Application of NAA and BA for improving the yield along with flavonoid and polyphenol content of *Eleutherine bulbosa* bulb

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Abstract
Eleutherine bulbosa (Mill.) urb. is a herbaceous bulbous plant, known for its high medicinal value. To improve bulb yield and quality, controlling bulb growth as well as flavononoid and polyphenol content by NAA and BA was the objective of this study. Apply NAA and BA separately at different concentrations (BA: 5 ppm, 10 ppm and 15 ppm; NAA: 5 ppm, 10 ppm and 15 ppm) on E.bulosa at 6 weeks of age. After 4 weeks of treatment, tuber diameter, fresh weight, dry weight, number of leaf sheaths, total sugar content, starch, polyphenol and flavonoid in bulb were determined. Then, combined treatment with NAA 10 ppm and BA 10 ppm (at weeks 6 and 10), after 6 weeks of treatment, bulb fresh weight, dry weight, flavonoid and polyphenol content and bulb yield per plant were determined. The results showed that the 10 ppm BA treatment had the highest bulb diameter, fresh weight, dry weight, polyphenol content compared to other treatments and controls, while the 10 ppm NAA treatment had the highest flavonoid content but dry weight and bulb diameter were lower than the 10 ppm BA treatment.

In all the treatments, the number of leaf sheaths was not different from that of the control. In the combined treatments, bulb fresh weight, dry weight and polyphenol content and bulb yield per pot were significantly increased in the BA (w6) + BA (w10) treatment, slightly increased compared to the control and lower than other treatments. In the treatment of NAA (w6) + NAA (w10), it had a high flavonoid content but the lowest polyphenol content compared to other treatments. The relationship between sugar, starch, polyphenol and flavonoid content is also discussed.

Keywords: *Eleutherine bulbosa*, polyphenol, flavonoid, BA, NAA, bulb yield.

Introduction

*Eleutherine bulbosa (E. bulbosa) i*s known to be a plant with high medicinal value due to the fact that the bulb contains many secondary compounds such as flavonoids, phenolic compounds, saponins, tannins, quines, triterpenoids, alkaloids, tannins, anthocyanins, glycosides, steroids/triterpenoids and naphthoquinone[7,12,18]. Research on *E. bulbosa* mainly focuses on biochemical analysis, effectiveness and value of biological compounds contained in medicinal herbs as well as in the application of compounds extract from bulb in the field and cosmetics[10,13,19]. Therefore, studies on the growth and accumulation of secondary compounds to increase the yield and quality of bulbs are very necessary[7]. The growth and accumulation of "bulb" is a complex process controlled by exogenous and endogenous factors, including plant growth regulators[2].

Auxin and cytokinin are the two most widely used plant growth regulators in the tuber growth process. In many cases, auxin has shown the ability to promote cell division and elongation and cytokinin has the ability positively to impact bulb growth[10]. BA (6-benzylaminopurine) and NAA (α-naphthylacetic) are synthetic cytokinin and auxin, which are commonly used exogenously to increase tuber yield[5,8] and accumulate polyphenol and flavonoid accumulation[21]. Therefore, it is essential to select the right concentrations and plant growth regulators having a positive effect on bulb growth and promote the accumulation of secondary compounds in bulb. For the above reasons, the aim of determining the concentration of NAA and BA individually or in combination appropriately for the growth and accumulation of polyphenols and flavonoids in the *E. bulbosa* bulb for increasing the yield and polyphenols and flavonoid content.

Material and Methods

Material: *E.bulbosa* mother bulbs bearing buds from the National Institute of Medical Materials and the 6-week-old from the mother bulbs, were grown in the experimental garden of the Plant Physiology Department, University of Sciences, Vietnam National University, Ho Chi Minh City (Fig. 1).

