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Biochemical parameters of *Gynura procumbens* Lour. on different types of culture *in vitro* media

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Abstract

Flavonoids, phenolic compounds, chlorophylls, carotenoids, and anthocyanins are vital secondary metabolites in plants, contributing to their growth, adaptation to environmental stressors, and medicinal properties. This study investigates the effects of different phytohormone concentrations on the biochemical composition of *Gynura procumbens* cultivated *in vitro*. Flavonoid content varied significantly among groups, with the highest levels observed in plants grown on medium supplemented with NAA at 2 mg/L, demonstrating a 70-90% increase compared to other treatments. Similarly, phenolic compounds were elevated by 28-31% under the same conditions, indicating improved plant resilience under stress. Chlorophyll a and b levels remained statistically consistent across groups, reflecting stable photosynthetic potential irrespective of phytohormone concentration. However, carotenoid synthesis peaked in plants grown on medium containing 2 mg/L BAP and 0.1 mg/L IAA, showing increases of 36-66% compared to other treatments. Anthocyanin content exhibited no significant variation between groups. These findings highlight the potential to optimize phytohormone concentrations in vitro to enhance specific phytochemical yields in *Gynura procumbens*, a plant with promising therapeutic applications.

Keywords: *Gynura procumbens* Lour., flavonoids, polyphenols, plant pigments.

1. INTRODUCTION

Gynura procumbens Lour., is rich in phytochemicals such as flavonoids, phenolic compounds, carotenoids, and anthocyanins. This species demonstrates a spectrum of pharmacological properties, including antihypertensive, cardioprotective, antihyperglycemic, hypoglycemic, fertility-enhancing, anticancer, antimicrobial, antioxidant, organ-protective, and anti-inflammatory activities (Shathi et al., 2022). The diverse pharmacological effects of *G. procumbens* are attributed to its rich phytochemical composition. This makes it a potent natural source of compounds with pharmacological properties, offering a foundation for the development of novel therapeutic agents.

A variety of medically significant chemicals were isolated from the plant, including phenolic compounds, polyphenols, flavonoids, saponins, tannins, terpenoids, and essential oils (Tan et al., 2016). Furthermore, the plant is rich in a diverse array of secondary metabolites, particularly flavonoids such as kaempferol, quercetin, rutin, myricetin, and stigmasterol (Pramita et al., 2018). A predominant portion of these extracted compounds function as phytoalexins, synthesized in

response to elicitor reactions, thereby endowing the plant with disease-resistance capabilities. Additionally, numerous flavonoids exhibit antioxidant bioactivities. These secondary metabolites are derived from various parts of the plant, including the roots, stems, leaves, flowers, and fruits.

The prioritization of optimizing growth conditions for a particular plant species is increasingly pertinent in current research. The plant tissue culture technique, instrumental in the identification and isolation of bioactive phytocompounds, finds extensive industrial applications. This methodology holds significant promise for diverse sectors, including the food, pharmaceutical, and cosmetic industries. Plant tissue culture involves the *in vitro* cultivation of plant tissue fragments (explants) in a synthetic environment under sterile conditions. The development of tissue culture media originated from nutrient solutions initially employed for whole plant cultivation, with Murashige and Skoog's medium being a commonly utilized example.

Therefore, a variety of phytohormone combinations are employed to enhance the production of biochemical compounds within plants. There are four primary groups of these hormones: auxins, cytokinins, gibberellins, and abscisic acid. Auxins and cytokinins, frequently used in tandem, are the most prevalent. Auxins are known to stimulate root and callus formation and growth, whereas cytokinins favor the development and growth of axillary and adventitious shoots. In this study, we will explore the effects of various auxin and cytokinin combinations.

2. MATERIALS AND METHODS

2.1. Materials and reagents

The following reagents were used in the experimental studies: $CaCO_3$, 96% ethyl alcohol, concentrated hydrochloric acid, 5% sodium nitrite, 10% aluminum chloride, 1M sodium hydroxide, standard quercetin solution, Folin-Ciocalteu reagent, 7.5% sodium carbonate, and gallic acid.

