-degrade, and when the plant has high sensitivity to 2,4-D compounds (Peterson et al., 2016).

Leaf area and stomatal density

The average leaf area of *A. tricolor* L. plants in NAA and 2,4-D treatment was different from that in

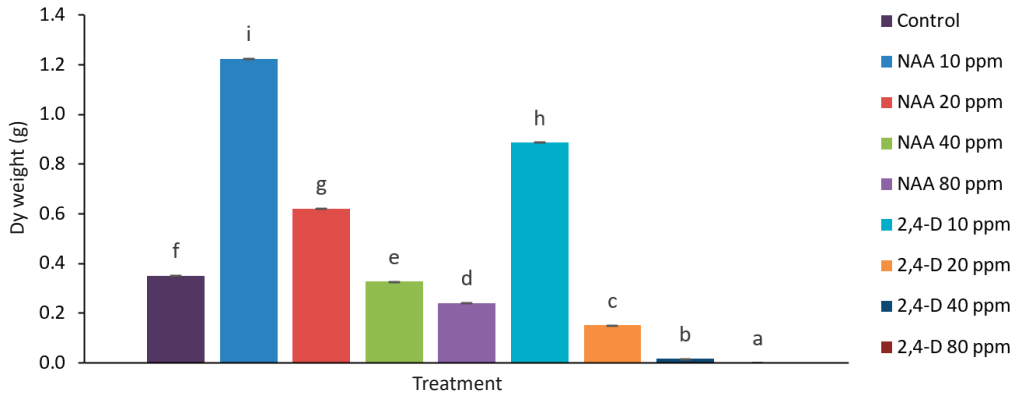

Figure 6. Dry weight of *Amaranthus tricolor* L. plants as affected by NAA and 2,4-D treatment at concentrations of 10, 20, 40, and 80 ppm after 8 weeks; Bars associated with different letters are statistically different as assessed by the Duncan Multiple Range Test (DMRT) at 5% level.

the control treatment, with NAA and 2,4-D treatments at a concentration of 10 ppm showing higher yields (Figure 7). The highest average leaf area was in the NAA treatments at a concentration of 10 ppm with a value of 10.45±0.89 cm² or almost three times wider than that in the control treatment, while the average leaf area in the 2.4-D treatment at a concentration of 10 ppm was 6.12±0.94 cm². The average leaf area in NAA treatment at a concentration of 20 ppm was not much different from that in the control treatment, while NAA treatment at concentrations of 40 and 80 ppm had a lower average leaf area when compared to the control treatment. The average leaf area in the 2.4-D treatment at a concentration of 20 ppm treatment was also lower when compared to the control treatment, although not significantly different. NAA and 2,4-D treatments at a concentration of 10 ppm increased the leaf area of *A. tricolor* L. plants, but treatment with higher concentrations did not increase the leaf area of the plant.

NAA and 2,4-D treatments at a concentration of 10 ppm significantly increased the average leaf area of *A. tricolor* L. plants due to the ability of auxin to increase cell growth, cell division, and induction of new leaf buds. Giving auxin at low concentrations can increase growth without disrupting the balance of auxin hormones with cytokinin so that leaf growth is faster with healthy leaf conditions. NAA treatment at higher concentrations inhibit leaf growth because the balance of auxin, cytokinin, and ethylene hormones is disturbed so that the average leaf area is smaller. In addition, excess accumulation of synthetic auxin can cause abnormalities in leaf shape shown in Figure 8.

The leaf morphology of *A. tricolor* L. in NAA treatment was similar to that in the control treatment