Method

Investigation of the effect of BA and NAA on the growth of *E. bulbosa*: *E.bulbosa* mother bulbs bearing buds are placed in pots with a volume of 20 cm x 21cm x20 cm containing organic soil placed in the experimental garden of Plant Physiology Department, light intensity of 10,000 – 25,000 lux, temperature 33 ± 5 ^{o}C, humidity 60 – 65 %. After 6 weeks of planting, the 6-week-old *E. bulbosa* plants grown from the original mother bulb were initially separated from the mother bulb, were then transferred to another pot to allow the plant to adapt for 3 days and then treated with BA and NAA at different concentrations including 7 experiments: Control (watered), BA 5 ppm, BA 10 ppm; BA

15 ppm, NAA 5 ppm; NAA 10 ppm; NAA 15 ppm. After 4 weeks of treatment, the following parameters were determined: diameter, fresh weight, dry weight, sheath length and number of leaf sheaths, total sugar content, starch content, respiration intensity, flavonoid and polyphenol content in bulb.

Investigation on the effect of the combination of plant growth regulators on *E. bulbosa* growth: *E. bulbosa* mother bulb bearing bud is grown using the above method for up to 6 weeks and then treated with a combination of plant growth regulators (PGRs) at 10 ppm BA and 10 ppm NAA. PGRs were sprayed through the leaves of the plants at weeks 6 and 10. The experiment consisted of 4 treatments (Table 1). After 6 weeks of treatment, the parameters for fresh weight, dry weight, bulb yield per plant, flavonoid and polyphenol content were determined.

Plants were planted in the experimental garden of the Plant Physiology Department with light intensity 10,000 – 25,000 lux, temperature 33 ± 5 ^{o}C, humidity of 60 – 65 %. The experiments were arranged randomly, each treatment was repeated 7 times with 3 plants in each pot. The treated plant growth regulators were sprayed on the leaves until wet. Spray continuously for 2 days at 4 pm.

Analysis of physiological and biochemical parameters
Determination of the diameter and length of the bulb: The diameter of bulb is measured with a ruler, clamped at the largest area of edema, the length of the bulb is measured with a ruler with units millimeters, measured from the position the root grows to the position where the leaf sheath separates.

Determination of fresh and dry weight of bulbs: *E. bulbosa* bulb were weighed using an analytical balance with an error of 0.01 mg of OHAUS (USA). Dry at 120 ^{o}C for 1 hour and then at 80 ^{o}C for about 72 hours until the weight remained unchanged to determine the dry weight[17].

Determination of total sugar and starch content of bulbs: Total sugar and starch content in *E. bulbosa bulb* were determined using the phenol-sulfuric acid method according to Combs et al[4] and González-Vázquez et al[6]. Absorbance was measured at a wavelength of 490 nm using a spectrophotometer, compared to the sucrose standard curve to obtain the total sugar content. Starch content was determined by comparison with standard D-Glucose from the pulp after the sugar extraction, drying at 60 ^{o}C and hydrolysis with 10 % sulfuric acid for 1 hour.

Measurement of respiratory intensity: Bulbosa tubers during treatment were determined using a Leaf Lab 2 oxygen electrode gas exchanger (Hansatech, UK) with an improved measuring chamber with a height of 6 cm. Oxygen exchange rate was calculated based on the amount of oxygen absorbed per gram of fresh weight per minute (μmol O_2/g/min).

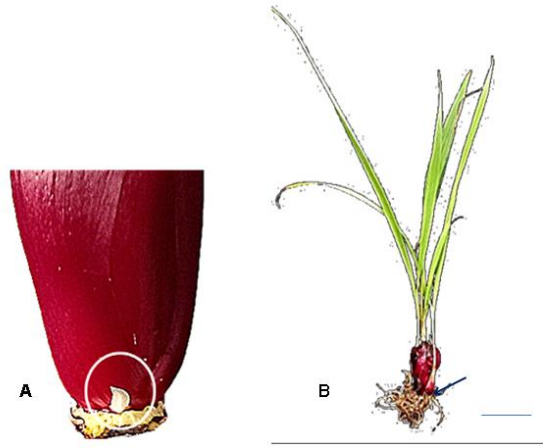

Figure 1: (A) *E.bulbosa* mother bulb bearing bud and (B) the 6-week-old from the mother bulb was grown in experimental garden with 5 cm cross bar

