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# Enhancing Morpho-phytochemical Properties of *Catharanthus roseus* L. var. 'Ocellatus' via Plant Growth Regulators

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**ABSTRACT**

*Catharanthus roseus* L. is a valuable medicinal plant renowned for its potent anticancer compounds, i.e., vincristine and vinblastine. The efficient mass production of this plant can be challenging due to limitations in conventional propagation methods. Tissue culture techniques offer a promising alternative, and optimizing plant growth regulator (PGR) treatments can play a crucial role in achieving successful *in vitro* propagation. In this study, we investigated the effects of several PGR combinations, i.e., NAA, BAP, and 2,4-D, on the morpho-phytochemical attributes and antioxidant activity of *in vitro*-grown *Catharanthus roseus* L. var. 'Ocellata'. The plants were arranged in a completely randomized design with three replications. The results demonstrated that applying PGRs, either alone or in combination, significantly improved the morphological characteristics. The NAA treatment caused the highest plant height, number of branches and leaves, and root diameter. Notably, the treatment with 2,4-D resulted in the highest photosynthetic pigment content compared to the control. Moreover, the treatments with NAA + BAP and NAA + BAP + 2,4-D exhibited the highest levels of total phenolic compounds (TPC) and total flavonoid content (TFC) in both roots and leaves. Regarding antioxidant activity, the DPPH radical scavenging assay revealed the highest radical scavenging percentages, i.e., 74.21% (NAA + 2,4-D) in leaf samples and 78.08% (NAA + 2,4-D) in root samples. These findings emphasize the potency of PGRs in optimizing tissue culture protocols for *Catharanthus roseus* L., thus facilitating the production of superior-quality plants with enriched medicinal properties. This study contributes to the advancement of sustainable and efficient cultivation of bioactive compounds from *Catharanthus roseus* L. using tissue culture techniques.

## Introduction

*Catharanthus roseus* L., commonly known as Madagascar periwinkle, is a medicinal plant renowned for its production of valuable anticancer compounds, vincristine, and vinblastine (Kabesh et al., 2015; Pereira et al., 2009). These compounds possess significance in treating various types of cancer, making *Catharanthus roseus* a highly sought-after plant in the pharmaceutical industry (Chen et al., 2018; Yamamura et al., 2017; Montagna et al., 2017; Gajalakshmiet al., 2013; Marcone et al., 1997; Pandey-Rai et al., 2006). However, conventional methods of propagation, such as sexual reproduction and cutting, exhibit low success rates and pose challenges for large-scale production (Bakrudeen et al., 2011). Tissue culture techniques have partly solved these problems and have emerged as a promising approach for the efficient

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and sustainable production of this valuable plant species (Sandhya et al., 2016).

Tissue culture, conducted under controlled laboratory conditions, provides an ideal environment conducive to propagating plant material rapidly and accurately while maintaining genetic uniformity and desired traits (Anand, 2010; Smetanska, 2008). In tissue culture, optimizing culture media components, including plant growth regulators (PGRs), can vitally assist in enhancing growth, development, and secondary metabolite production under in vitro-grown plants (Roberts, 2012).

PGRs, such as auxins and cytokinin, and their combinations, reportedly regulate various physiological processes in plants (Yong et al., 2009). They influence cell division, differentiation, and organogenesis, thus affecting the growth and development of tissue-cultured plants (Mitra et al., 2016). Moreover, PGRs have reportedly amended the biosynthesis of secondary metabolites, including phenolic components, flavonoids, and alkaloids, which are responsible for the therapeutic properties of medicinal plants (Derbassi et al., 2022; Ochatt et al., 2022).

In this study, our objective was to investigate the effects of different PGR treatments on the morphological and phytochemical characteristics of *in vitro*-grown *Catharanthus roseus* L. var. 'Ocellata' plants. We sought to optimize the tissue culture conditions to increase the production of valuable bioactive compounds with potential therapeutic applications. By evaluating various combined and separate PGR treatments, including NAA (Naphthalene acetic acid), BAP (6-benzylaminopurine), and 2,4-D (2,4-dichlorophenoxyacetic acid), we aimed to elucidate the specific roles of these regulators in enhancing plant growth, biomass accumulation, and secondary metabolite synthesis.

## Materials and Methods

### *Plant materials and explant preparation*

Nodal shoot explants (with two pairs of leaves) were obtained from healthy *Catharanthus roseus* L. plants grown in the research greenhouse at the University of Zanjan. The explants were immediately transferred to the Horticulture Biotechnology and Tissue Culture Laboratory for further processing.

### *Sterilization protocol*

The explants were subjected to a sterilization protocol to eliminate any surface contaminants. The protocol involved washing the explants with running tap water for 5 min, followed by immersion in a solution of 70% ethanol for 1 min, and subsequently in 5% sodium hypochlorite for 10 min. The explants were then rinsed three times with sterile distilled water (Hernández-Domínguez et al., 2004).

### *Culture medium and treatment setup*

After sterilization, the explants were placed on a culture medium based on Murashige and Skoog (MS) (Classic Murashige and Skoog, 1962) medium supplemented with different PGRs as treatments. The PGR treatments were NAA (1 mg $L^{-1}$), (1 mg $L^{-1}$) + BAP (1 mg $L^{-1}$), NAA (1 mg $L^{-1}$) + 2,4-D (0.5 mg $L^{-1}$), NAA (1 mg $L^{-1}$) + BAP (1 mg $L^{-1}$) + 2,4-D (0.5 mg $L^{-1}$), BAP (1 mg $L^{-1}$), BAP (1 mg $L^{-1}$) + 2,4-D (0.5 mg $L^{-1}$), 2,4-D (0.5 mg $L^{-1}$). A control treatment without any PGRs was also included. The research was conducted as a completely randomized design (CRD) with three replications.

### *Incubation and growth conditions*

Following the treatment setup, the explants were incubated in a growth chamber at a temperature of 25 °C under a 16 h photoperiod provided by white fluorescent lights with a photon flux density of 70 µmol $m^{-2}$ $s^{-1}$.

### *Measurement of morphological traits*

After 90 days of growth in the tissue culture environment, various morphological traits were measured, including plant height, stem diameter, intermediate length, root diameter, root length, number of branches (NB), number of leaves, fresh yield, dry yield, tissue water content (shoot and root water content), tissues dry matter content (shoot and root dry matter content), shoot fresh yield, root fresh yield, shoot dry yield, and root dry yield.

