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Evaluation of Metallic Nanoparticles and Plant Growth Regulators Affecting *Catharanthus roseus* L. Performance under Water-Deficit Stress

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ABSTRACT

This study aimed to evaluate the effects of nanoparticle fertilizers and plant growth regulator (PGR) treatments on *Catharanthus roseus* (L.) plants under water-deficit stress. Plant growth and yield were evaluated in two consecutive years (2018-2019 and 2019-2020). This experiment was performed as a split factorial plot arranged in a randomized complete block design (RCBD) with three replications. The main factor was irrigation at three levels (full irrigation, mild, and severe water-deficit stress). The subfactors included priming with PGRs (not priming, auxin, gibberellic acid, and cytokinin) and foliar application of nanoparticles (water, nano Zn particles, nano Mg particles, and nano Zn particles + nano Mg particles). The results showed that water-deficit stress decreased total chlorophyll content, plant height, number of flowers, and shoot dry weight. However, plants primed with PGRs had higher plant height, lateral branches, and shoot dry weights than the control. The highest vinblastine (0.0091%) and agmatine content (0.667%) occurred in response to severe water-deficit stress and ZnO (1 mg L^{-1}) + MgO (0.5 mg L^{-1}). Moreover, stem diameter and lateral branches had the highest value without priming with plant growth regulators but with ZnO application (1 mg L^{-1}) + MgO (0.5 mg L^{-1}). In contrast, priming with gibberellic acid and foliar application of ZnO (1 mg L^{-1}) + MgO (0.5 mg L^{-1}) increased the agmatine content. The current study suggested nanoparticles and PGRs can enhance *Catharanthus roseus* tolerance to water-deficit conditions.

Introduction

Environmental factors such as moisture, temperature, nutrients, and radiation can affect secondary metabolite composition, physical appearance, growth, and plant development by changing metabolic and physiological processes. Due to extreme nutritional and salt accumulation, arable lands are becoming threatened with the possibility of turning into wastelands. Salt stress occurs more in arid and semi-arid regions as a result of high evaporation (high temperature) and low rainfall, associated with poor soil and water management. Stress situations cause membrane metabolic and disorganization toxicity. Reactive

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oxygen species (Hosseini et al., 2023a) can emanate from hydrogen peroxide (H2O2), superoxide anion (O2-), and hydroxyl radicals, as the first consequence of stress taking place in mitochondria and chloroplasts. Plants have advanced differences in their apparatus to protect themselves from the impacts of oxidative stress such as enzymatic and non-enzymatic antioxidants. In most situations, the activity of the antioxidant defense system increases but usually decreases in severe stress conditions (Hosseini et al., 2023b). Osmotic adjustment is often regarded as a reaction of plants to salt stress for the protection of plants by synthesizing and/or accumulation of compatible osmolytes solutes in the cytoplasm, such as proline, glycine betaine, free amino acids, and soluble sugars. Meanwhile, the hydrolyzation of protein under drought stress is a reason for high proline contents.

In the last decade, treating plants with plant growth regulators (PGRs) has been an effective way to improve plant resistance and reduce stress injury. Phytohormones can moderate plant physiological processes and have an important role in ameliorating plant defense systems against unfavorable environmental conditions. Among these substances, gibberellic acid, auxin, and cytokinin are worth mentioning. Cytokinin assumes a protective role and acts as an internal growth regulator or plant growth inhibitor (Khan et al., 2012). Auxin compounds play a key role in growth, development, and response to environmental stresses (Keshavarz et al., 2016). Also, they are key messenger compounds in the induction process that lead to the accumulation of secondary metabolites (Keshavarz and Khodabin, 2019). Cytokinin reportedly increased plant tolerance to water-deficit stress with chlorophyll accumulation, and its application decreased lipid peroxidation (malondialdehyde and hydrogen peroxide) while promoting the activities of ascorbate peroxidase, glutathione reductase, and antioxidant enzyme systems such as superoxide dismutase, peroxidase, catalase, and non-enzymatic compounds (proline and soluble sugars) (Farooq et al., 2013).

To mitigate or compensate for drought stress and increase crop fitting and yield, the effects of micronutrient application have received attention from researchers (Keshavarz et al., 2018). In this context, many reports have demonstrated that zinc (Zn) acts as an activator for many enzymes such as aldolase, isomerase, transferase, and DNA polymerase (Zhang et al., 2023). Moreover, Zn presents fundamental roles in several critical cellular functions such as protein metabolism, gene expression, elicitation of plant resistance mechanisms against environmental stresses, structural and functional integrity in biomembranes, and indole acetic acid (IAA) metabolism (Karami et al., 2016). Zn neutralizes the impact of drought stress by increasing protein and chlorophyll biosynthesis and improving the yield potential of photosystem II (Chavoush et al., 2019). Farahani et al. (2020) indicated that the application of zinc improves seed yield due to an increase in carbohydrate metabolites.

Magnesium (Mg) is an important micronutrient, which plays a vital role in the structure of photosynthesis II, providing required electrons for photosynthetic activity (Pourjafar et al., 2016). It is also involved in activating nitrogen metabolizing enzymes, RNA polymerase, and enzymes for the biosynthesis of fatty acid and gibberellic acid (Movahhedy-Dehnavy et al., 2009). Previous research demonstrated the beneficial effects of Mg on water-deficit stress responses, such as an increase in plant water-use efficiency, stomatal conductance, viability of pollens, photosynthesis rate, and leaf chlorophyll content (Millaleo et al., 2013). Movahhedi Dehnavi et al. (2008) reported that Zn and Mg foliar spraying can compensate for the adverse effects of drought stress on flowering developmental stages. Ajmal et al. (2023) argued that Zn foliar application resulted in a faster uptake of nitrogen, phosphorous, and potassium. Zn and Mg foliar spraying enhanced seed production and yield components in canola (Kalantar et al., 2015), soybean (Karami et al., 2016), and *Vigna radiata* (L.) (Ajmal et al., 2023). High phosphorus content in the soil, high pH, lime, soil moisture, cold temperatures, and increased levels of HCO3 in the root-soil system are several causes of Zn and Mg unavailability in the soil (Lima et al., 2013).

Many soils in Iran are calcareous and have low Zn and Mg availability. Foliar application of these elements as nanoparticles seems to be an effective method to alleviate Zn and Mg deficiency in plants. Therefore, this study compared the effects of water-deficit stress and nanoparticle application of PGRs to alleviate the effects of water-deficit and determine their relationships with biochemical and agronomical traits in *Catharanthus roseus* plants.