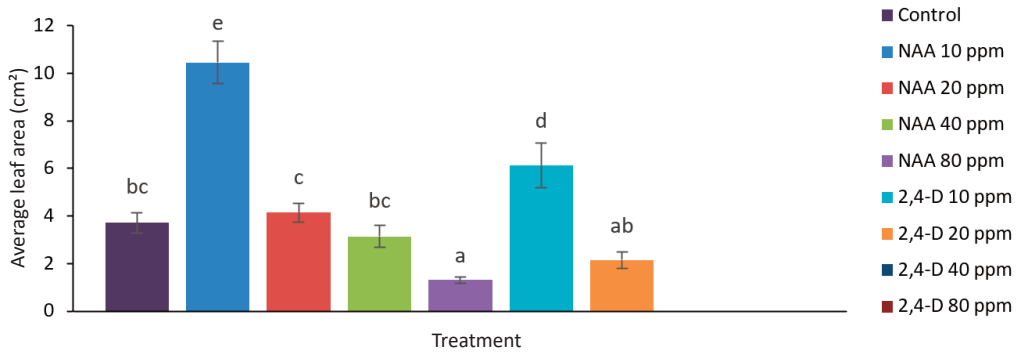

Figure 7. Dry weight of *Amaranthus tricolor* L. plants as affected by NAA and 2,4-D treatment at concentrations of 10, 20, 40, and 80 ppm after 8 weeks; Bars associated with different letters are statistically different as assessed by the Duncan Multiple Range Test (DMRT) at 5% level.

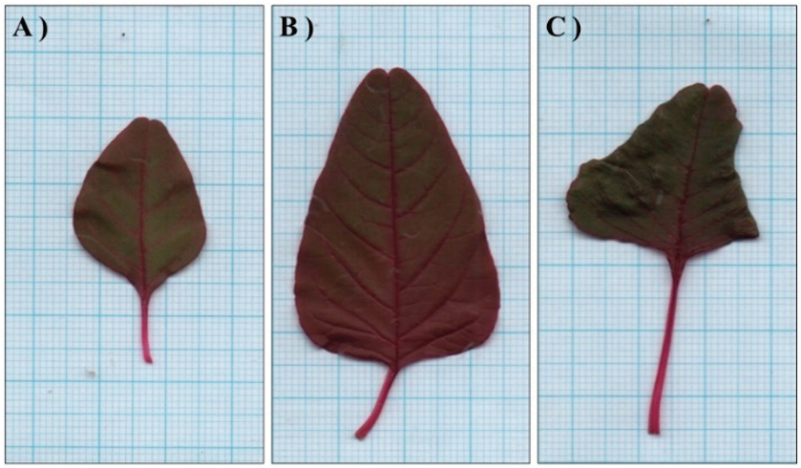

Figure 8. Leaf morphology of *Amaranthus tricolor* L. as affected by A) control treatment; B) NAA 10 ppm treatment; C) 2,4-D 10 ppm treatment

but leaves treated with 2,4-D had wrinkles and folded on the leaf margin. This is due to the performance of NAA and 2,4-D in the division and elongation of plant cells. NAA can induce cell elongation and cell division at low concentrations, whereas 2,4-D is only able to induce cell division without cell elongation, so new cells induced by 2,4-D become irregular and tend to accumulate (Campanoni and Nick, 2005). NAA-treated leaves tended to be wider with leaf morphology similar to the control treatment, while 2,4-D-treated leaves tended to be slightly narrow with wavy leaf edges, indicating a buildup of irregularly arranged cells, and tended to be fragile as a result of 2,4-D treatment. The stomatal density in the abaxial and adaxial parts of the leaf is shown in Figure 9.

Stomatal density in NAA and 2,4-D treatments showed lower values when compared to control treatments although the differences were not significant between treatments (Figure 9). The lowest adaxial side stomatal density was in the NAA treatment at a concentration of 80 ppm, with a value of 59.89±8.93 units/mm² or about 60% of the stomatal density in the control treatment. The lowest abaxial side stomatal density was found in NAA treatment at a concentration of 40 ppm and 2,4-D treatment at a concentration of 10 ppm, with a value of 67.87±7.99 units/mm². Increased synthetic auxin concentrations decreased stomatal density on leaves although the results were not significant between concentration treatments.

The decrease in stomatal density in *A. tricolor* L. leaves with synthetic auxin treatment is due to auxin activity that regulates the differentiation, development, and distribution of stomata on leaves. Auxins inhibit the process of differentiation of epidermal cells into guard cells to regulate the distribution of stomata on leaves, especially in sprouts and young plants, to reduce gas and moisture exchange (Balcerowicz and Hoecker, 2014). Low stomatal density leads to lower gas and water vapor exchange, resulting in lower potential water loss through transpiration. Higher concentrations of auxins lead to a decrease in the number of stomata, which can increase the plant's ability to regulate water in leaves.