Table 1
Treatment method of the combination of plant growth regulators

Treatments	Treatment method	
	Week 6	Week 10
Control	Water	Water
NAA (W6) + NAA (W10)	NAA	NAA
BA (W6) + BA (W10)	BA	BA
NAA (W6) + BA (W10)	NAA	BA
BA (W6) + NAA (W10)	BA	NAA

Determination of total phenolic content and flavonoid content: Polyphenol and flavonoid of bulb dried powder were extracted in 60 % ethanol at ratio of 1:35 (w/v) (at pH 3.0) for one hours at 50 °C, then the extract was filtered and allowed to dry[11]. Polyphenol content (PC) was determined spectrophotometrically using the Folin–Ciocalteu method described by Shi et al[18] who used color reaction with Folin-Ciocalteu reagent and 20 % Na_2CO_3 with gallic acid as the standard, then measured OD at 765 nm. Total flavonoid content (TFC) was determined by aluminium chloride spectrophotometry as described by Atanassova et al[1]. They used a color reaction with 5 % $NaNO_2$ and 10 % $AlCl_3$, OD measured at 510 nm. Rutin was used as a standard. Polyphenol and flavonoid contents in bulb were expressed as mg/g dry weight.

Statistical Analysis: Data were analyzed using SPSS (Statistical Package for the Social Sciences) 20.0 software for Windows. Statistical differences were significant at 95 % level and statistical results were expressed as means ± standard deviations and accompanying text samples.

Results

Effects of BA and NAA individually on the growth and accumulation of *E. bulbosa* bulb: After 2 weeks of treatment with *E. bulbosa* plants at 6-week-old, diameter, fresh weight, dry weight and length of bulb in all treatments were higher than the control and the highest in the 10 ppm BA treatment. In the BA treatments, the bulb diameter, fresh weight and dry weight increased rapidly when increasing BA concentration up to 10 ppm, but then decreased when increasing BA amount up to 15 ppm, while bulb length increased with BA treatment at 5 ppm and remained unchanged when increasing BA treatment concentration up to 15 ppm. In the NAA treatments, bulb diameter, fresh weight and length started to increase at 5 ppm NAA and remained constant at 10 ppm NAA but then decreased at 15 ppm NAA, while the dry weight remained increased sharply at 10 ppm NAA and then decreased at 15 ppm NAA (Fig. 2 and table 2).

Flavonoid and polyphenol contents were higher in all treatments than in the controls. Polyphenol content increased most strongly in the 10 ppm BA treatment, while the flavonoid content increased sharply in 10 ppm in the NAA treatment. In the BA treatment, the content of flavonoid and polyphenol content gradually increased with the BA treatment concentration of 10 ppm and remained constant at the BA concentration of 15 ppm. In NAA treatment, the flavonoid content gradually increased up to 10 ppm NAA and remained constant at 15 ppm NAA but polyphenol content did not differ between treatments. In the NAA treatments, the flavonoid content gradually increased to the NAA treatment concentration of 10 ppm and remained constant at the NAA concentration of 15 ppm, but the polyphenol content did not differ between the treatments.

Total sugar content decreased in all treatments and decreased most strongly in treatments BA 10 ppm and NAA 10 ppm, however the starch content tended to increase slightly or remained unchanged compared to the control (Table 2). Respiration intensity increased significantly in the 10 ppm BA treatment and in the 10 ppm NAA treatment compared to control and other treatments (Table 3).