Materials and Methods

Study site and experimental design

This study was conducted in a research field at Tehran University, Varamin, Iran (latitude 35.21E; longitude 51.38N, with an altitude of 1000 meters above sea level) in the 2018-2020 crop seasons. This region has a semi-arid climate, with an annual average temperature of 14.2 °C and an average

rainfall of 244 mm (Fig. 1).
Before planting, several soil samples were collected and tested for their properties. Based on the soil analysis, soil Mg and Zn contents were relatively low. Also, irrigation water samples indicated insufficient Mg and Zn levels. The experimental site had clay loamy soil, with pH 7.8 and 7.7, EC 4.6 and 4.8 dS m^{-1}, organic matter 1.28% and 1.31%, with available nitrogen 14.9 and 17.8 ppm, and available phosphorus 41.8 and 39.6 ppm. We arranged a randomized complete block design (RCBD) in a split factorial plot with three replications. Each experiment included forty-eight experimental units, including three irrigation treatments, four PGRs, seed priming, and four foliar applications of Zn and Mg nanoparticles.

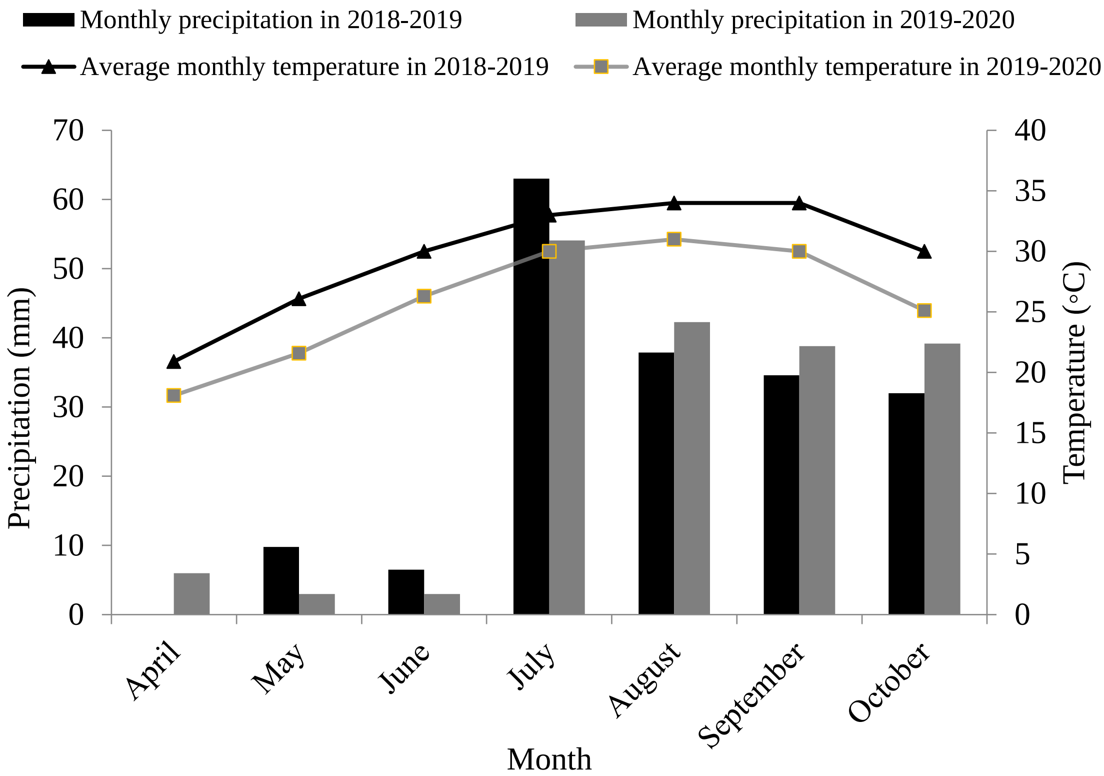

Fig. 1. Average monthly temperature and precipitation during the period of April-October in 2018-2019 and 2019-2020.

Plant treatments
For each treatment, we cultured one-hundred periwinkle seeds at one-centimeter distances apart from each other. When the plants reached the 3-4 leaf stage, we transferred each seedling from the nursery bed to the farm (Sepehri et al., 2013). The main factor consisted of three irrigation treatments: irrigation at 75% (full irrigation), 60% (mild water-deficit stress), and 40% (severe water-deficit stress) (Keshavarz Mirzamohammadi et al., 2021a). The water-deficit stress treatments were applied at the 4-6 leaf stage at the time of plant establishment. Irrigation levels were applied using a tensiometer device manufactured by Ecomatik (EQ15 SN:02385, Germany). The subplots included foliar application with ZnO and MgO [F1: water, F2: nano Zn particles (1 mg L^{-1}), F3: nano Mg particles (0.5 mg L^{-1}) and F4: nano Zn particles (1 mg L^{-1}) + nano Mg particles (0.5 mg L^{-1})] and PGRs [non-application as control; auxin (50 ppm), gibberellic acid (50 ppm) and cytokinin (50 ppm)]. Zinc oxide (ZnO) nanoparticles appeared as whitish crystals of a hexagon shape with 5.61 g cm^{-3} density. Manganese oxide (MnO) nanoparticles were in liquid form and had 4.5 g cm^{-3} density. For priming treatments, we primed the seeds for 6 h in priming solutions. Also, the control samples were primed using distilled water only. The foliar application of nanoparticles was done twice, first when the plants were 68 days old and then at a second time when they were 78 days old. Meanwhile, the soil surface was covered by polyethylene bags to prevent the foliar treatment from reaching the soil.

Harvest and parameters analysis
Three-month-old uniform *C. roseus* plants were collected after careful consideration of the marginal effect. The samples were fixed in liquid nitrogen and

kept at –80 °C in a refrigerator. The total chlorophyll content (mg g^{-1} FW) and anthocyanin content (mM cm^{-1}) were measurable according to Lichtenthaler (1987) and Krizek et al. (1993), respectively. Total alkaloids content (%) was determined using a spectrophotometer (Model Varian SpectrAA-400) and by a standard curve according to Fazel et al. (2008). After harvesting, the plant height (cm), stem diameter (mm), lateral branch ($plant^{-1}$) and number of flowers (plant-1) were measured. Then, the plant was air-dried in a shady, well-ventilated room (20-25 °C) for 7 days until reaching a constant dry matter, which was reported as shoot dry weight (g $plant^{-1}$) and consisted of total aboveground biomass (Keshavarz et al., 2018). The essential oil was extracted from 20 g of dried leaves by hydrodistillation in a Clevenger-type device for two h. Vinblastine, vincristine, and agmatine in *C. roseus* leaves were measured using high-performance liquid chromatography (HPLC) according to Hisiger and Jolicoeur (2007). Vinblastine, vincristine, and agmatine standards in ultrapure water were purchased from Sigma Aldrich (Burlington, USA).