2.2. Cultivation of plant material

The introduction of the plant into *in vitro* culture was as follows: shoots of Gynura were washed in a 0.1% solution of mercuric chloride, and then washed three times with sterile water. Subsequently, the plants were cultivated at a temperature of +25 °C, relative humidity was in the range of 70 -75%, illumination was 2000 lux, and the photoperiod was 16 hours. After about 4 weeks, regenerating plants developed from axillary buds, which were used for further microclonal propagation. The *in vitro* culture was grown on a medium for woody crops, namely modified Murashige-Skoog medium (MS media), with the addition of different concentrations of hormones (see table 1).

Table 1. Concentrations of phytohormones for Gynura cultivation

Name of phytohormones	Phytohormone code	Phytohormone concentration, mg/l	Abbreviation
6-Benzylaminopurine/ Indole-3-acetic acid	A	0.8 / 0.02	0.8 BAP / 0.02 IAA
1-Naphthaleneacetic acid	B	2	2 NAA

6-Benzylaminopurine/ Indole-3-acetic acid	C	2 / 0.02	2 BAP / 0.02 IAA
6-Benzylaminopurine/ Indole-3-acetic acid	D	2 /0.1	2 BAP / 0.1 IAA

2.3. Preparation of plant extracts for the experiment

The plant samples, previously frozen in liquid nitrogen, are removed and weighed. After weighing, the material is placed in test tubes and filled with boiling water in a ratio of 1:10, respectively. The tubes are tightly closed and placed in a water bath, boiled for 25 minutes. The resulting extract is filtered through a pleated filter and used for quantitative determination of the test substances.

2.4. Determination of flavonoid content in plant material

The principle of the colorimetric method for the determination of flavonoids is based on the fact that $AlCl_3$ forms stable complexes with the C4 keto group or the C3 or C5 hydroxyl group of flavones and flavanols, which have a maximum absorbance at 510 nm. The extracts required for the measurement are prepared from the test samples, and solutions are pre-prepared: 5% sodium nitrite, 10% aluminum chloride, 1M sodium hydroxide, and a standard quercetin solution. Additionally, 7 samples for the calibration chart are prepared containing the following components: quercetin standard solution, 5% sodium nitrite, and 10% aluminum chloride. The resulting solutions are mixed thoroughly and the absorbance is determined against the control sample at 510 nm using a ULAB 102 UV spectrophotometer. From the data obtained, the flavonoid content is calculated according to the corresponding formula (Bijy et al., 2013).

2.5. Determination of polyphenol content

The colorimetric method for determining the total content of phenolic substances is based on the use of the Folin-Ciocalteu reagent, which is a mixture of phosphoric-molybdenum and phosphoric-tungstic acids that is reduced by oxidation of phenols to a mixture of oxides. This reaction produces a blue color, which is proportional to the number of phenolic substances. The Folin-Ciocalteu method is commonly used to determine phenolic compounds in wine, juices, fruits, and vegetables. To determine polyphenols, an extract from plants is used, which is prepared beforehand. Test samples contain 50 µl of the plant preparation and 150 µl of water. After that, 1 ml of Folin-Ciocalteu reagent diluted 1:100 is added to each sample, mixed and incubated for 8 minutes. Then add 7.5% sodium carbonate, mix and incubate for 15 minutes at 45 °C. The absorbance is determined against the control sample at a wavelength of 765 nm using a ULAB 102 UV spectrophotometer. Also, along with the test samples, 6 tubes for the calibration graph are prepared: standard solution of gallic acid, Folin-Ciocalteu reagent, and 7.5% sodium carbonate. All actions are carried out similarly to the experimental samples. The polyphenol content is calculated according to the corresponding formula (Nazish Siddiqui et al. 2013).

2.6. Determination of pigment concentration

For pigment extraction, leaves were homogenized with ice-cold 96% ethanol (1:10, w:v) in the

presence of $CaCO_3$ to prevent pheophytinization. The homogenates were centrifuged at 8000×g for 10 min (4 °C). Supernatants were collected and the pigments were repeatedly extracted twice from pellets with 1 mL ice-cold 96% ethanol and extracts were pooled. The concentrations of pigments were measured spectrophotometrically. Specific absorption coefficients for chlorophyll *a*, chlorophyll *b*, and total carotenoids were used (Husak et al., 2020). Molecular mass of carotenoids 570 was used for calculation. The anthocyanin concentration was measured as described previously (Gitelson et al. 2001; Semchuk et al. 2009).