Figure 2: *Eleutherine bulbosa* in treatments after 4 weeks with bulb (A-G), bulb cross section (A1-G1): Control (A, A1), BA 5 ppm (B,B1); BA 10 ppm (C,C1); BA 15 ppm (D,D1) ; NAA 5 ppm (E,E1); NAA 10 ppm (F,F1) and NAA 15 ppm (G,G1), 1 cm cross bar

Table 2
Change in bulb diameter, fresh weight (FW), dry weight (DW), Bulb length and the number of leaf sheaths of *E. bulbosa* in treatments.

Treatments	Bulb diameter (mm)	Bulb fresh weight (g)	(g)DW. g^{-1} FW	Bulb length (cm)	Number of leaf sheaths
Control	10.60 ± 0.06^{d}	1.54 ± 0.06^{c}	0.19 ± 0.03^{d}	3.17 ± 0.07^{c}	6.33 ± 0.03^{a}
BA 5 ppm	11.21 ± 0.10^{c}	1.72 ± 0.09^{b}	0.26 ± 0.03^{c}	4.30 ± 0.12^{a}	6.67 ± 0.03^{a}
BA 10 ppm	**12.50 ± 0.06^{a}**	**1.96 ± 0.02^{a}**	**0.47 ± 0.03^{a}**	**4.12 ± 0.12^{a}**	**6.68 ± 0.04^{a}**
BA 15 ppm	11.73 ± 0.03^{b}	1.74 ± 0.03^{b}	0.38 ± 0.02^{b}	4.33 ± 0.09^{a}	6.67 ± 0.03^{a}
NAA 5 ppm	11.67 ± 0.06^{b}	**0.93 ± 0.02^{a}**	0.36 ± 0.03^{b}	**4.13 ± 0.06^{ab}**	**6.33 ± 0.03^{a}**
NAA 10 ppm	**11.83 ± 0.07^{b}**	0.92 ± 0.05^{a}	**0.49 ± 0.02^{a}**	4.11 ± 0.15^{ab}	6.33 ± 0.08^{a}
NAA 15 ppm	11.23 ± 0.12^{c}	0.68 ± 0.08^{b}	0.39 ± 0.02^{b}	3.03 ± 0.07^{c}	6.00 ± 0.20^{a}

The average numbers in the column with different letters are meaningful at $p \leq 0.05$.

Table 3
Total sugar content (TSC), starch content (StC), flavonoids, polyphenols content and respiration intensity (RI) of *E. bulbosa* bulb after 4 weeks of treatment with BA or NAA at the 6-week-old plant stage.

Treatments	TSC (mg.g^{-1} DW)	StC (mg.g^{-1} DW)	Flavonoid (mg.g^{-1} DW)	Polyphenol (mg.g^{-1} DW)	RI ($\mu LO_2.g^{-1}FW.h^{-1}$)
Control	22.23 ± 0.07^{a}	77.24 ± 1.5^{c}	1.93 ± 0.10^{d}	3.07 ± 0.05^{d}	43.50 ± 1.09^{d}
BA 5 ppm	19.75 ± 1.15^{b}	82.68 ± 1.34^{ab}	2.41 ± 0.01^{c}	3.59 ± 0.08^{b}	40.56 ± 1.46^{d}
BA 10 ppm	13.02 ± 0.50^{e}	84.26 ± 1.21^{ab}	4.53 ± 0.02^{b}	4.66 ± 0.11^{a}	65.27 ± 0.02^{a}
BA 15 ppm	16.48 ± 0.50^{c}	84.41 ± 1.98^{ab}	4.55 ± 0.01^{b}	4.60 ± 0.11^{a}	50.28 ± 0.72^{c}
NAA 5 ppm	17.73 ± 0.19^{c}	77.27 ± 2.57^{c}	4.29 ± 0.04^{b}	3.15 ± 0.12^{c}	49.15 ± 0.02^{c}
NAA 10 ppm	15.24 ± 0.18^{d}	82.87 ± 2.18^{ab}	5.44 ± 0.14^{a}	3.21 ± 0.11^{c}	60.61 ± 0.04^{b}
NAA 15 ppm	15.15 ± 0.29^{d}	85.97 ± 2.86^{a}	5.50 ± 0.07^{a}	3.24 ± 0.06^{c}	48.85 ± 0.57^{c}

The average numbers in the column with different letters are meaningful at $p \leq 0.05$. Effects of combined BA and NAA treatment on the growth and accumulation of E. bulbosa bulb

Table 4
Change in bulb fresh weight, dry weight, flavonoid and polyphenol content and bulb yield per plant of *Eleutherine bulbosa* after 6 weeks in treatments NAA –BA hay BA – BA in week 6^{th} and week 10^{th}.