Statistical analysis

Data variance during the two years was analyzed using ANOVA and the LSD mean-wise comparison test ($P \leq 0.05$). For the analysis, SAS 9.1 and S-PLUS ver. 6.1 software executed Principal Component Analysis (PCA) based on a biplot and clustering analysis.

Results

Principal component analysis (PCA) and cluster analysis (CA)

PCA demonstrated that the first and second components had an eigenvalue higher than one, thus being selected as effective components (Fig. 2). The first and second components had the highest relative variance, 27.92 and 21.44%, respectively, and accounted for 49.36% of the total variance. The biplot obtained from the first and second components showed that under well-watered conditions, T2, T9, and T12 were placed in the same groups due to their high affinity and correlated significantly with Y1, Y4, and Y8. In addition, T7, T10, T14, T15, and T16, under well-watered conditions, were placed in the same group due to their neighborhood and showed a strong correlation with Y, Y5, Y7, and Y10. The results of PCA showed that under mild drought stress, T2, T3, and T4 were placed in one set due to their high affinity based on the measured traits and had a high correlation with Y1, Y3, Y4, and Y5. Moreover, T5, T6, T7, T8, and T9 under mild drought conditions, were placed in the same group due to their neighborhood and showed a strong correlation with Y7, Y10, and Y11. Under severe water-deficit stress, treatment of T3, T4, T5, T8, T9 and T10 formed an adjacent group, and were related with Y2, Y7, Y8, Y9, Y10 and Y11. Moreover, T6, T11, T12, T13, T14, and T16, under severe drought conditions, formed one group that was strongly associated with Y7, Y10, and Y11.

Analysis of variance and comparison of mean values

The combined analysis of variance on the data showed that the effect of irrigation regimes, PGRs, and micronutrients were statistically significant on all studied characteristics, except for total alkaloid content (Table 1). The interactions between irrigations and PGRs were also statistically significant on plant height, stem diameter, number of lateral branches, shoot dry weight, vinblastine, vincristine, and agmatine (%) (Table 1).

Total chlorophyll content

The total chlorophyll content responded differently to the main effects of irrigation regimes, PGRs, and micronutrients (Table 1). The results showed that mild and severe water-deficit stress reduced the total chlorophyll content in plants compared to those treated with well-watered conditions (Table 2). The highest total chlorophyll content (2.42 mg g^{-1} FW) occurred under well-watered conditions while mild and severe water-deficit stress decreased total chlorophyll content by 14.04% and 27.68%, respectively. Total chlorophyll content showed a variation response to PGRs application ranging from 2.03 (mg g^{-1} FW) in non-application treatments to 2.13 (mg g^{-1} FW) in the gibberellic acid treatment. Regarding micronutrient application, the maximum chlorophyll content occurred in response to the ZnO + MgO treatment, with an average of 2.11 mg g^{-1} FW that increased by 2.84%, 0.94%, and 1.89%, compared to the non-application treatment, ZnO, and MgO treatments (Table 3).

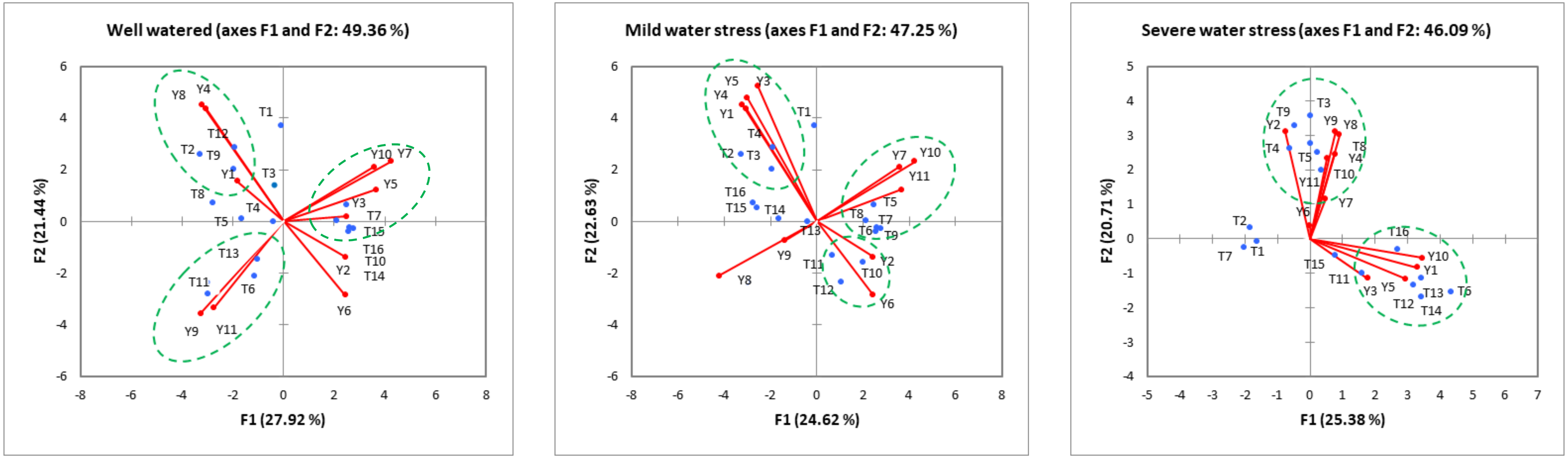

Fig. 2. Results of biplot of the first and second components based on principal component analysis. Y1: total chlorophyll; Y2: plant height; Y3: stem diameter; Y4: number of lateral branches; Y5: number of flowers; Y6: shoot dry weight; Y7: anthocyanin; Y8: total alkaloid; Y9 vinblastine; Y10: vincristine; Y11: agmatine. T1: no hormone application × no micronutrient application; T2: no hormone application × ZnO; T3: no hormone application × MgO; T4: no hormone application × ZnO + MgO; T5: auxin × no micronutrient application; T6: auxin × ZnO, T7: auxin × MgO; T8: auxin × ZnO + MgO; T9: gibberellic acid × no micronutrient application; T10: gibberellic acid × ZnO, T11: gibberellic acid × MgO; T12: gibberellic acid × ZnO + MgO; T13: cytokinin × no micronutrient application; T14: cytokinin × ZnO, T15: cytokinin × MgO; T16: cytokinin × ZnO + MgO.

Table 1. Combined analysis of variance on some physiological and agronomic traits of periwinkle herb (*Catharanthus roseus* L.) as affected by irrigation regimes, plant hormone, and nano particle treatments.