2.7. Statistics

Each treatment was performed in two replications with six samples in each. Data are presented as means ± S.E.M. Statistical analysis was performed using StatOptima computer program (version 2.5) with ANOVA followed by a Dunkan test. The probability value of $P < 0.05$ was considered to be statistically significant.

3. RESULTS AND DISCUSSION

In plants, flavonoids have many functions, such as regulating cell growth, attracting insect pollinators, and protecting against biotic and abiotic stresses. For example, plant flavonoids can work as signaling molecules, UV filters, and play several functional roles in resistance to drought, heat, and freezing. Flavonoids can prevent damage to plants by viruses, fungi, bacteria, and herbivores, function as chemical messengers in connection with (or in association with) mycorrhiza and bacteria, and contribute to the color of flowers to attract pollinators (Shen et al., 2022). The study revealed no significant differences in flavonoid content among groups A, C, and D (Fig. 1). However, group B, cultivated with 2 mg/L NAA, exhibited a marked increase of 90%, 70%, and 80% compared to groups A, C, and D, respectively. This suggests that the presence of NAA enhances flavonoid biosynthesis, likely by promoting enzymatic pathways associated with flavonoid metabolism.

Research on flavonoids, auxins, and cytokinins reveals complex interactions in plant development and symbiosis. Flavonoids can modulate auxin transport and accumulation, influencing root development and nodulation in legumes (Ng et al., 2015; Kurepa et al., 2023). In Medicago truncatula, cytokinin signaling through CRE1 regulates flavonoid production, which in turn affects auxin transport during nodule initiation (Ng et al., 2015). Bradyrhizobium japonicum, a nitrogen-fixing bacterium, can synthesize auxins and cytokinins, but exposure to flavonoids like genistein and naringenin reduces phytohormone production (Leonova, 2015). This suppression may redirect bacterial metabolism towards nodulation mechanisms rather than secondary metabolite synthesis (Leonova, 2015). The auxin-cytokinin balance, influenced by flavonoids, plays a crucial role in plant terrestrialization by regulating shoot/root growth ratios in response to environmental stresses (Kurepa et al., 2023). These findings highlight the intricate relationships between flavonoids, auxins, and cytokinins in plant-microbe interactions and adaptation to terrestrial environments.