### *Measurement of photosynthetic pigments*

Chlorophyll a, chlorophyll b, and carotenoid contents were evaluated according to a method described by Arnone (1949).

### *Measurement of phytochemicals, determination of total phenolic and flavonoid content*

Phytochemical attributes such as total phenolic content (TPC) and total flavonoid content (TFC) were assayed. Methanolic extracts of shoot and root tissues were prepared and the TPC was evaluated using the Folin-Ciocalteu reagent method and calibrated against Gallic acid standards, following a relevant protocol (Singleton and Rossi, 1965). The results were expressed as mg of Gallic acid equivalents (GAE mg $g^{-1}$) per g of dry weight extract. A spectrophotometer measured the absorbance at

765 nm.

The spectrophotometric colorimetric method (Kähkönen et al., 1999) determined the TFC. The reaction mixture was prepared in a test tube by adding 500 µL of the extract solution, 100 µL of 10% aluminum chloride solution, 100 µL of 1 M potassium acetate solution, and 2.8 mL of distilled water. The samples were incubated for 40 min at room temperature, and the absorbance was measured at 415 nm using a spectrophotometer (T-60, PG Instrument UK). Quercetin was used to plot the standard curve, and the results were expressed as milligrams of quercetin equivalents (QE mg $g^{-1}$) per gram of extract.

### *Antioxidant capacity determination*

The antioxidant capacity of the plant extracts was assessed using three different methods: DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay, reducing power assay, and phosphomolybdenum assay (TAC assay).

### *DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay*

The DPPH radical scavenging assay was performed according to a relevant method (Hatano et al., 1988). A stock solution of DPPH was prepared by dissolving 4 mg in 100 mL of methanol and kept at 20 °C for further use. A mixture of 250 µL of plant samples and 750 µL of DPPH aliquot was incubated for 15 min at room temperature in the dark. Optical density was measured at a wavelength of 517 nm. The antioxidant capacity was calculated using the following equation:

$$Free\ radical\ scavenging\ activity\ (\%) = \frac{Control\ absorbance\ -\ Sample\ absorbance}{Control\ absorbance} \times 100$$

### *Reducing power assay*

Reducing power activity was determined using a method described in the literature (Oyaizu, 1986). A mixture of 0.5 mL plant extract, 0.5 mL 0.2 M phosphate buffer (pH 6.6), and 0.5 mL potassium ferricyanide (10 mg $L^{-1}$) was incubated at 50 °C for 20 min. Then, 0.5 mL of trichloroacetic acid (TCA) (100 mg $L^{-1}$) was added to the solution, and the mixture was centrifuged at 3000 rpm for 10 min. Then, 0.5 mL of the supernatant was diluted with 0.5 mL of pure water and mixed with 0.1 mL of FeCl3 (0.1%). The optical density was measured at 700 nm after 10 min. Methanol (80%) was used as a blank, and a solution mixture without plant extract served as the control under the same preparation conditions for each plant sample.

### *Phosphomolybdenum assay or total antioxidant capacity (TAC) assay*

Total antioxidant capacity of plant samples was determined using the phosphomolybdenum assay as described in the literature (Prieto et al., 1999). The phosphomolybdenum reagent solution was prepared by mixing 1 mL of $Na_3PO_4$ (28 mM), $H_2SO_4$ (0.6 M), and ammonium molybdate (4 mM) with 0.3 mL of the plant extract. The reaction mixture was heated at 95 °C in a water bath for 90 min, covered with silver foil to avoid direct light exposure. After cooling them down to room temperature, the absorbance was measured at 695 nm using a spectrophotometer. All treatments occurred in triplicates.

$$\%\ TAC\ inhibition = \frac{Abs\ sample\ -\ Abs\ control}{Abs\ sample} \times 100$$

### *Statistical analysis*

All data were subjected to one-way analysis of variance (ANOVA) using SAS statistical analysis software version 9.4. Data were expressed as mean values ± standard deviation (SD), and significant differences were determined among the treatment groups using LSD tests ($P \leq 0.05$).

## Results

### *Morphological attributes*

Full plantlet regeneration of the typical shoot explants on MS medium supplemented with various PGRs occurred in the third month of in vitro culture (Figs. 1 and 2).

Compared to the control, all measured morphological traits showed significant differences in all treatments. Regarding most of the desired traits, the PGRS treatments improved morphological attributes (Table 1). The highest mean values of height (71.75 mm), number of lateral branches (5), number of leaves (40), and internode length (9.71 mm) occurred in response to the 1 mg $L^{-1}$ NAA treatment. Regarding plant diameter, root diameter, and length, maximum values (4.67 mm, 3.67 mm, and 115.97 mm) occurred in response to the 1 mg $L^{-1}$ BAP + 0.5 mg $L^{-1}$ 2,4-D, 1 mg $L^{-1}$ NAA + 1 mg $L^{-1}$ BAP, and 0.5 mg $L^{-1}$ 2,4-D treatments, respectively. Meanwhile, the smallest root diameter and length (0.53 mm and 10.66 mm) were in the control group. The fresh and dry yield and its components, such as fresh and dry yield of the roots and shoots, had the

highest significant values (8662.43 mg, 709.7 mg, 3051.66 mg, 5610.77 mg, 236.5 mg, and 473.2 mg) in MS medium supplemented with 1 mg $L^{-1}$ NAA + 1 mg $L^{-1}$ BAP (Table 1).