Source of variation	df	TChl	PH	SD	LB	No. F	SDW	Anto	TAlk	Vinb	Vinc	Agma
Year	1	0.0002^{ns}	29.6^{ns}	0.01^{ns}	1.68^{ns}	30.02^{ns}	3.05^{ns}	0.0007^{ns}	0.007^{ns}	0.00000002^{ns}	0.0000001^{ns}	0.0007^{ns}
year×Replication	4	0.0001	4.9	0.002	0.84	4.1	0.47	0.0001	0.0009	0.00000004	0.0000002	0.0001
Irrigation (IR)	2	10.8**	10725**	65.9**	324**	11147**	6444**	1.1**	12.05**	0.0006**	0.19**	3.7**
IR×Year	2	0.000001^{ns}	0.26^{ns}	0.0001^{ns}	0.031^{ns}	0.33^{ns}	0.04^{ns}	0.000007^{ns}	0.0001^{ns}	0.00000004^{ns}	0.0000001^{ns}	0.00009^{ns}
IR× year ×Replication	8	0.0002	1.73	0.01	0.51	2.5	1.54	0.0002	0.001	0.00000004	0.000003	0.00006^{ns}
Plant hormone (H)	3	0.14**	4152**	101.2**	419**	1150**	4921**	0.013**	0.031^{ns}	0.00001**	0.003**	0.82**
Micro nutrient (MN)	3	0.046**	831**	6.38**	222**	1185**	655**	0.05**	0.18**	0.00001**	0.001**	0.3**
H×IR	6	0.001^{ns}	53**	0.72**	17**	10^{ns}	70**	0.0003^{ns}	0.001^{ns}	0.0000006**	0.0003**	0.12**
MN×IR	6	0.0003^{ns}	9.5^{ns}	0.04^{ns}	3.2**	6.8^{ns}	6.18^{ns}	0.0005^{ns}	0.0054^{ns}	0.0000006**	0.0001^{ns}	0.032**
MN×H	9	0.001^{ns}	21.7^{ns}	0.11*	2.39*	6.09^{ns}	6.42^{ns}	0.0013^{ns}	0.011^{ns}	0.0000001^{ns}	0.00004^{ns}	0.007**
MN× H ×IR	18	0.00003^{ns}	3.43^{ns}	0.002^{ns}	0.9^{ns}	2.42^{ns}	0.55^{ns}	0.0001^{ns}	0.0008^{ns}	0.00000007^{ns}	0.000007^{ns}	0.001^{ns}
H×Year	3	0.0000001^{ns}	0.1^{ns}	0.0003^{ns}	0.04^{ns}	0.03^{ns}	0.02^{ns}	0.00000008^{ns}	0.0000005^{ns}	0.000000001^{ns}	0.00000001^{ns}	0.00001^{ns}
Year × MN	6	0.0003^{ns}	3.18^{ns}	0.002^{ns}	0.43^{ns}	3.09^{ns}	2.1^{ns}	0.0002^{ns}	0.003^{ns}	0.00000004^{ns}	0.000007^{ns}	0.0001^{ns}
Year × IR × MN	6	0.0000001^{ns}	0.001^{ns}	0.00001^{ns}	0.001^{ns}	0.0003^{ns}	0.0005^{ns}	0.000000001^{ns}	0.00000003^{ns}	0.000000001^{ns}	0.000000001^{ns}	0.000003^{ns}
Year × IR × MN	9	0.00000009^{ns}	0.006^{ns}	0.00001^{ns}	0.007^{ns}	0.01^{ns}	0.001^{ns}	0.0000001^{ns}	0.000001^{ns}	0.000000001^{ns}	0.000000001^{ns}	0.000002^{ns}
IR × H × MN	9	0.00000004^{ns}	0.0005^{ns}	0.000009^{ns}	0.0002^{ns}	0.0001^{ns}	0.00004^{ns}	0.00000001^{ns}	0.0000002^{ns}	0.000000001^{ns}	0.000000001^{ns}	0.0000002^{ns}
Year × IR × H × MN	18	0.00000003^{ns}	0.00008^{ns}	0.000006^{ns}	0.00008^{ns}	0.00008^{ns}	0.00001^{ns}	0.000000001^{ns}	0.00000002^{ns}	0.000000001^{ns}	0.000000001^{ns}	0.00000004^{ns}
Error	174	0.0008	17.0	0.051	191.2	17.06	11.6	0.0009	0.01	0.0000001	0.00007	0.001
CV (%)		1.42	6.4	3.86	13.5	7.0	8.2	5.0	9.92	5.8	10.9	9.81

TChl: total chlorophyll; PH: plant height; SD: stem diameter; LB: number of lateral branches; No. F: number of flowers; SDW: shoot dry weight; Anto: anthocyanin; TAl l: total alkaloid; Vinb: vinblastine; Vinc: vincristine; Agma: agmatine.

**, * and ns indicate significance ($p \leq 0.01$), ($p \leq 0.05$), and no significance, respectively.

Table 2. Main effects of irrigation regimes and plant hormone foliar treatments on total chlorophyll content, number of flowers, flavonoids, and total alkaloid content in periwinkle herbs (*Catharanthus roseus* L.).

Irrigation regimes	Total chlorophyll (mg g^{-1} FW)	Number of flowers (Plant^{-1})	Anthocyanin (mM cm^{-1})	Total alkaloid (%)
Well-watered	2.42^{a}	70.4^{a}	0.51^{c}	0.87^{c}
Mild water-deficit stress	2.08^{b}	57.3^{b}	0.63^{b}	1.27^{b}
Severe water-deficit stress	1.75^{c}	49.1^{c}	0.73^{a}	1.58^{a}
Plant hormone				
No application	2.03^{d}	53.6^{d}	0.62^{b}	-
Auxin	2.06^{c}	58.8^{c}	0.61^{c}	-
Gibberellic acid	2.13^{a}	62.8^{a}	0.64^{a}	-
Cytokinin	2.1^{b}	60.9^{b}	0.62^{b}	-

Means followed by similar letters in columns are not significantly different at 5% probability level by the Least Significant Difference test.

Table 3. Main effects of nano particles foliar application on some studied traits of periwinkle herb (*Catharanthus roseus* L.).