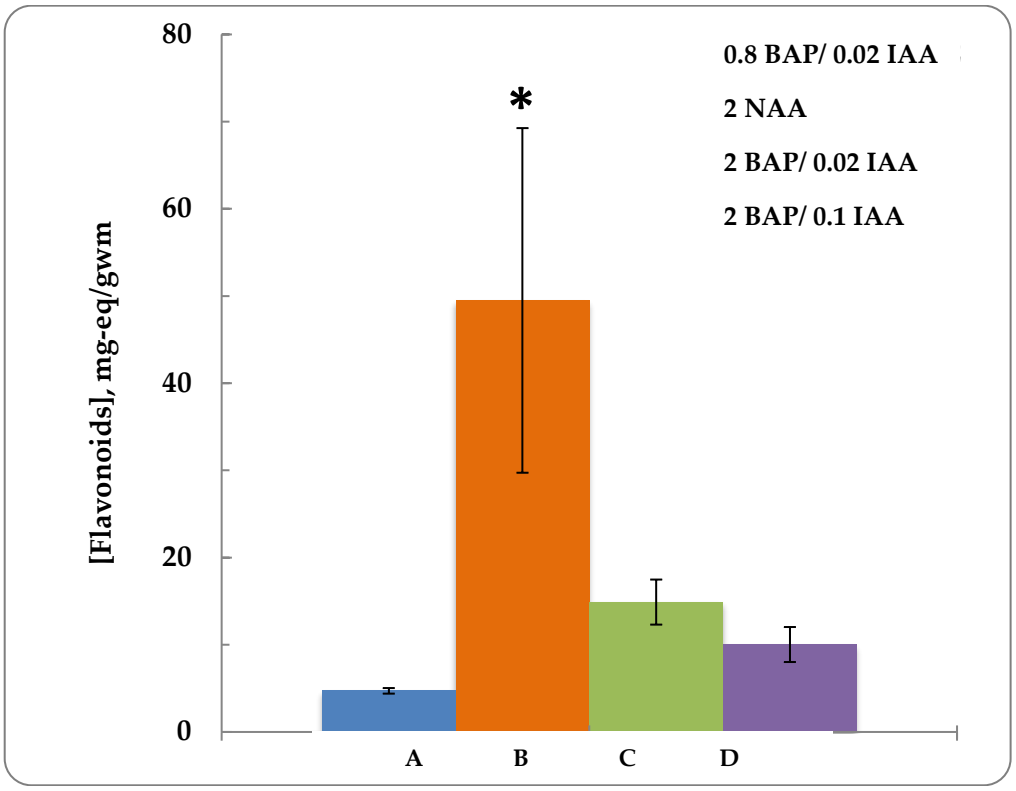

Figure 1. The effect of different concentrations of phytohormones on the flavonoid content in *G. procumbens*. * - significantly different from the groups with $P < 0.05$. n = 4-6

Phenolic compounds, or polyphenols, are widespread and involved in key metabolic and physiological processes in plants. Polyphenols, for example, are involved in signal transduction from root to shoot and also help in nutrient mobilization. They are produced under optimal and suboptimal conditions in plants and play a crucial role in development, including signal transduction, cell division, hormonal regulation, regulation of photosynthetic activity, germination and reproduction rates. Plants that exhibit increased polyphenol synthesis under abiotic stresses usually show better adaptation to limited environments (Belščak-Cvitanović et al., 2018).

The polyphenol content in the extracts of *G. procumbens* leaves was significantly higher in group B by 28% and 31% compared to groups A and D, respectively (Fig. 2). This highlights the role of NAA in enhancing polyphenol synthesis, possibly through its influence on stress-related metabolic pathways. Plants with elevated polyphenol levels generally exhibit better tolerance to abiotic stress, further underscoring the importance of optimizing growth conditions to enhance these compounds. Gynura species, particularly *G. procumbens, G. bicolor, and G. divaricata*, are medicinal plants rich in phenolic compounds with antioxidant properties (Chen et al., 2015; Qiu et al., 2018). These plants contain various polyphenols, including caffeoylquinic acids, p-coumaroylquinic acids, and flavonoids like kaempferol and quercetin (Chen et al., 2015; Qiu et al., 2018). Cultivation conditions affect the polyphenol content in Gynura species, with longer irrigation cycles (72 hours) resulting in higher total polyphenol concentrations in G. procumbens (Kyu-Hoi et al., 2020). Interestingly, polyphenols may influence plant growth by inhibiting polar auxin transport, as demonstrated in tomato plants (Marigo & Boudet, 1977). The antioxidant activity of Gynura extracts correlates with their total phenolic content, with plants from different origins showing varying levels of antioxidant capacity (Chen et al., 2015; Qiu et al., 2018). These findings highlight the potential of Gynura species for medicinal development and their importance as nutritious vegetables in Asian countries.