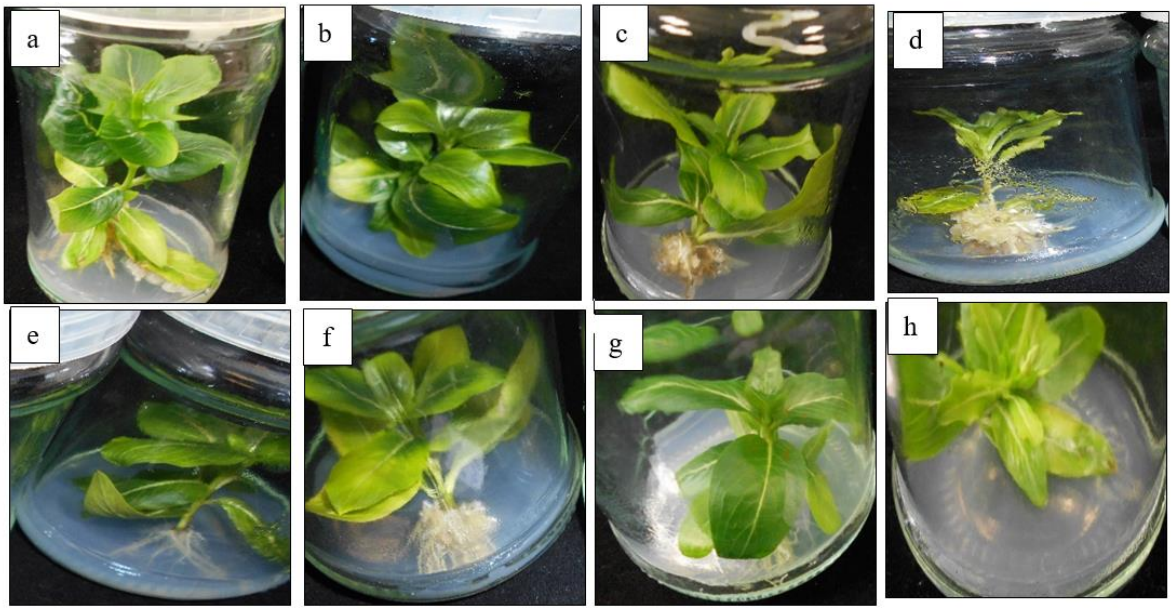


**Fig. 1.** Shoot regeneration of the typical shoot explant of *C. roseus* L. var. 'Ocellatus' on MS medium supplemented with different PGRs (a: NAA, b: NAA + BAP, c: NAA + 2,4-D, d: NAA + BAP + 2,4-D, e: BAP, f: BAP + 2,4-D, g: 2,4-D, h: control). NAA: 1-Naphthaleneacetic acid, BAP: 6-Benzylaminopurine, 2,4-D: 2,4-Dichlorophenoxyacetic acid.

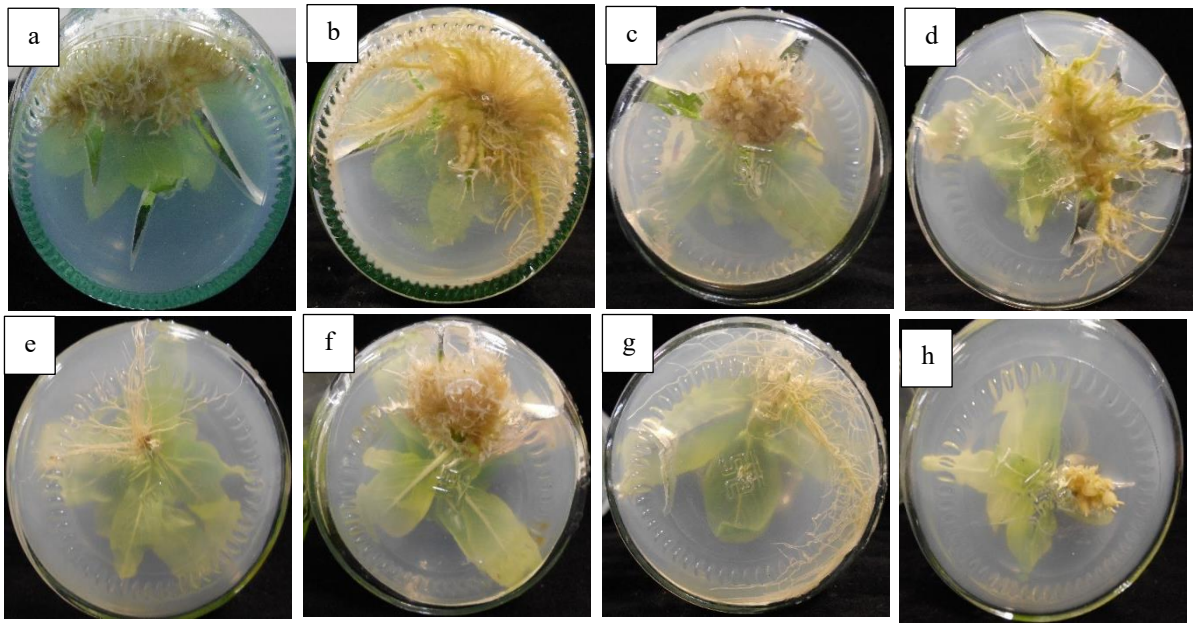


**Fig. 2.** Root system regeneration of the typical shoot explant of *C. roseus* L. var. 'Ocellatus' on MS medium supplemented with different PGRs (a: NAA, b: NAA + BAP, c: NAA + 2,4-D, d: NAA + BAP + 2,4-D, e: BAP, f: BAP + 2,4-D, g: 2,4-D, h: control). NAA: 1-Naphthaleneacetic acid, BAP: 6-Benzylaminopurine, 2,4-D: 2,4-Dichlorophenoxyacetic acid.