The NAA and 2,4-D treatments led to a decrease in stomatal density in the leaves of *A. tricolor* L. with no significant different effect. This is due to a similar type of synthetic auxins, and both easily enter the leaves, causing a similar effect. Higher concentrations of NAA and 2,4-D lead to a greater decrease in stomatal density as the accumulation of auxins leads to a stronger slowing effect of guard cell differentiation. The stomatal density as affected by NAA and 2,4-D treatments was slightly reduced with insignificant

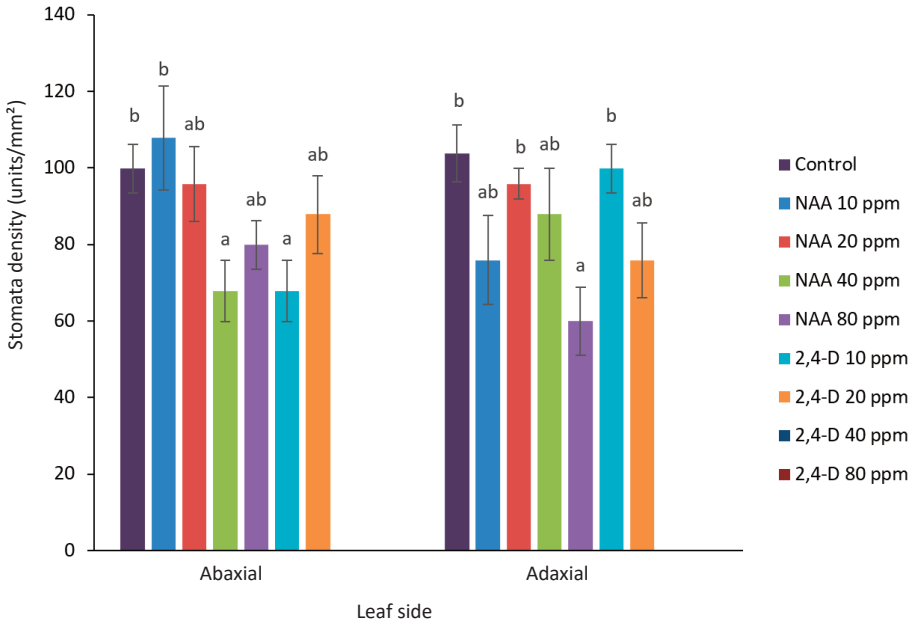

Figure 9. Stomatal density of *Amaranthus tricolor* L. leaves as affected by NAA and 2,4-D treatment at concentrations of 10, 20, 40, and 80 ppm after 8 weeks. Bars associated with different letters are statistically different as assessed by the Duncan Multiple Range Test (DMRT) at 5% level.

differences compared to controls, indicating that there are factors other than auxins that play a role in stomata formation.

Chlorophyll and carotenoid content

Chlorophyll content in NAA and 2,4-D treatments was generally increased when compared to control treatments (Figure 10). The highest chlorophyll content was recorded in the samples treated with NAA at a concentration of 10 ppm, with a value of 539.75±39.55 µg/g or twice that in the control treatment. The lowest chlorophyll content was found in the samples treated with NAA at a concentration of 80 ppm with a value of 158.59±16.33 µg/g. The chlorophyll content in NAA treatment at concentrations of 20 and 40 ppm and 2,4-D treatment at concentrations of 10 and 20 ppm was slightly higher than the chlorophyll content in the control treatment although the difference was not significant.