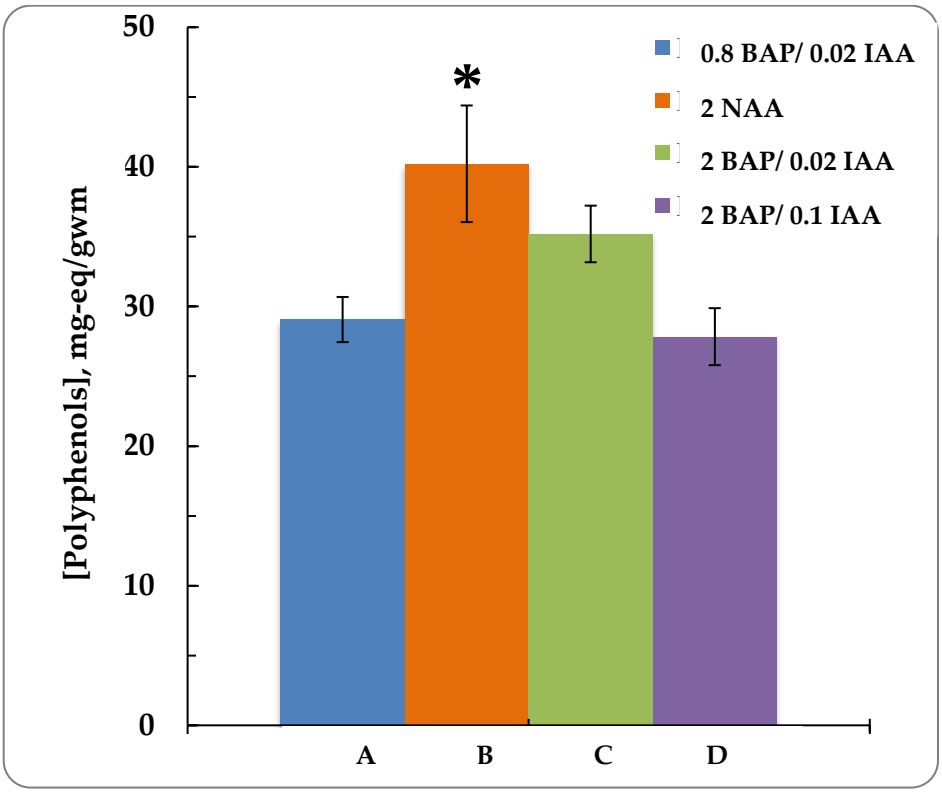

Figure 2. The effect of different concentrations of phytohormones on the polyphenol content in *G. procumbens*. * - significantly different from the groups with $P < 0.05$. n = 4-6

Chlorophylls, carotenoids, anthocyanins are the four main classes of biological pigments produced in plants (Pareek et al., 2017; Takashi, 2020).

Chlorophylls are the main pigments responsible for plant photosynthesis. The other three are auxiliary pigments and secondary metabolites, which have much more diverse structures and functions in plants and greater potential nutritional and medicinal benefits in the diet. The stability of chlorophyll content suggests that the plant's primary photosynthetic machinery remains unaffected under these experimental conditions. Research on plant growth regulators reveals complex interactions between auxins, cytokinins, and chlorophyll biosynthesis. Auxins generally inhibit chlorophyll accumulation, with auxin-deficient mutants showing increased chlorophyll content and improved photosynthetic efficiency (Luo et al., 2023). This inhibition occurs through ARF7-IAA14-mediated repression of chlorophyll biosynthesis genes like PORA and GUN5 (Luo et al., 2023). Conversely, cytokinins promote chlorophyll biosynthesis and inhibit its degradation (Almeida & Rodrigues, 2016; Kobayashi et al., 2012). The transcription factors HY5 and GLKs play crucial roles in regulating root greening, with HY5 being essential for chlorophyll biosynthesis gene expression and GLKs acting as potent activators (Kobayashi et al., 2012). Endophytic bacteria isolated from *G. procumbens* leaves have been found to produce cytokinin-like compounds, potentially contributing to the plant's growth and development (Bhore et al., 2010). These findings highlight the intricate balance of plant hormones in regulating chlorophyll metabolism and plastid differentiation. The study showed no significant differences in the chlorophyll *a* and *b* content across all treatment groups (Fig. 3a, b). This indicates that variations in phytohormone concentrations did not impact the photosynthetic potential of *G. procumbens*.