**Table 1.** Morphological traits of *C. roseus* var. 'Ocellatus' on MS medium supplemented with different PGRs.

| Treatments | | Height (mm) | NB | Plant Diameter (mm) | NL | IL (mm) | RD (mm) | RL (mm) |
|---|---|---|---|---|---|---|---|---|
| 1 mg $L^{-1}$ NAA | | 71.75 ± 0.09$^{a}$ | 5 ± 0$^{a}$ | 2.75 ± 0.01$^{c}$ | 40 ± 1$^{a}$ | 9.71 ± 0.29$^{a}$ | 3.04 ± 0.58$^{a}$ | 22.76 ± 0.29$^{d}$ |
| 1 mg $L^{-1}$ NAA + 1 mg $L^{-1}$ BAP | | 68.96 ± 18.5$^{ab}$ | 3 ± 1$^{b}$ | 3.29 ± 0.9$^{bc}$ | 29 ± 8.7$^{b}$ | 7.55 ± 1.21$^{abc}$ | 3.67 ± 0.59$^{a}$ | 70.14 ± 9.5$^{b}$ |
| 1 mg $L^{-1}$ NAA + 0.5 mg $L^{-1}$ 2,4-D | | 57.06 ± 0.6$^{bcd}$ | 1.66 ± 0.57$^{cd}$ | 2.71 ± 0.04$^{c}$ | 25 ± 1$^{b}$ | 7.72 ± 0.05$^{ab}$ | 0.46 ± 0.05$^{b}$ | 24 ± 4$^{d}$ |
| 1 mg $L^{-1}$ NAA + 1 mg $L^{-1}$ BAP + 0.5 mg $L^{-1}$ 2,4-D | | 41.02 ± 1.5$^{e}$ | 2 ± 0$^{c}$ | 3.5 ± 0.68$^{bc}$ | 22.33 ± 5.03$^{b}$ | 5.49 ± 0.96$^{cd}$ | 3.01 ± 1.9$^{a}$ | 24.17 ± 3.7$^{cd}$ |
| 1 mg $L^{-1}$ BAP | | 62.18 ± 5.05$^{abc}$ | 3 ± 0$^{b}$ | 2.92 ± 0.19$^{c}$ | 28.33 ± 1.5$^{b}$ | 7.48 ± 2.08$^{bc}$ | 0.74 ± 0.12$^{b}$ | 45.28 ± 26.56$^{c}$ |
| 1 mg $L^{-1}$ BAP + 0.5 mg $L^{-1}$ 2,4-D | | 45.01 ± 2.8$^{de}$ | 1 ± 0$^{d}$ | 4.67 ± 0.4$^{a}$ | 23 ± 3$^{b}$ | 6.08 ± 0.82$^{bcd}$ | 1.44 ± 0.43$^{b}$ | 8.50 ± 1.2$^{d}$ |
| 0.5 mg $L^{-1}$ 2,4-D | | 51.61 ± 5.7$^{cde}$ | 1.33 ± 0.57$^{cd}$ | 3.21 ± 0.05$^{bc}$ | 24.66 ± 5.03$^{b}$ | 5.17 ± 2.06$^{d}$ | 0.76 ± 0.09$^{b}$ | 115.97 ± 18.1$^{a}$ |
| Control | | 24.49 ± 3.1$^{f}$ | 1 ± 0$^{d}$ | 3.89 ± 0.41$^{ab}$ | 22.66 ± 2.3$^{b}$ | 2.29 ± 0.99$^{e}$ | 0.53 ± 0.03$^{b}$ | 10.66 ± 7.17$^{d}$ |
| LSD | | 12.57 | 0.79 | 0.79 | 7.37 | 2.18 | 1.34 | 21.26 |
| Significant | df | | | | | | | |
| Treatment | 7 | ** | ** | ** | ** | ** | ** | ** |
| CV | | 13.76 | 20.28 | 13.58 | 15.85 | 19.63 | 15.83 | 15.50 |

Data was expressed as means ± standard deviation (n = 3). Different superscript letters in the same column indicate significant differences according to LSD ($p \leq 0.05$), ** and * are significant at the 0.01 and 0.05 levels. (Nb: number of branches, Nl: number of leaves, IL: intermediate length, RD: root diameter, RL: root length). Data are mean values of n = 3. Mean values followed by different letters indicate they are significantly different by the LSD test ($p<0.05$). NAA: 1-Naphthaleneacetic acid, BAP: 6-Benzylaminopurine, 2,4-D: 2,4-Dichlorophenoxyacetic acid.

**Table 1.** (Continued)

| Treatments | FY (mg) | DY (mg) | SH TW (%) | RTW (%) | DRM (%) | DSHM (%) | SHFY (mg) | RFY (mg) | SHDY (mg) | RDY (mg) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 mg $L^{-1}$ NAA | 7021.9 ± 7.8$^{c}$ | 411.23 ± 0.45$^{c}$ | 94.42 ± 2.25$^{a}$ | 93.98 ± 0.08$^{a}$ | 6.04 ± 0.08$^{e}$ | 5.58 ± 2.25$^{h}$ | 2550.16 ± 5.15$^{c}$ | 4471.7 ± 5.8$^{c}$ | 142.26 ± 0.3$^{d}$ | 268.96 ± 0.15$^{c}$ |
| 1 mg $L^{-1}$ NAA + 1 mg $L^{-1}$ BAP | 8662.43 ± 8.6$^{a}$ | 709.7 ± 1.1$^{a}$ | 92.25 ± 1.31$^{e}$ | 91.56 ± 1.56$^{c}$ | 8.43 ± 1.56$^{c}$ | 7.75 ± 1.31$^{d}$ | 3051.66 ± 10.02$^{a}$ | 5610.77 ± 17.9$^{a}$ | 236.5 ± 0.8$^{a}$ | 473.2 ± 1.6$^{a}$ |
| 1 mg $L^{-1}$ NAA + 0.5 mg $L^{-1}$ 2,4-D | 8028.2 ± 9.1$^{b}$ | 527.66 ± 1.2$^{b}$ | 93.43 ± 0$^{b}$ | 93.42 ± 0$^{b}$ | 6.57 ± 0$^{d}$ | 6.57 ± 0$^{g}$ | 2930 ± 10$^{b}$ | 5098.2 ± 2$^{b}$ | 192.4 ± 1.3$^{b}$ | 335.2 ± 0.17$^{b}$ |
| 1 mg $L^{-1}$ NAA + 1 mg $L^{-1}$ BAP + 0.5 mg $L^{-1}$ 2,4-D | 4993 ± 28.7$^{d}$ | 318.7 ± 2.6$^{d}$ | 92.43 ± 0.54$^{d}$ | 94.17 ± 0.71$^{a}$ | 5.82 ± 0.71$^{e}$ | 7.56 ± 0.54$^{e}$ | 1603.1 ± 6.5$^{e}$ | 3389.9 ± 27.4$^{d}$ | 121.2 ± 0.5$^{e}$ | 197.5 ± 2.3$^{d}$ |
| 1 mg $L^{-1}$ BAP | 1602.97 ± 14.27$^{g}$ | 132.86 ± 1.4$^{g}$ | 1.43 ± 1.62$^{f}$ | 94 ± 2.47$^{a}$ | 5.99 ± 2.47$^{e}$ | 8.56 ± 1.62$^{c}$ | 1429.6 ± 10.5$^{f}$ | 173.37 ± 4.08$^{g}$ | 122.4 ± 1.2$^{e}$ | 10.39 ± 0.35$^{g}$ |
| 1 mg $L^{-1}$ BAP + 0.5 mg $L^{-1}$ 2,4-D | 3038.30 ± 48.37$^{e}$ | 302.83 ± 4.8$^{e}$ | 90.93 ± 0.78$^{h}$ | 88.75 ± 1.2$^{d}$ | 11.24 ± 1.2$^{b}$ | 9.07 ± 0.78$^{a}$ | 1783.76 ± 11.3$^{d}$ | 1254.5 ± 37.10$^{e}$ | 161.76 ± 0.9$^{c}$ | 141.06 ± 3.9$^{e}$ |
| 0.5 mg $L^{-1}$ 2,4-D | 1970.30 ± 11.7$^{f}$ | 171.7 ± 1.5$^{f}$ | 91.24 ± 2.07$^{g}$ | 91.47 ± 2.13$^{c}$ | 8.52 ± 2.13$^{e}$ | 8.75 ± 2.07$^{b}$ | 1606.46 ± 10.1$^{e}$ | 363.8 ± 5.3$^{f}$ | 140.6 ± 1.04$^{d}$ | 31.03 ± 1.16$^{f}$ |
| Control | 864.53 ± 16.45$^{h}$ | 64.76 ± 0.6$^{h}$ | 93.11 ± 1.67$^{c}$ | 86.31 ± 5.18$^{e}$ | 13.68 ± 5.18$^{a}$ | 6.88 ± 1.67$^{f}$ | 787.53 ± 15.2$^{g}$ | 77 ± 1.5$^{h}$ | 54.23 ± 0.76$^{f}$ | 10.53 ± 0.40$^{g}$ |
| LSD | 38.7 | 3.81 | 0.04 | 0.35 | 0.35 | 0.04 | 17.8 | 30.83 | 1.6 | 3.08 |
| Significant df | | | | | | | | | | |
| Treatment 7 | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** |
| CV | 0.49 | 0.66 | 0.02 | 0.22 | 2.49 | 0.35 | 0.52 | 0.69 | 0.64 | 0.97 |