Treatments	Bulb fresh weight (g)	Bulb dry weight (g)	Flavonoid (mg/g DW)	Polyphenol (mg/g DW)	Bulb yield (g)/pot
Control	10.55 ± 0.02^{d}	4.92 ± 0.15^{c}	5.51 ± 0.05^{d}	4.75 ± 0.07^{c}	221.55 ± 3.69^{c}
NAA (w6) + NAA (w10)	11.79 ± 0.02^{c}	5.73 ± 0.07^{b}	$7.72 \pm 0,12^{a}$	4.81 ± 0.07^{c}	253.59 ± 3.060^{b}
BA (w6) + BA (w10)	13.00 ± 0.05^{a}	6.23 ± 0.18^{a}	6.05 ± 0.08^{c}	6.35 ± 0.08^{a}	303.06 ± 6.36^{a}
NAA (w6) + BA (w10)	12.39 ± 0.04^{b}	5.82 ± 0.09^{b}	6.40 ± 0.13^{b}	5.08 ± 0.05^{b}	260.19 ± 1.68^{b}
BA (w6)+ NAA (w10)	12.46 ± 0.01^{b}	5.92 ± 0.03^{b}	6.38 ± 0.13^{b}	6.66 ± 0.06^{a}	261.66 ± 2.16^{b}

The average numbers in the column with different letters are meaningful at $p \leq 0.05$.

In all treatments, the parameters of bulb fresh weight, dry weight and bulb yield per pot were higher than that of the control and the highest in the combination treatment of BA (w6-w10). Flavonoid content was highest in the NAA (w6) + NAA (w10) treatment and lowest in the BA (w6) + BA (w10) treatment, while the polyphenol content was the opposite. In the combined treatments of BA (w6) + NAA (w10) or NAA (w6) + BA (w10), dry weight was not different from NAA (w6) + NAA (w10) but was lower than that of BA (w6) + BA(w10). The bulb fresh weight and polyphenol content were higher when only treated with NAA (w6) + NAA (w10) but lower than BA (w6) + BA (w10). Conversely, flavonoid content was higher when treated with only BA (w6) + BA (w10) but was lower than NAA (w6) + NAA (w10) (Table 4).

Discussion

Eleutherine bulbosa plant at the stage of 6 weeks old, after 2 weeks of treatment, in the BA treatment with concentrations of 10 ppm and 15 ppm had the highest bulb diameter, fresh weight and dry weight is in the treatments and control. However, in the 15 ppm BA treatment, the dry weight was lower than the 10 ppm BA treatment. This shows that, BA, as well as other plant hormones, play a dual role at low and high concentrations[21]. Different concentrations have different effects on tuber formation, so when BA is treated

at different concentrations at a medium concentration, cytokinin stimulates cell expansion, increasing tuber diameter, but if the high BA concentration is increased to 15 ppm, all growth and accumulation indicators begin to decrease or tend to decrease (table 2).

Thus, in *E. bulbosa*, BA treatment at a concentration of 10 ppm will stimulate bulb growth, so this can be considered as a signal to improve the growth process of "bulb", stimulating a wide increase in tubers as studied by Tang et al[19]. According to Rao et al[16], cytokinin effect depends on nature of secondary metabolites in the species and in *E. bulbosa* bulb, BA signifcantly increased polyphenol và flavonoid content in the treatment with BA 10 ppm. This may explain that BA strongly induced the expression of favonoid biosynthesis-related genes similar to that of Zhang et al[22] in Mulberry by BA or Ravanfar et al[15] in red cabbage using Zeatin.