Nano particles	TChl (mg g^{-1} FW)	PH (cm)	No. F ($plant^{-1}$)	SDW (g $plant^{-1}$)	Anto (mM cm^{-1})	TAlk (%)	Vinc (%)
No application	2.05^{d}	60.6^{c}	54.4^{d}	37.8^{d}	0.59^{d}	1.17^{c}	0.072^{d}
ZnO (1 mg L^{-1})	2.09^{b}	64.7^{b}	60.7^{b}	42.4^{b}	0.63^{b}	1.24^{b}	0.076^{c}
MgO (0.5 mg L^{-1})	2.07^{c}	62.9^{b}	57.1^{c}	40.02^{c}	0.62^{c}	1.24^{b}	0.079^{b}
ZnO (1 mg L^{-1}) + MgO (0.5 mg L^{-1})	2.11^{a}	68.7^{a}	63.6^{a}	44.8^{a}	0.66^{a}	1.30^{a}	0.083^{a}

TChl: total chlorophyll; PH: plant height; SD: stem diameter; LB: number of lateral branches; No. F: number of f lowers; SDW: shoot dry weight; Anto: anthocyanin; TAll: total alkaloid; Vinc: vincristine. Means followed by si milar letters in columns are not significantly different at 5% probability level by the Least Significant Difference test.

Stem diameter

Combined analyses across both years revealed that the interaction between irrigation and PGR significantly affected the stem diameter. The well-watered irrigation regime produced the highest stem diameter across all PGRs, and it was higher than mild and severe water-deficit stress by 17.61% and 23.50%, respectively. The maximum (8.73%) and minimum (4.32%) stem diameter occurred in the absence of PGR application under well-watered conditions and auxin application under severe water-deficit stress conditions, respectively (Table 4). It should be noted that the stem diameter (on average across irrigation regimes) decreased by 35.41%, 30.89%, and 21.97%, respectively, when the plants were treated with auxin, gibberellic acid, and cytokinin, compared to non-treated plants. Auxin, gibberellic acid, and cytokinin decreased the stem diameter by 36%, 28.02%, and 22.6%, respectively. Indeed, the thickest (7.98 mm) and thinnest (4.6 mm) stem diameters occurred in the absence of PGR application × ZnO + MgO and auxin application without micronutrients (Table 5). Among PGR treatments, the stem diameter grew 6.22 mm in response to ZnO (1 mg L^{-1}) + MgO (0.5 mg L^{-1}), which was higher than the non-application, auxin, and cytokinin by 23.9%, 4.22%, and 13.32%.

Lateral branches

When averaged across PGR application, the maximum lateral branch (9.79 $plant^{-1}$) occurred in plants of the well-watered condition. Meanwhile, mild and severe water-deficit stress decreased the lateral branch count by 26.9% and 36.1% (Table 4). Among all treatments, no PGR application under well-watered conditions (14.18 $plant^{-1}$) had the highest lateral branch $plant^{-1}$ (Table 4). Regarding the interaction between irrigation levels and micronutrient application (Table 6), the maximum lateral branch count occurred in the ZnO + MgO treatment under well-watered conditions with an average of 12.03 $plant^{-1}$. Among the micronutrient treatments, plants grown under well-watered conditions had the maximum lateral branches that increased by 26.9% and 36.1%, compared to mild and severe water-deficit stress.

Averaged across PGRs, nanoparticles increased the number of lateral branches by 34.8%, 18.8%, and 41.6%, respectively, compared to the control (Table 5). Indeed, the highest (13.35) and lowest (3.25) number of lateral branches occurred in the absence of PGRs × nano Zn + nano Mg and auxin × no nano particles application (Table 5). Among nanoparticle treatments, the number of lateral branches was 10.9 $plant^{-1}$ in the gibberellic acid treatment, which was higher than in the non-application, auxin, and cytokinin treatment groups by 23.9%, 4.22%, and 13.32%, respectively.

Flower count

Main comparisons indicated 70.4 flowers $plant^{-1}$ in well-watered conditions, and a decrease of 18.61% and 30.38% occurred when the plants received mild and severe water-deficit stress (Table 2). The flower count became 62.8 $plant^{-1}$ in response to gibberellic acid foliar application and decreased by 14.72%, 6.88%, and 3.04%, respectively, when the plants received no PGR, auxin, and cytokinin, respectively (Table 2). The results showed that the application of MgO + ZnO significantly increased the flower count, reaching 63.6 flowers $plant^{-1}$, and a decrease by 14.53%, 4.58%, and 10.26% occurred in the flower count $plant^{-1}$ in the non-treated application, ZnO, and MgO, respectively (Table 3).

Shoot dry weight

The results showed that the application of MgO + ZnO significantly increased shoot dry weight by 44.8 g $plant^{-1}$. A decrease by 15.6%, 5.3%, and 10.6% occurred in shoot dry weight when the plants were not treated with ZnO and MgO (Table 3). Although a significant decrease occurred in shoot dry weight when the plants were exposed to water-deficit stress, the application of plant growth regulators boosted the shoot dry weight in all irrigation regimes (Table 4). The mean comparison indicated that the shoot dry weight (on average across PGRs) was 49.58 g plant^{-1} at well-watered irrigation and decreased by 18.07% and 32.89% when mild and severe water-deficit stress occurred, respectively. Plants treated with gibberellic acid under well-watered conditions and non-treatment of PGRs under severe water-deficit stress produced the maximum shoot dry weight (59.1 g plant^{-1}) and minimum shoot dry weight (24.9 g plant^{-1}), respectively (Table 4).

Anthocyanins

Anthocyanin content increased significantly in response to mild and severe water-deficit stress by 19.04% and 30.1%, compared to well-watered conditions, respectively (Table 2). The anthocyanin content became 0.64 mM cm^{-1} by the gibberellic acid foliar application and decreased by 3.1%, 4.6%, and 3.1% in plants treated with no PGR, auxin, and cytokinin, respectively (Table 2). Regarding the micronutrient treatment, the highest anthocyanin content occurred in response to ZnO + MgO (0.66 mM cm^{-1}), increasing by 4.5%, 6.06%, and 10.6% in plants treated with ZnO, MgO, and non-application treatments, respectively (Table 3).

Total alkaloid content

Only the main effects of irrigation regimes and micronutrient treatments were significant on the total alkaloid content of plants (Table 1). Unfavorable conditions (mild and severe water-deficit stress) increased the total alkaloid content (Table 2). By decreasing water availability, the alkaloid contents increased by 31.4% and 44.9% under mild and severe water-deficit stress, respectively, compared to well-watered conditions (Table 2). Moreover, the lowest (1.17%) alkaloid content occurred from the non-micronutrient treatment and increased in response to the micronutrient application. Although no significant differences occurred between the effects of ZnO and MgO treatments (Table 3), the highest total alkaloid content occurred in response to the ZnO (1 mg L^{-1}) + MgO (0.5 mg L^{-1}), with an average of 1.3 mM cm^{-1}.