Data was expressed as means ± standard deviation (n = 3). Different superscript letters in the same column indicate significant differences according to LSD ($p \leq 0.05$), ** and * are significant at the 0.01 and 0.05 levels. (FY: fresh yield, DY: dry yield, SHTW: shoot tissue water content, RTW: root tissue water content, DRM: dry root matter, DSHM: dry shoot matter, SHFY: shoot fresh yield, RFY: root fresh yield, SHDY: shoot dry yield, RDY: root dry yield. Data are mean values of n = 3. Mean values followed by different letters indicate they are significantly different by the LSD test ($p$<0.05). NAA: 1-Naphthaleneacetic acid, BAP: 6-Benzylaminopurine, 2,4-D: 2,4-Dichlorophenoxyacetic acid.

### *Photosynthetic pigments*

Data analysis indicated that MS medium supplemented with 0.5 mg $L^{-1}$ 2,4-D produced the highest chlorophyll a, chlorophyll b, and carotenoid content (2.62, 0.63, 0.36 mg $g^{-1}$ FW). The lowest mean values for these variables (1.18, 0.26, 0.20 mg $g^{-1}$ FW, respectively) occurred in the control (Table 2).

**Table 2.** Photosynthetic pigments content of *C. roseus* L. var. 'Ocellatus' on MS medium supplemented with different PGRs.

| Treatments | Chlorophyll a (mg $g^{-1}$ FW) | Chlorophyll b (mg $g^{-1}$ FW ) | Carotenoid (mg $g^{-1}$ FW) |
|---|---|---|---|
| 1 mg $L^{-1}$ NAA | 2.05 ± 0.25 $^{ab}$ | 0.48 ± 0.11$^{ab}$ | 0.32 ± 0.04$^{ab}$ |
| 1 mg $L^{-1}$ NAA + 1 mg $L^{-1}$ BAP | 1.97 ± 0.64$^{ac}$ | 0.43 ± 0.14$^{ac}$ | 0.29 ± 0.07$^{ac}$ |
| 1 mg $L^{-1}$ NAA + 0.5 mg $L^{-1}$ 2,4-D | 1.47 ± 0.11$^{bc}$ | 0.36 ± 0.02$^{bc}$ | 0.22 ± 0.01$^{bc}$ |
| 1 mg $L^{-1}$ NAA + 1 mg $L^{-1}$ BAP + 0.5 mg $L^{-1}$ 2,4-D | 1.78 ± 0.75$^{ac}$ | 0.36 ± 0.13$^{bc}$ | 0.27 ± 0.1$^{ac}$ |
| 1 mg $L^{-1}$ BAP | 1.99 ± 0.57$^{ac}$ | 0.41 ± 0.12$^{bc}$ | 0.29 ± 0.06 $^{ac}$ |
| 1 mg $L^{-1}$ BAP + 0.5 mg $L^{-1}$ 2,4-D | 1.81 ± 0.44$^{ac}$ | 0.35 ± 0.07$^{bc}$ | 0.27 ± 0.05 $^{ac}$ |
| 0.5 mg $L^{-1}$ 2,4-D | 2.6 ± 0.42$^{a}$ | 0.63 ± 0.16$^{a}$ | 0.36 ± 0.06$^{a}$ |
| Control | 1.18 ± 0.39$^{c}$ | 0.26 ± 0.08$^{c}$ | 0.2 ± 0.07 $^{c}$ |
| LSD | 0.8501 | 0.2007 | 0.1153 |
| Significant df | | | |
| Treatment 7 | * | * | ns |
| CV | 26.34 | 27.91 | 23.68 |

Data are mean values of n = 3. Mean values followed by different letters indicate they are significantly different by the LSD test ($p$<0.05). NAA: 1-Naphthaleneacetic acid, BAP: 6-Benzylaminopurine, 2,4-D: 2,4-Dichlorophenoxyacetic acid.