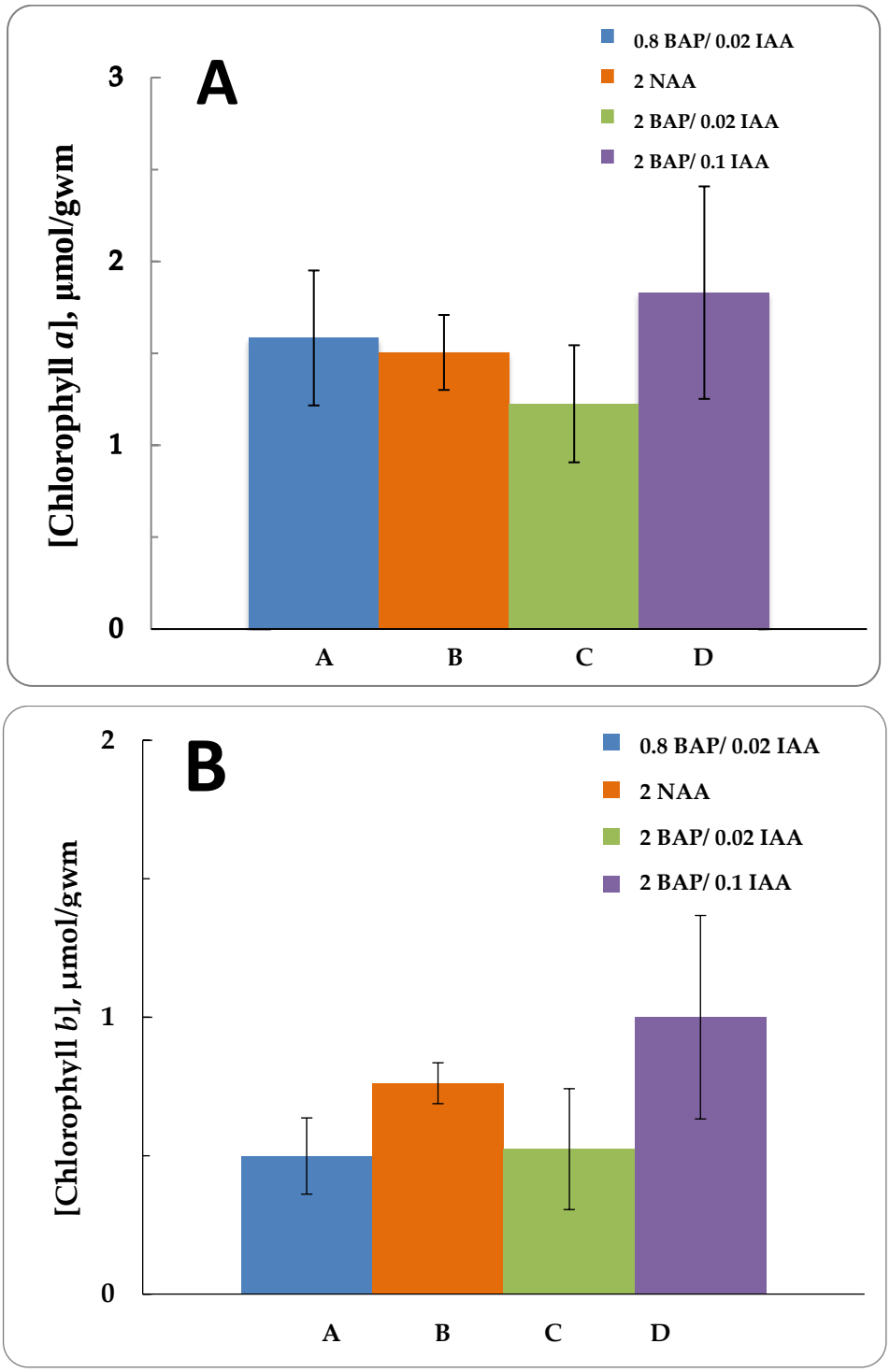

Figure 3. Chlorophyll *a* and *b* contents in *G. procumbens* leaves under the influence of different concentrations of phytohormones. n=4-6

Carotenoids are isoprenoids that are most commonly found in plants and microorganisms. They are important components of photosystems and give flowers and fruits their yellow-red color.

The carotenoid content was significantly higher in group D (2 mg/L BAP and 0.1 mg/L IAA), with increases of 47%, 36%, and 66% compared to groups A, B, and C, respectively (Fig. 4). This highlights the synergistic effect of BAP and IAA in promoting carotenoid biosynthesis, possibly by upregulating key enzymes in the carotenoid pathway.