Total chlorophyll content in NAA treatment at a concentration of 10 ppm was significantly higher than total chlorophyll content in control treatment. This occurs because auxin triggers leaf growth and chlorophyll biosynthesis in leaves. A slight increase in total chlorophyll pigment also occurred in the NAA treatment at concentrations of 20 and 40 ppm although the total pigment values were not significantly different when compared to controls. Giving NAA at a concentration of 80 ppm significantly reduced the chlorophyll content in plants because NAA at high concentrations can cause the amount of auxin to exceed the growth ability of plants, resulting in a shortage of resources (Uddin et al., 2023). In addition, high amounts of synthetic auxin cause changes in the balance of auxin hormones with cytokinin, ABA, and ethylene, causing a stress response and an increase in reactive oxygen species (ROS) that decreases the amount of chlorophyll pigment in plants. NAA treatment at a concentration of 10 ppm was effective in significantly increasing the content of chlorophyll a and b compared to the control treatment.

The 2,4-D treatment did not result in a significant effect on total chlorophyll when compared to control. This happens because 2,4-D compounds tend to increase the amount of chlorophyll pigments and other pigments that are easily damaged by light, resulting in the accumulation of photolabile pigments that are easily oxidized (Dayan and Zaccaro, 2012). This easily oxidized pigment will trigger an increase in reactive oxygen species (ROS), which will damage chlorophyll pigments and similar pigments that are labile to light. In addition, 2,4-D can bind to enzymes associated with chlorophyll biosynthesis so that the amount of chlorophyll pigment with 2,4-D treatment is lower compared to that in NAA treatment at the same concentration. The inhibitory effect on the chlorophyll biosynthesis is characterized by symptoms

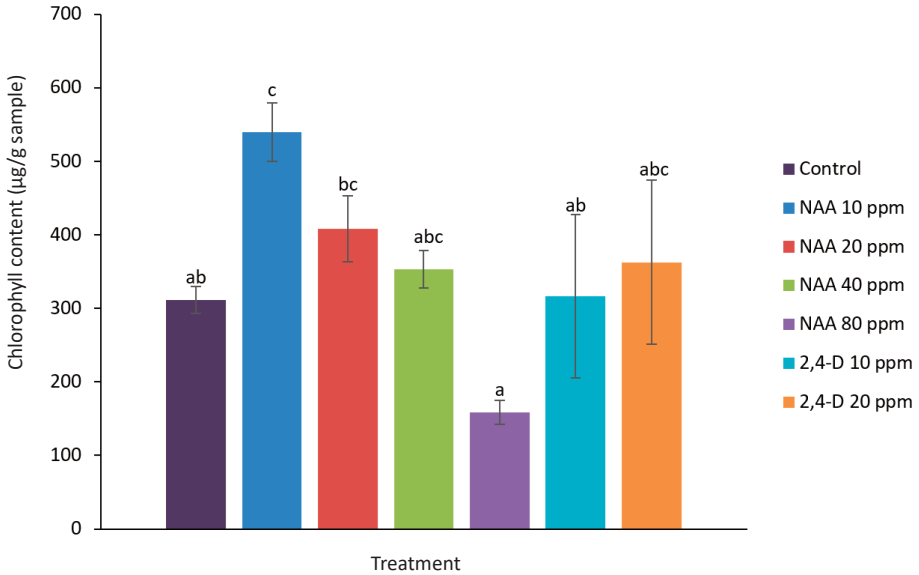

Figure 10. Chlorophyll content of *Amaranthus tricolor* L. as affected by NAA and 2,4-D treatment at concentrations of 10, 20, 40, and 80 ppm after 8 weeks; Bars associated with different letters are statistically different as assessed by the Duncan Multiple Range Test (DMRT) at 5% level.