### *Phytochemical attributes*

TFC varied among the treatment groups and had significant differences (Table 3), ranging from 128.82 (1 mg $L^{-1}$ BAP + 0.5 mg $L^{-1}$ 2,4-D) to 36.95 (1 mg $L^{-1}$ NAA + 0.5 mg $L^{-1}$ 2,4-D) in leaf samples and from 24.50 (1 mg $L^{-1}$ NAA + 1 mg $L^{-1}$ BAP + 0.5 mg $L^{-1}$ 2,4-D) to 0.7 (control) in root samples (Fig. 3A). Maximum TPC in the methanolic extract of the leaf and root samples (respectively, 394.5 and 306.17) occurred in response to 1 mg $L^{-1}$ NAA + 1 mg $L^{-1}$ BAP treatment (Fig. 3B).

**Table 3.** Phytochemical traits of *C. roseus* L. var. 'Ocellatus' on MS medium supplemented with different PGRs.

| SOV | DF | Mean Square (MS) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | TFL | TFR | TPL | TPR | DPPHL | DPPHR | TAC L | TACR | PFRAP L | PFRAPR |
| Treatment | 7 | 2913.20$^{**}$ | 216.71$^{**}$ | 10559.42$^{**}$ | 23363.27$^{**}$ | 26.35$^{*}$ | 600.06$^{**}$ | 67.39$^{**}$ | 297.70$^{**}$ | 122.67$^{**}$ | 422.96$^{**}$ |
| Error | 16 | 21.03 | 1.42 | 24.10 | 1.42 | 10.14 | 2.34 | 2.86 | 2.34 | 7.87 | 7.04 |
| LSD | | 7.93 | 2.06 | 8.49 | 2.06 | 5.51 | 53.16 | 2.92 | 2.65 | 4.85 | 4.59 |
| CV | | 4.92 | 16.35 | 1.94 | 16.35 | 4.52 | 17.83 | 1.96 | 2.27 | 4.90 | 3.28 |

Data are mean values of n = 3 and are significantly different by the LSD test ($p$<0.05). ** and * are significant at the 0.01 and 0.05 levels. Total flavonoid content in the leaf and root samples (TFL, TFR), total phenol content in the leaf and root samples (TPL, TPR), DPPH in the leaf and root samples (DPPPHL, DPPHR), total antioxidant capacity in the leaf and root samples (TACL, TACR), and PFARAP assay in the leaf and root samples (PFRAPL, PFRAPR). NAA: 1-Naphthaleneacetic acid, BAP: 6-Benzylaminopurine, 2,4-D: 2,4-Dichlorophenoxyacetic acid.

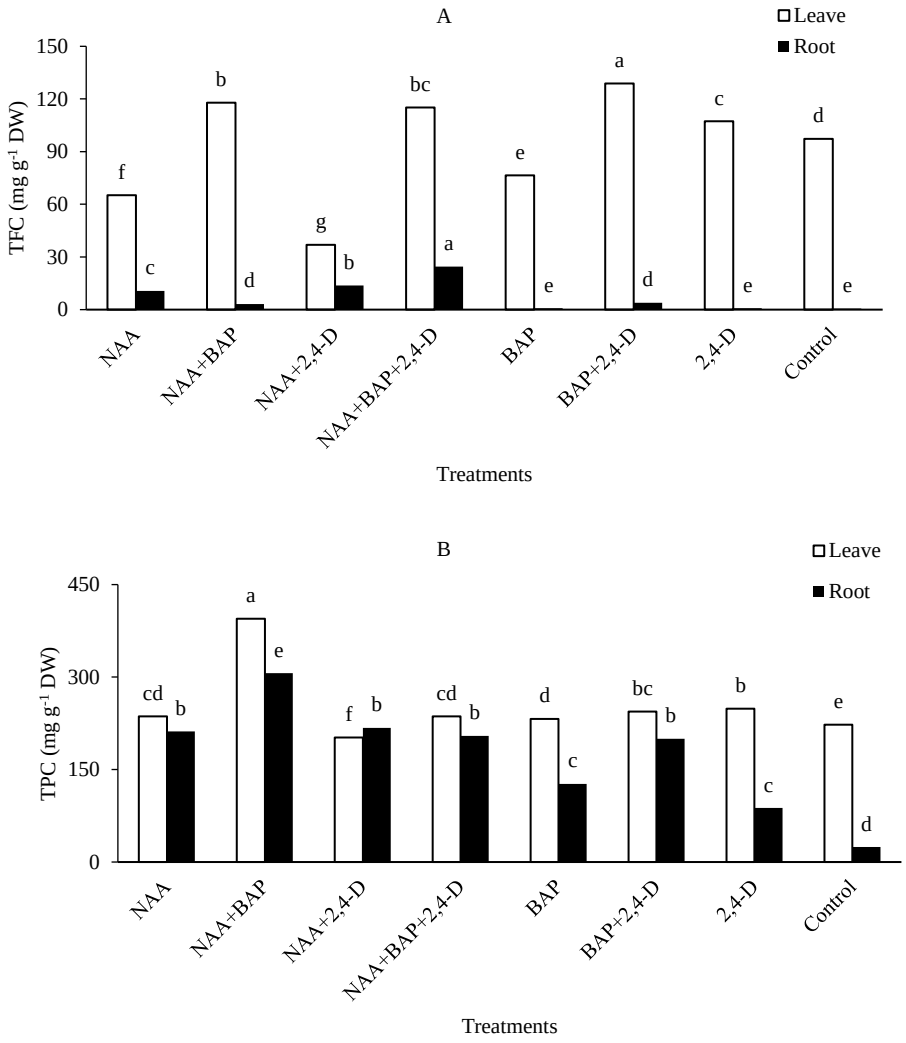


**Fig. 3.** Total flavonoid content (A) and total phenol content (B) of *C. roseus* L. var. 'Ocellatus' on MS medium supplemented with different PGRs. Data are mean values of n = 3. Mean values followed by different letters indicate they are significantly different by the LSD test ($p<0.05$). NAA: 1-Naphthaleneacetic acid, BAP: 6-Benzylaminopurine, 2,4-D: 2,4-Dichlorophenoxyacetic acid.

### *Antioxidant capacity*

All three antioxidant assays revealed significant differences among the treatments in both leaf and root samples (Table 3).