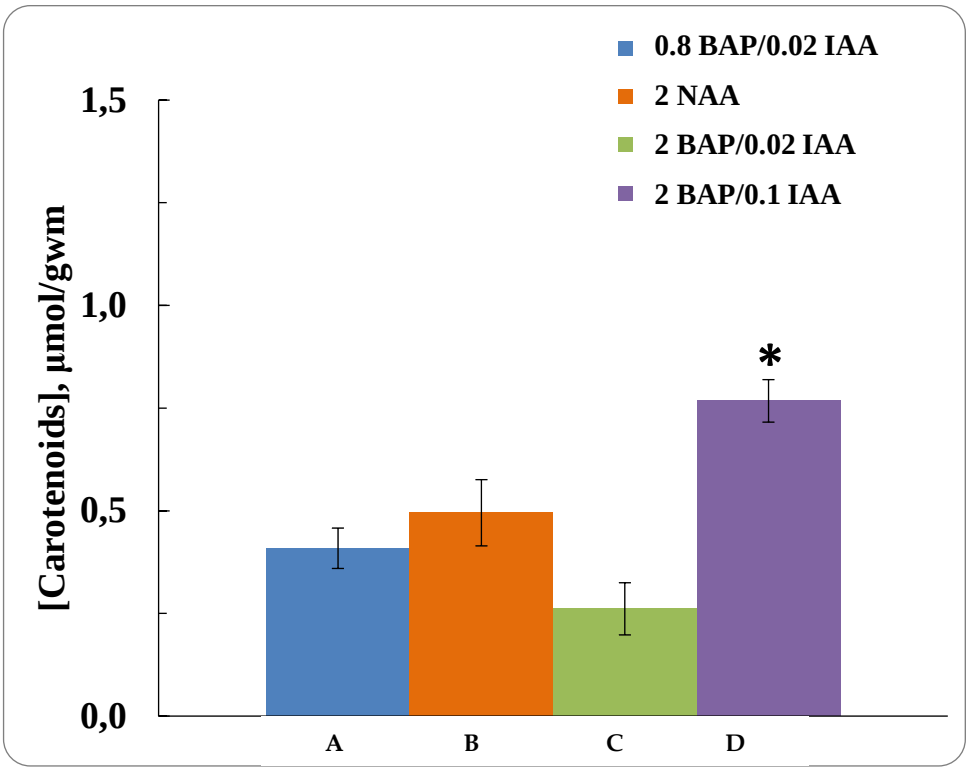

Figure 4. Carotenoid content in *G. procumbens* leaves under the influence of different concentrations of phytohormones. * - significantly different from the groups with $P < 0.05$. n=4-6

Anthocyanins, water-soluble flavonoids responsible for a wide spectrum of colors in plants, showed no significant differences among the treatment groups (Fig. 5). The lack of variation suggests that the selected phytohormone concentrations do not strongly influence anthocyanin synthesis in *G. procumbens* under the conditions tested.