Vinblastine

The vinblastine value increased by postponing the irrigation (Table 4). The vinblastine content was 0.003275 mg g^{-1} DW in well-watered conditions (averaged by PGRs), and the enhancement was obtained in vinblastine content when mild and severe water-deficit stress was applied. In all irrigation levels, the application of gibberellic acid made a significant increase in vinblastine content (Table 4). Maximum vinblastine content (0.0091 mg g^{-1} DW) occurred under severe water-deficit stress and the application of ZnO + MgO caused an average vinblastine content (0.0090 mg g^{-1} DW), followed by MgO application under the same irrigation status (Table 6). In the case of vinblastine affected by irrigation regimes and micronutrient treatments, the vinblastine content increased in response to ZnO + MgO in each irrigation level (Table 6).

Vincristine

The micronutrient treatment significantly affected the vincristine content in *C. roseus* leaves (Table 3). The highest amount of vincristine was measured in response to 1 mg L^{-1} ZnO + 0.5 mg L^{-1} MgO, which had increased by 8.43%, 4.81%, and 13.25%, respectively, compared to plants treated with ZnO (1 mg L^{-1}), MgO (0.5 mg L^{-1}), and the non-application. The vincristine content in *C. roseus* plants was significantly affected by irrigation regimes × PGR treatment. Plant exposure to water-deficit stress increased the vincristine content compared to the well-watered conditions by 51.21% and 72.05%, respectively (Table 3). Moreover, it appears that the values of vincristine increased in response to the PGR application, so the highest vincristine content in *C. roseus* occurred in response to the application of gibberellic acid under severe water-deficit conditions (Table 4). In all irrigation levels, there was no significant difference between auxin and cytokinin applications.

Agmatine

When averaged across the PGR applications, the maximum agmatine content (0.53%) occurred in plants treated with severe water-deficit stress conditions. Meanwhile, mild water-deficit stress and well-watered conditions decreased agmatine content by 43.39% and 73.5% (Table 4). Among all treatments, the absence of PGR application under well-watered conditions (0.103%) caused the lowest agmatine percentage (Table 4). The application of ZnO + MgO caused an increase in agmatine percentage in water-deficit conditions (Table 6). The non-application treatment under well-watered conditions caused the lowest agmatine value (0.11%), whereas ZnO + MgO had the highest agmatine content (0.66%) under severe water-deficit conditions (Table 6).

Table 4. Two-way interaction of irrigation regime × hormone foliar application on some studied traits of periwinkle herb (*Catharanthus roseus* L.).

Irrigation	Plant hormone	PH (cm)	SD (mm)	LB ($Plant^{-1}$)	SDW (g $plant^{-1}$)	Vinb (%)	Vinc (%)	Agma (%)
Well-watered	No application	62.2^{e}	8.73^{a}	14.18^{a}	37.2^{f}	0.0030^{i}	0.032^{h}	0.103^{j}
	Auxin	78.6^{b}	5.62^{f}	6.43^{e}	45.9^{d}	0.0033^{h}	0.034^{h}	0.122^{i}
	Gibberellic acid	81.2^{a}	6.03^{e}	8.37^{c}	59.1^{a}	0.0036^{g}	0.039^{g}	0.201^{g}
	Cytokinin	71.3^{d}	6.80^{c}	10.18^{b}	56.5^{b}	0.0033^{h}	0.035^{h}	0.157^{h}
Mild water-deficit stress	No application	56.2^{fg}	7.18^{b}	10.10^{b}	30.8^{g}	0.0053^{f}	0.066^{f}	0.211^{g}
	Auxin	71.4^{d}	4.63^{i}	4.71^{g}	37.8^{ef}	0.0057^{e}	0.069^{ef}	0.250^{f}
	Gibberellic acid	74.2^{c}	4.96^{h}	6.18^{e}	48.3^{c}	0.0064^{d}	0.081^{d}	0.426^{c}
	Cytokinin	63.6^{e}	5.61^{f}	7.61^{d}	46.08^{d}	0.0059^{e}	0.071^{e}	0.332^{e}
Severe water-deficit stress	No application	44.8^{i}	6.67^{d}	8.54^{c}	24.9^{h}	0.0078^{c}	0.115^{c}	0.368^{d}
	Auxin	55.9^{g}	4.32^{j}	4.75^{g}	30.9^{g}	0.0084^{b}	0.122^{b}	0.412^{c}
	Gibberellic acid	58.7^{f}	4.6^{i}	5.38^{f}	39.3^{e}	0.0091^{a}	0.141^{a}	0.765^{a}
	Cytokinin	51.7^{h}	5.21^{g}	6.35^{e}	38.2^{ef}	0.0086^{b}	0.123^{b}	0.600^{b}

PH: plant height; SD: stem diameter; LB: number of lateral branches; SDW: shoot dry weight; Anto: anthocyanin; Vinb: vinblastine; Vinc: vincristine; Agma: ag matine. Each parameter with the same letter is not significantly different according to LSD test at the 5% level of probability. Means followed by similar letters in columns are not significantly different at 5% probability level by the Least Significant Difference test.

Table 5. Two-way interaction of plant hormone × nano particles foliar application on stem diameter, number of lateral branches and agmatine content of periwinkle herb (*Catharanthus roseus* L.).

Plant hormone	Nano particles	SD (mm)	LB ($Plant^{-1}$)	Agma (%)
Control	No application	7.12^{d}	8.69^{ef}	0.156^{j}
	ZnO (1 mg L^{-1})	7.69^{b}	11.83^{b}	0.216^{i}
	MgO (0.5 mg L^{-1})	7.32^{c}	9.87^{cd}	0.242^{h}
	ZnO (1 mg L^{-1}) + MgO (0.5 mg L^{-1})	7.98^{a}	13.35^{a}	0.296^{fg}
Auxin	No application	4.60^{l}	3.25^{k}	0.206^{i}
	ZnO (1 mg L^{-1})	4.97^{k}	6.17^{i}	0.245^{h}
	MgO (0.5 mg L^{-1})	4.71^{l}	4.71^{j}	0.286^{g}
	ZnO (1 mg L^{-1}) + MgO (0.5 mg L^{-1})	5.15^{ij}	7.07^{h}	0.308^{ef}
Gibberellic acid	No application	5.00^{k}	4.88^{j}	0.378^{d}
	ZnO (1 mg L^{-1})	5.22^{i}	7.35^{gh}	0.444^{c}
	MgO (0.5 mg L^{-1})	5.06^{jk}	6.34^{i}	0.468^{b}
	ZnO (1 mg L^{-1}) + MgO (0.5 mg L^{-1})	5.51^{h}	8.02^{fg}	0.567^{a}
Cytokinin	No application	5.51^{h}	5.83^{i}	0.280^{g}
	ZnO (1 mg L^{-1})	6.01^{f}	9.20^{de}	0.321^{e}
	MgO (0.5 mg L^{-1})	5.69^{g}	7.07^{h}	0.383^{d}
	ZnO (1 mg L^{-1}) + MgO (0.5 mg L^{-1})	6.27^{e}	10.10^{c}	0.469

SD: stem diameter; LB: number of lateral branches; Agma: agmatine. Each parameter with the same letter is not significantly different according to the LSD test a t the 5% probability level.