Regarding DPPH, the highest radical scavenging was 74.21% (1 mg $L^{-1}$ NAA + 0.5 mg $L^{-1}$ 2,4-D) and 78.08% (1 mg $L^{-1}$ NAA + 0.5 mg $L^{-1}$ 2,4-D) in the leaf and root samples, respectively (Fig. 4A). The results varied from 74.21 to 65.66 in the leaves and from 78.08 to 38.34 in the roots.

The TAC assay indicated that maximum scavenging (89.33% and 87.9%) occurred in response to 1 mg $L^{-1}$ NAA + 1 mg $L^{-1}$ BAP and 1 mg $L^{-1}$ NAA + 1 mg $L^{-1}$ BAP + 0.5 mg $L^{-1}$ 2,4-D in the leaf and root samples, respectively (Fig. 4B). The comparison of mean values showed that the reducing power activity in the leaves and root samples were affected by the 0.5 mg $L^{-1}$ 2,4-D + 1 mg $L^{-1}$ BAP and 1 mg $L^{-1}$ NAA + 1 mg $L^{-1}$ BAP + 0.5 mg $L^{-1}$ 2,4-D treatments, thus having the highest significance level (62.34 and 62.68 in the leaves and roots, respectively) (Fig. 4C).

### Pearson's correlation coefficient

Pearson's correlation coefficient of TAC, TPC, DPPH, TAC, and RP from the roots were analyzed and the results indicated that a positive significant correlation was observed (Table 4). The highest correlation between phytochemical traits and antioxidant activity assays was related to the TPC with TAC (R2 = 0.849) and DPPH (R2 = 0.845). The correlation coefficient between DPPH and TAC was 0.966.

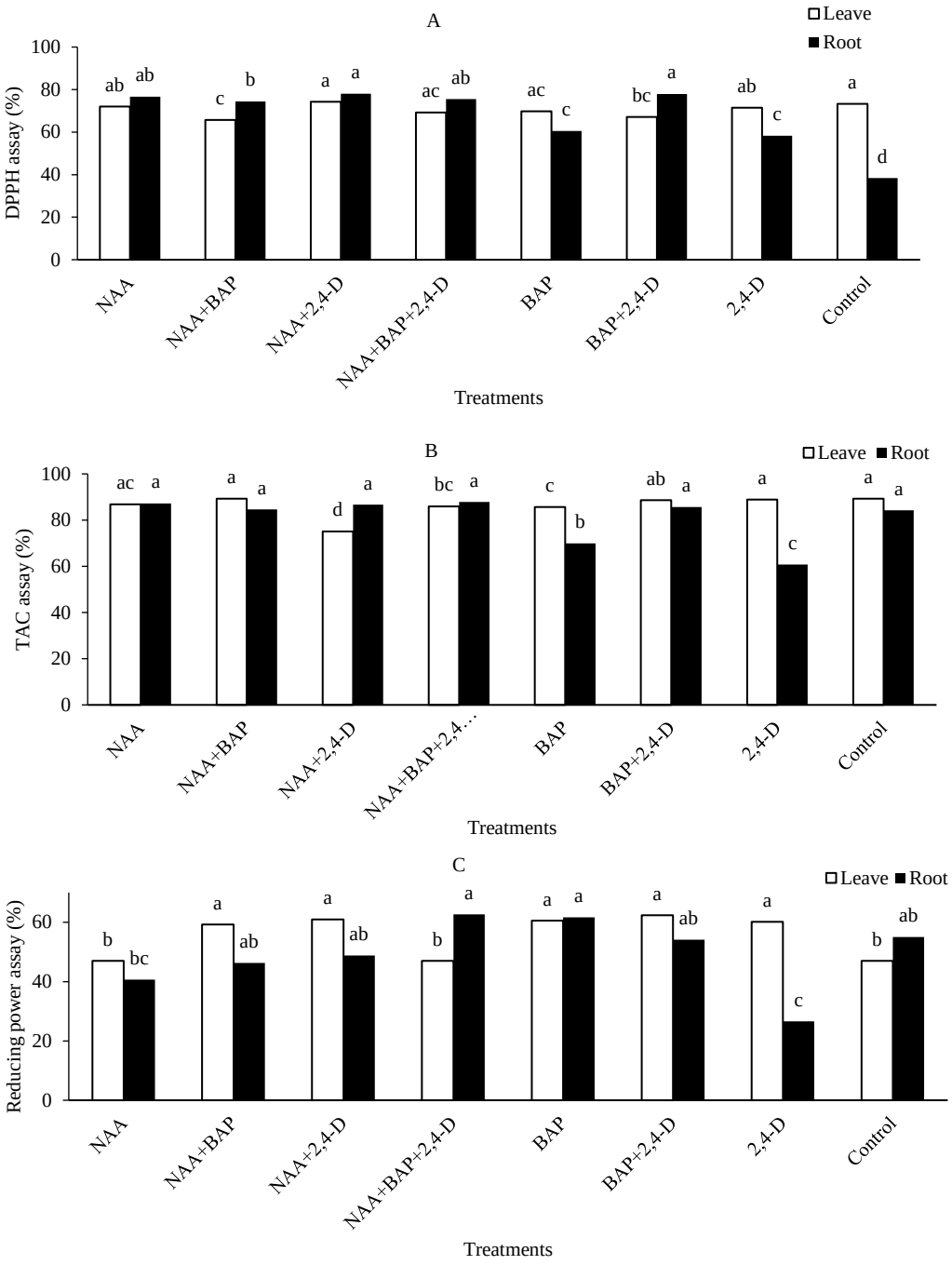


**Fig. 4.** Antioxidant assays: DPPH assay (A), TAC assay (B), and reducing power assay (C) for *C. roseus* L. var. 'Ocellatus' on MS medium supplemented with different PGRs. Data are mean values of n = 3. Mean values followed by different letters indicate they are significantly different by the LSD test ($p<0.05$). NAA: 1-Naphthaleneacetic acid, BAP: 6-Benzylaminopurine, 2,4-D: 2,4-Dichlorophenoxyacetic acid.