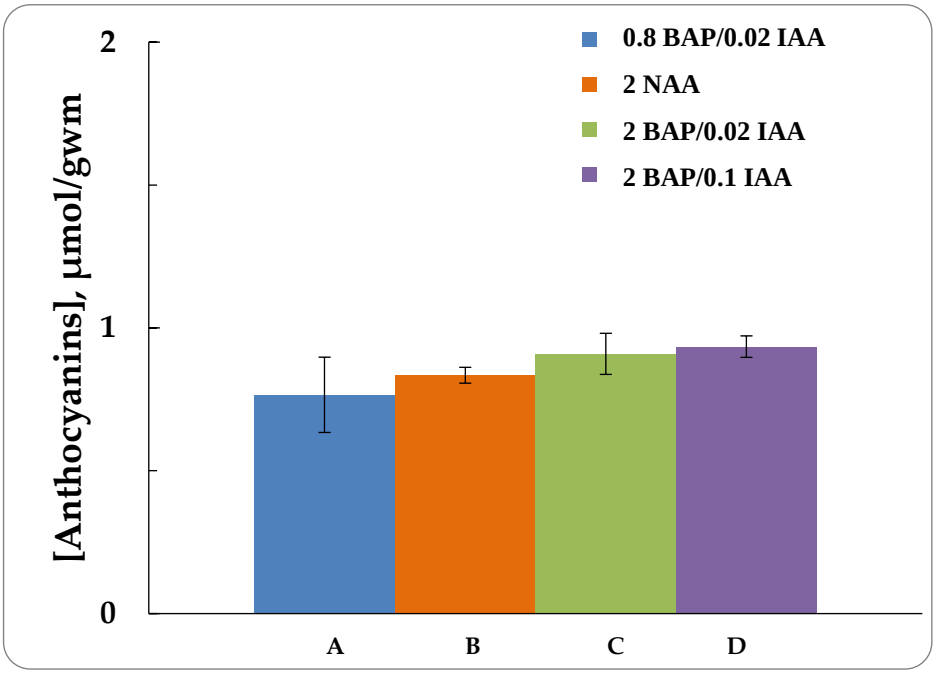

Figure 5. Anthocyanin content in *G. procumbens* leaves under the influence of different concentrations of phytohormones. n=4-6

Research on carotenoids, cytokinins, and auxins in various plant species has revealed complex interactions. In *Chlorella pyrenoidosa*, cytokinins strongly stimulated α- and β-carotene production, while both cytokinins and auxins reduced xanthophyll content (Czerpak & Bajguz, 2014). In Ricinus cell cultures, auxin and cytokinin controlled chloroplast differentiation, while auxin removal triggered chromoplast formation and rhodoxanthin accumulation (Gemmrich & Kayser, 1984). The genus Gynura, known for its ethnomedicinal uses, contains various phytochemicals including carotenoids (Bari et al., 2021). Endophytic bacteria isolated from *G. procumbens* leaves were found to produce cytokinin-like compounds, potentially influencing the plant's growth and development (Bhore et al., 2010). These studies highlight the intricate relationships between plant hormones and carotenoid biosynthesis, as well as the potential role of endophytic bacteria in plant growth regulation through hormone production.

4. CONCLUSION

This study demonstrates that the in vitro cultivation of *Gynura procumbens* under specific phytohormone conditions can optimize the production of certain phytochemicals. Specifically, the application of 2 mg/L NAA significantly enhances flavonoid and phenolic compound content, while the combination of 2 mg/L BAP and 0.1 mg/L IAA maximizes carotenoid synthesis. These findings provide valuable insights into the metabolic regulation of phytochemicals in *Gynura procumbens*, with implications for both agricultural practices and pharmacological applications. Future research should explore the underlying molecular mechanisms driving these changes to further refine in vitro cultivation strategies.

Conflict of interest

The authors declare that they have no conflict of interest.

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Ольга Булій, Дмитро Шебунчак, Віктор Гусак. Біохімічні показники *Gynura procumbens* Lour. на різних типах поживних середовищ в умовах *in vitro*. *Журнал Прикарпатського університету імені Василя Стефаника. Біологія*, Том 11 (2024), С.104-С.114.

Флавоноїди, фенольні сполуки, хлорофіли, каротиноїди та антоціани є життєво важливими вторинними метаболітами рослин, що сприяють їх росту, адаптації до стресових факторів навколишнього середовища та мають лікувальні властивості. У цій роботі досліджено вплив різних концентрацій фітогормонів на біохімічний склад *Gynura procumbens*, культивованої *in vitro*. Вміст флавоноїдів значно відрізнявся між групами, причому найвищий рівень спостерігався у рослин, вирощених на середовищі з додаванням NAA у концентрації 2 мг/л, демонструючи збільшення на 70-90% порівняно з іншими варіантами обробки. Аналогічно, вміст фенольних сполук був підвищений на 28-31% за тих самих умов, що свідчить про покращену стійкість рослин до стресу. Вміст хлорофілів *a* і *b* залишався статистично однаковим у всіх групах, що свідчить про стабільний фотосинтетичний потенціал незалежно від концентрації фітогормонів. Однак синтез каротиноїдів досягав піку в рослинах, вирощених на середовищі, що містило 2 мг/л БАП та 0,1 мг/л ІОК, демонструючи збільшення на 36-66% порівняно з іншими варіантами обробки. Вміст антоціанів не мав суттєвих відмінностей між групами. Ці результати підкреслюють потенціал оптимізації концентрації фітогормонів *in vitro* для підвищення питомої фітохімічної продуктивності *Gynura procumbens*, рослини з багатообіцяючим терапевтичним застосуванням.

Ключові слова: *Gynura procumbens* Lour., гинура, флавоноїди, поліфеноли, рослинні пігменти.