Table 6. Two-way interaction of irrigation regime × nano particles foliar application affecting the number of lateral branches, vinblastine, and agmatine content of periwinkle herbs (*Catharanthus roseus* L.).

Irrigation regimes	Nano particles	LB ($Plant^{-1}$)	Vinb (%)	Agma (%)
Well-watered	No application	7.19^{e}	0.0030^{k}	0.111^{k}
	ZnO (1 mg L^{-1})	10.98^{b}	0.0033^{j}	0.139^{j}
	MgO (0.5 mg L^{-1})	8.96^{c}	0.0034^{j}	0.154^{j}
	ZnO (1 mg L^{-1}) + MgO (0.5 mg L^{-1})	12.03^{a}	0.0036^{i}	0.180^{i}
Mild water-deficit stress	No application	5.34^{g}	0.0053^{h}	0.231^{h}
	ZnO (1 mg L^{-1})	7.99^{d}	0.0056^{g}	0.281^{g}
	MgO (0.5 mg L^{-1})	6.31^{f}	0.0060^{f}	0.325^{f}
	ZnO (1 mg L^{-1}) + MgO (0.5 mg L^{-1})	8.96^{c}	0.0063^{e}	0.384^{e}
Severe water-deficit stress	No application	4.46^{h}	0.0078^{d}	0.424^{d}
	ZnO (1 mg L^{-1})	6.94^{e}	0.0082^{c}	0.500^{c}
	MgO (0.5 mg L^{-1})	5.72^{fg}	0.0088^{b}	0.555^{b}
	ZnO (1 mg L^{-1}) + MgO (0.5 mg L^{-1})	7.91^{d}	0.0091^{a}	0.667^{a}

LB: number of lateral branches; Vinb: vinblastine; Agma: agmatine. Each parameter with the same letter is not signif icantly different according to the LSD test at the 5% probability level. Means followed by similar letters in columns a re not significantly different at 5% probability level by the Least Significant Difference test.

By increasing the stress intensity, agmatine content increased in all micronutrient levels. PGRs as foliar applications led to significant changes in agmatine content when used in combination with all micronutrient applications (Table 5). Gibberellic acid caused the highest agmatine percentage, with an average of 0.45%. However, among the PGRs, using 1 mg L^{-1} ZnO + 0.5 mg L^{-1} MgO increased the agmatine content by 38.50%, 24.84%, and 15.52%, compared to the non-application, ZnO, and MgO, respectively (Table 6).

Discussion

Catharanthus roseus (L.) is an important medicinal plant with unique characteristics due to its medicinal compounds (Farouk et al., 2022). Environmental stresses, especially nutrient deficiency and toxicity, significantly affect physiological and phytochemical properties in medicinal plants (Hosseini et al., 2023a). In the present experiment, water-deficit stress caused a decrease in total chlorophyll content, and using PGRs such as jasmonic acid caused an increase in total chlorophyll content compared to its non-application. It seems that water-deficit stress affects the photosynthetic properties and causes a decrease in the performance of the photosynthetic apparatus, thus disrupting carbon fixation (Keshavarz Mirzamohammadi et al., 2021a). On the other hand, water-deficit stress causes disturbances in the absorption of micro and macro elements, thus suppressing the performance of other physiological characteristics (Chandrasekaran, 2022). Disturbance in the absorption of nutrients, reduction of carbon fixation, and other adverse effects result from plant exposure to water-deficit stress. These effects can decrease plant growth and reduce shoot dry weight (Keshavarz Mirzamohammadi et al., 2021b). One of the essential ways to improve growth conditions for plants in the face of environmental stress is to use PGRs such as auxin, gibberellic acid, and cytokinin. Auxin protects plants against abiotic stresses by interfering with the expression of various genes (Li et al., 2019; Rah Khosravani et al., 2017). Gibberellic is an essential compound in cells, serves as a messenger, and plays a role in plant cell responses. However, cytokinins are cell regulators that have functions in different growth periods, such as seed development, root growth, fertility, fruit ripening, and plant aging (Khalvandi et al., 2019). In response to water-deficit stress, PGRs can activate various genes in the plant, thus assisting in neutralizing stress (Wang et al., 2021). Accordingly, the application of PGRs, especially gibberellic acid, increased the shoot dry weight under water-deficit stress in the current experiment. Also, the results of the present experiment showed that gibberellic acid increased the total chlorophyll in the periwinkle herb. Gibberellic acid also increased the plant height in water-deficit stress and well-watered conditions in the present experiment. Gibberellic acid has reportedly improved photosynthesis by increasing chlorophyll content, thus improving carbon fixation and shoot dry weight. Gholamreza et al. (2019) showed that water stress reduced the growth and yield of dry matter in peppermint medicinal plants, and the application of PGRs reduced the adverse effects of stress, thus being consistent with the results of our experiment. In the present experiment, auxin and gibberellic acid increased the plant height under water-deficit stress conditions. These PGRs can partially maintain chlorophyll synthesis and photosynthetic activity while the plants are under stress, thus improving food absorption, assisting with cell division, and increasing total chlorophyll content. Also, the increase in plant height correlates with more leaves and stems, thus making the shoot dry weight heavier (Jahani et al., 2021).

In stress conditions, the accumulation of compatible soluble substances plays a role in maintaining cellular osmotic balance (Hosseini et al., 2023a). Water-deficit stress caused a remarkable accumulation of agmatine and total alkaloids in the leaf tissue. The biosynthesis of secondary metabolites and compatible solutes assists plants in improving defense mechanisms against stress conditions. *C. roseus* plants showed another defense system pathway through the biosynthesis of anthocyanin, vinblastine, and vincristine. Although water-deficit stress affected plant growth, it improved the accumulation of alkaloids that can benefit medicinal industries. The stimulatory effects of ZnO + MgO on total alkaloids and vincristine were likely because Zn and Mg regulated a series of physiological pathways, including nitrogen and carbon fixation, thus inducing some PGRs signaling processes and contributing to secondary biosynthesis promotion. Moreover, nutrients as nanoparticles are more available due to the higher reactivity of MnNPs compared to bulk Mn, thus leading to higher translocation efficiency in these nutrients compared to ionic Mn salt.

The anthocyanin content correlated with total alkaloid, vincristine, and agmatine under mild and severe water-deficit stress in the PCA (Fig. 2). Our findings agree with Keshavarz et al. (2018) on mint plants under water-deficit conditions.