## Discussion

Fazal et al. (2020) reported that in the culture medium of *Prunella vulgaris* augmented with various NAA concentrations, maximum biomass production was detected, and NAA was the best treatment for fresh biomass production. As a root regulator substance, auxin is suitable for controlling root development and its anatomy (Olatunji et al., 2017; Overvoorde et al., 2010), thus offering to encourage lateral root development, biosynthesis, polar transport, and signal transduction of auxin are crucial (Sun et al., 2017). Cytokinin is another central regulator of lateral root development

(Laplaze et al., 2007; Marhavý et al., 2011; Marhavý et al., 2014; Moreira et al., 2013). Several reports indicated the increasing role of combined auxins and cytokinins in fresh and dry root masses from *in vitro* plants (Danova et al., 2018). Morphogenesis of *Artemisia alba in vitro plants* was affected by exogenous cytokinins. According to Purohit et al. (2020) and Bakhshipour et al. (2019), auxin and cytokinins are suitable for plant growth enhancement. Cytokinin via scavenging pin-formed (PIN) proteins leads to intervening with polar auxin transport and regulates lateral root development (Marhavý et al., 2011; Marhavý et al., 2014). Additional PGRs directly or indirectly contribute to cell fate decisions and cooperate differentially in lateral root development (Chandler and Werr, 2015; Schaller et al., 2015).

**Table 4.** Pearson's correlation coefficient of TFC, TPC, DPPH, TAC, and RP from the roots.

| | Total flavonoid content | Total phenol content | DPPH assay | TAC assay | Reducing power assay |
|---|---|---|---|---|---|
| Total flavonoid content | 1 | 0.389* | 0.556** | 0.633*** | 0.434* |
| Total phenol content | | 1 | 0.845*** | 0.849*** | 0.505* |
| DPPH assay | | | 1 | 0.966*** | 0.551** |
| TAC assay | | | | 1 | 0.615** |
| Reducing power assay | | | | | 1 |

Total flavonoid content (TFC), total phenol content (TPC), total antioxidant capacity (TAC), and DPPPH (1,1-diphenyl-2-picrylhydrazyl) for the radical scavenging assay. *, ** and ns indicate significance at the 0.05 and 0.01 levels and non-significance, respectively.

Kulus (2020) revealed that IAA (at 1 mg $L^{-1}$) stimulated the synthesis of chlorophyll a and carotenoids in *Lamprocapnos spectabilis*, and similar results were obtained in previous research (Sarami et al., 2017), indicating that IAA was more effective than BAP on photosynthetic pigments. Changes in the chlorophyll content by PGRs could be related to growth rate and primary and secondary metabolic activities (Lichtenthaler, 1987). Kaviani (2014) and Parsaeimehr et al. (2010) reported the positive effects of auxin-cytokinin, separately or in combinations, on increasing the photosynthetic pigment content of *Ephedra, Eustoma grandiflorum* L. and *Saccharum officinarum* L. Furthermore, auxin-cytokinin revealed an increased cell division rate and chlorophyll content by the decrease in different enzymatic functions involved in chlorophyll biosynthesis. The involvement of various processes in the metabolism of chlorophyll and the multidimensionality of PGRs impacts is the reason for variable results (Bakhshipour et al., 2019).

These results were confirmed by a previous report (Fazal et al., 2020). Flavonoids are one of the largest groups of secondary metabolites belonging to polyphenols and play different roles in treatments by having a strong ability to degrade reactive oxygen species (Matkowski, 2008). In soybean (*Glycine max* L. Merr) plants, associations were studied between PGR treatments and the expression level of different genes involved in the biosynthesis of total phenolic, flavonoid, and isoflavones. Results showed that the 6-BA treatment caused the highest expression, followed by NAA and GA3 treatments (Guo et al., 2018). PGRs noticeably influence the production, distribution, and accumulation of secondary metabolites, such as phenolics, and enhance the expression levels of associated genes (Radic, 2016). PGRs, especially a combination of applied auxin and cytokinins, can lead to an accumulation of flavonoids in cell culture. Their roles in the biosynthesis of flavonoids are well documented (Bota and Deliu, 2015; El-Shafey et al., 2016; Ji et al., 2015; Murthy et al., 2014).

PGRs have shown significant effects on antioxidant scavenging, TPC, and TFC of micro-propagated *Vaccinium arctostaphylos* L. plants, and cytokinins treatments improved the antioxidant activity of in vitro-grown plants (Bakhshipour et al., 2019). Moreover, the extract of in vitro-grown *Coleonema pulchellum* L. plants showed high scavenging activity against DPPH radical (Baskaran et al., 2014). Another study that emphasized the roles of PGRs in the antioxidant activity of in vitro-grown plants was reported by Abbasi et al. (2019).

In plants, synergistic or additive functions between several growth hormones, such as auxins and cytokinins, by producing ethylene followed by an induced systemic resistance (ISR) mechanism have led to the appearance of total antioxidant capacity (Cary et al., 1995; Heinrich 2008; Van Wees et al., 2000; Woeste et al., 1999). Thus, growth hormones in the medium might affect the cells to generate ISR (Mustafa and Verpoorte, 2007) and increase antioxidant activity. Biochemical pathways and physiological processes in plants can be influenced by PGRs as they function. This signaling component can change plant metabolism and bioactive compounds (Mitra et al., 2016).

Aryal et al. (2019), Asem et al. (2020), Gan et al. (2017),

and Kainama et al. (2020) reported similar results about the presence of a strong correlation between TPC and different antioxidant activity assays. They suggested that bioactive compounds such as TPC and TFC play a scavenging role and exhibit antioxidant activity.

## Conclusion

An increase in demand for medicinal plants in recent years has led to the use of biotechnology techniques for the mass production of these plants in a uniform manner. Tissue culture techniques with ideal conditions for the micro-propagation of medicinal plants have excelled in producing these plants. An important and effective factor for *in vitro* production of medicinal plants is the use of PGRs or phytohormones. In addition to creating suitable growing conditions for plant production, these substances improve the phytochemical properties of medicinal plants. Here, the positive role of PGRs alone or in combination with each other enhanced the morphological, physiological, phytochemical properties, and antioxidant activity in the medicinal plant *Catharanthus roseus* L. Collectively, 1 mg $L^{-1}$ NAA alone or in combination with 1 mg $L^{-1}$ BAP improved the morphological attributes. Regarding phytochemical properties, 0.5 mg $L^{-1}$ 2,4-D treatments alone or combined with 1 mg $L^{-1}$ BAP resulted in optimal outcomes.

### Conflict of Interest

The authors indicate no conflict of interest in this work.

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