Auxins can elongate stem cells, enhance bulb growth and provide the dual functions of hormones in a similar way to cytokinins [2,21], thereby promoting bulb development shown in tables 2 and 3. Treatment with 10 ppm NAA is suitable for improving growth parameters, however, increasing the concentration to 15 ppm will reduce these parameters, but will still retain the ability to accumulate flavonoid. In addition, the treatment with 10 ppm NAA had the highest flavonoid content and the polyphenol content was not different from the control. Thus, in addition to its the role of promoting growth, cell elongation and increasing bulb diameter and length, NAA also plays a role in increasing flavonoid accumulation in *E. bulbosa*.

This can be explained by the fact that flavonoid may inhibit polar auxin transport, leading to auxin accumulation and possibly inducing of flavonoid synthesis[14]. Therefore, flavonoid content in *E.bulbosa* bulb increased. These results also showed that auxin interacts with the flavonoid 3-O-glucosyltransferase enzyme from the flavonoid synthesis pathway, increasing starch content in bulb and increasing flavonoid production[22]. There is a relationship between the sugar, flavonoid and polyphenol content in Eleutherine bulbosa bulb. In BA 10 pmm treatment, flavonoid and polyphenol content increased as sucrose content decreased and respiration intensity increased (Table 3).

This suggests the role of cytokinin in promoting flavonoids and polyphenols via the regulation of sugar accumulation, as evidenced by the fact that when respiration intensity increases, sugars are broken down, creating precursors and energy for other processes in the cell, including the biosynthesis of flavonoids and polyphenols in *E. bulbosa*. Most of the differentially regulated metabolites are involved in sugar, flavonoid, polyphenol mechanism[23].

In all treatments, there were increased growth parameters compared to the control without changing the number of leaf sheaths. Thus, auxin and cytokinin only have an effect on the process of division, expansion and accumulation in *E. bulbosa* cells without increasing the number of leaves.

On the other hand, in *E. bulbosa*, when bulb diameter, fresh weight and bulb length increase, dry weight increases (Table 2). In other words, auxin and cytokinin are two hormones involved in this process as in Lycoris[21]. Auxin and cytokinin influenced the production of secondary metabolites through secondary pathway biosynthetic enzymes of the polyphenols and flavonoids[3]. In all combination treatments, the bulb fresh weight, dry weight and bulb yield per pot were higher than the control and highest in the BA (w6) + BA (w10) combination treatment, with an increase, highest polyphenol content (Table 4).

In addition, the polyphenol content in combined experiments when BA treatment was effective at week 6 was higher than that at week 10. This once again proves that the role of BA, in addition to increasing the yield of *E.bulbos*a bulb as well as other tuberous crops[5], is to increase the total phenol content in *E.bulbosa bulb*. Although the NAA (w6)+ NAA (w10) treatment had bulb yield not as high as the BA (w6)+ BA (w10) treatment, it had the highest flavonoid content. Additionally, any combination containing NAA will increase the flavonoid content. This once again confirms the role of NAA in the accumulation of flavonoid compounds in *E.bulbosa* bulb.

Conclusion

After 4 weeks, the treatment with BA and NAA individually showed the highest diameter, fresh weight, dry weight and polyphenol content compared to other treatments and controls. The treatment with NAA 10 ppm had the highest flavonoid content while the treatment with BA 10 ppm had the highest polyphenol content.

After 6 weeks of combined treatment, the of BA (w6) + BA (w10) treatment with bulb fresh weight, dry weight and polyphenol content and bulb yield per pot all increased significantly, but the flavonoid content increased slightly compared to the control and lower than that of other treatments. In the treatment experiment, NAA (w6) + NAA (w10) had the highest flavonoid content but the lowest polyphenol content compared to other treatments.

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