Agmatine content improved in response to gibberellic acid application in all irrigation levels (Table 4), which probably contributes to a better defense mechanism. Keshavarz et al. (2020) reported that secondary compounds are one of the defense processes in plants that deal with ROS. In the current study, agmatine, vinblastine, vincristine, and total alkaloids increased in content because of water-deficit stress and further increased when using PGRs or micronutrient treatments. Moreover, the PCA showed a positive correlation among anthocyanins, vincristine, and agmatine under mild water-deficit conditions. This observation can be due to a lower leaf area and a reduction in the vegetative biomass, which leads to higher gland density per unit of area. The enhancement in alkaloids suggested that the application of PGRs, especially gibberellic acid, led to profound effects on the physiological processes of plants. The authors documented that Mg as NPs can stimulate metabolic processes such as the biosynthesis of alkaloids (Kiani et al., 2023), which explained that MgNPs can be a biocompatible boost for plants to modulate abiotic stress while continuously supplying Mg2+. Nano particles help the plant to have better and faster access to nutrients. For instance, nutrients that appear as NPs (Ag, Zn, Mn, Fe, and Cu) could also act as antioxidants to inhibit ROS production and lipid peroxidation. A reported increase in alkaloid content significantly improved the plant defense system (Bisht and Rayamajhi, 2016; Khan et al., 2019; Alamdari et al., 2020).

In the present experiment, plant height and shoot dry weight decreased in response to water stress, but applying gibberellic acid increased the plant height and shoot dry weight. It seems that the application of PGRs improved physiological processes such as photosynthesis, respiration, and absorption of nutrients in the plant, thus alleviating the effect of water-deficit stress in *Catharanthus roseus* L. plants. Therefore, gibberellic acid alleviated the intensity of stress. The results on water-deficit stress and salicylic acid affecting the proline content are consistent with previous results by Radi et al. (2019) on *Thymus vulgaris*.

The total alkaloids increased in response to water-deficit stress. However, ZnO (1 mg L^{-1}) + MgO (0.5 mg L^{-1}) increased the total alkaloid activity, which means that stress conditions and nutrient availability increased these compounds. The increase in total alkaloids in water-deficit stress conditions was due to the activation of this enzyme through the excessive production of ROS (Keshavarz, 2020; Hosseini et al., 2023b). It seems that micronutrients increased the production of this enzyme by boosting the expression of genes related to total alkaloid synthesis (Chavoushi et al., 2019), which increased the capacity of the antioxidant system. In the current experiment, micronutrients increased the agmatine content in all irrigation regimes. Meanwhile, severe water-deficit stress caused the highest agmatine content.

Alkaloids can protect proteins and cell structures against water-deficit stress by triggering the production of more antioxidant enzymes (Panche et al., 2020), so their primary biological role is to protect organisms against oxidative damage. The biochemical function of alkaloid metabolites is to reduce ROS and convert more free hydrogen peroxide molecules to water molecules (Mahendra et al., 2023). The stimulation of ROS production in plants is a response to water stress. However, an excessive increase in ROS and direct interactions with numerous macromolecules cause oxidative damage and can decrease plant growth and performance (Hosseini et al., 2021). ROS are inevitable products of anaerobic metabolism, produced in electron transport chain reactions where mitochondria, chloroplasts, and peroxisome organelles exist (Moradi-Ghahderijani et al., 2017). ROS can destroy proteins, biological membranes, and DNA and finally cause cell death. Micronutrients reportedly increased antioxidant power by acting on SOD and H_2O_2, thus protecting plant cells against oxidative stress (Hosseini et al., 2023a). In Table 7, the main components of *C. roseus* L. differ depending on the treatments, stress severity, geographical location, soil type, climate, altitude above sea level, and water availability. Even the season and the sampling time, for example, before or after flowering, affect the chemical structure of the prominent components. Another important influencing factor is the genetic structure of the plant. Therefore, each genetic or environmental factor may specifically affect the biosynthesis of secondary metabolites in each plant species.

In the present experiment, specific levels of water-deficit stress increased vinblastine, vincristine, and agmatine contents in periwinkle. Hosseini et al. (2023b) reported that secondary metabolite contents increased by the effect of water-deficit stress, and the production of MDA in mint plants (*Mentha* spp.) can be a suitable indicator for this increase. The level of alkaloids was an indicator of harmful free radicals for the cell membrane under water-deficit conditions, so vinblastine and vincristine can be benchmarks that determine the extent of oxidative damage under drought-stress conditions (Sepehri et al., 2013).

Table 7. Total alkaloid, vinblastine, and vincristine contents reported in other studies.

Item	Maximum content	Reference
Total Alkaliod content	12 (mg g^{-1} DW)	Hassan et al. (2021)
	8 (mg g^{-1} DW)	Abdul Jaleel et al. (2007)
	7 (mg g^{-1} DW)	AL-Huqail and Ali (2021)
	14 (mg g^{-1} DW)	Ali et al. (2021)
Vinblastine	300 (µg g^{-1} DW)	Chang et al. (2014)
	130 (µg g^{-1} DW)	Sahi et al. (2022)
	699 (µg g^{-1} DW)	Thi Bach Vu et al. (2022)
	10 (µg g^{-1} DW)	Ababaf et al. (2021)
	513 (µg g^{-1} DW)	Ahmadzadeh et al. (2021)
Vincristine	65 (µg g^{-1} DW)	Sahi et al. (2022)
	6 (µg g^{-1} DW)	Ababaf et al. (2021)
	1238 (µg g^{-1} DW)	Ahmadzadeh et al. (2021)

Conclusion

This experiment showed that vinblastine, vincristine, agmatine, anthocyanin, and total alkaloid content in periwinkle (*Catharanthus roseus* L.) varied depending on the level of oxidative stress injury and subsequent tolerance to water-deficit conditions with hormone priming and nanoparticle treatments. This study showed that gibberellic acid (50 ppm) had a maximum effect on the studied traits. Also, nano Zn particles (1 mg L^{-1}) + nano Mg particles (0.5 mg L^{-1}) had higher total anthocyanins, alkaloids, and vincristine contents than the other nanoparticle concentrations. Thus, the interaction between gibberellic acid and nanoparticles in periwinkle can ameliorate drought stress and improve the quantity and quality of alkaloid contents.

Author contributions

BS designed and performed the experiment. H-R, T-M, and FG wrote the manuscript. MO and PK helped in revising the manuscript and improved the language. All authors read and approved the final manuscript and agreed to the published version of the manuscript.

Conflict of Interest

The authors indicate no conflict of interest in this work.

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