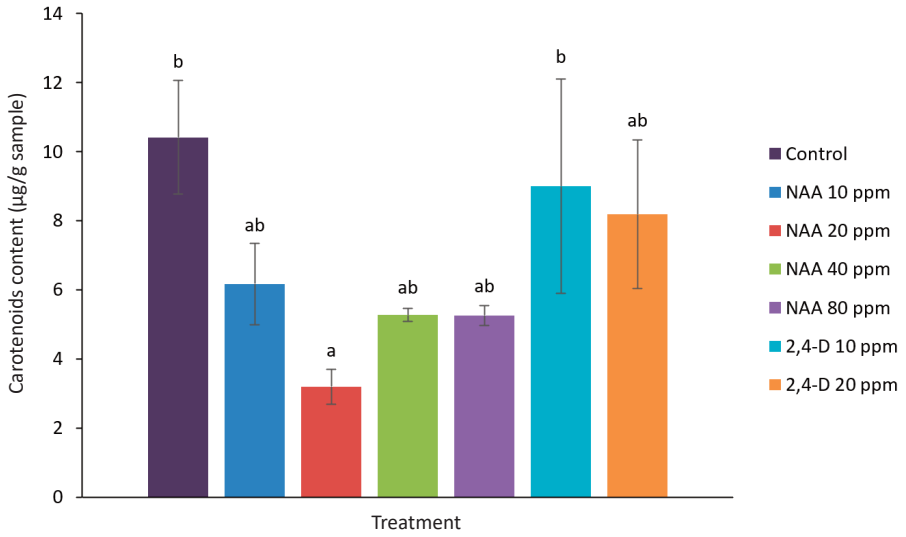

Figure 11. Carotenoid content of *Amaranthus tricolor* L. as affected by NAA and 2,4-D treatment at concentrations of 10, 20, 40, and 80 ppm after 8 weeks; Bars associated with different letters are statistically different as assessed by the Duncan Multiple Range Test (DMRT) at 5% level.

of rolling the edges of the leaves and the surface of the leaves that become wrinkled at the edges (Dayan and Zaccaro, 2012). The effect of NAA and 2,4-D on the carotenoid content is shown in Figure 11.

Carotenoid content in NAA and 2,4-D treatments was generally lower when compared to control treatments (Figure 11). The highest carotenoid content was in the control treatment of 10.42±1.64 µg/g, while the lowest carotenoid content was in the NAA treatment at a concentration of 20 ppm with a value of 3.19±0.51 µg/g. The carotenoid content decreased with the increasing concentrations of NAA and 2,4-D although the difference in carotenoid content between concentrations was not significant. The carotenoid content in NAA treatment was lower when compared to that in 2,4-D treatment.

Carotenoid pigments function as one of the light-capturing pigments with wavelengths of 400–550 nm, a precursor for the biosynthesis of ABA and strigolactones, as well as one of the photoprotective compounds (Hermanns et al., 2020). Carotenoids can protect other pigments such as chlorophyll when there is an accumulation of ROS and compounds that are reactive to light. Changes in carotenoid content in plants will have an impact on the content of chlorophyll and other pigments in leaves, so carotenoids and chlorophyll can be indicators of photosynthesis and photooxidation disorders in leaves (Dayan and Zaccaro, 2012).

The decrease in carotenoid content in NAA and 2,4-D treatment plants indicates that this synthetic auxin affects carotenoid biosynthesis in plants. Excess auxin will increase the amount of ROS in plants, causing a decrease in carotenoid pigments due to oxidation of carotenoid pigments and conversion of carotenoids to ABA as an indicator of stress in plants (Dayan and Zaccaro, 2012). A decrease in the number of carotenoids in higher synthetic auxin treatment indicates that auxin accumulation indirectly leads to increased ABA and excess ROS formation in leaves. In addition, the decreased content of carotenoids causes plant cell metabolic disorders characterized by leaf aging and decreased plant biomass even though the water content in plants is not affected by synthetic auxins.

CONCLUSIONS

The results indicate that NAA decelerates seed germination of *Amaranthus tricolor* L. in a short time, while 2,4-D inhibits seed germination up to seven days. NAA treatment increases plant height, fresh and dry weight, leaf area, and chlorophyll yet it does not affect stomatal density. Meanwhile, 2,4-D treatment inhibits shoot growth, decreases fresh and dry weight, causes wrinkled leaves, and reduces carotenoid content. The effective treatment of synthetic auxin in improving the growth of *A.*

tricolor L. is NAA at a concentration of 10 ppm, while 2,4-D at concentrations of 20 ppm inhibits germination and growth of *A. tricolor* L., and its application at concentrations above 40 ppm is lethal to *A. tricolor* L.

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