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ORIGINAL RESEARCH

Enhancing Germination and Growth of Chrysanthemum Synthetic Seeds Through Iron Oxide Nanoparticles and Indole-3-Acetic Acid: Impact of Treatment Duration on Metabolic Activity and Genetic Stability

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Background: This study investigated the effects of pure iron oxide nanoparticles (Fe_3O_4 NPs), citrate-stabilized iron oxide nanoparticles (Fe_3O_4CA NPs), and indole-3-acetic acid (IAA), applied at various time regimes, on the germination, growth, and ex vitro development of chrysanthemum synthetic seeds. The genetic and metabolic stability of the plants was also assessed.

Methods: Nodal segments of *Chrysanthemum* × *morifolium* /Ramat./ Hemsl. 'Richmond', with a single axillary bud, were encapsulated in 3% calcium alginate with the addition of IAA (1 $mg \cdot L^{-1}$) and/or NPs (7.7 $mg \cdot L^{-1}$). The synthetic seeds were cultured in vitro for 30 or 60 days on a water-agar medium and then transplanted to the greenhouse for further analyses.

Results: Results indicated that IAA and Fe_3O_4CA NPs applied singularly significantly enhanced germination rates (83.33–92.18%) compared with the IAA- and NP-free control (56.67–64.18%), regardless of treatment time. The simultaneous use of IAA and Fe_3O_4CA NPs promoted longer shoot development after 30 days of treatment but showed negative effects after extended exposure. The same combination improved rooting efficiency compared to IAA alone. Supplementation with NPs improved acclimatization rates for younger plants but had variable effects on older plants. Leaf growth metrics were enhanced with Fe_3O_4CA NPs in plants after 30 days of treatment, yet no significant differences were observed in leaf dimensions after 60 days. The content of flavonoids, anthocyanins, and chlorophyll was affected by the exposure duration. Biochemical analyses revealed increased total polyphenol content and antioxidant capacity (FRAP, ABTS) in treated plants, particularly with IAA and Fe_3O_4CA NPs. Start codon targeted (SCoT) analyses showed no polymorphisms among treated plants, confirming their genetic stability.

Conclusion: The study found that the combination of IAA and Fe_3O_4CA NPs improved germination and shoot development in chrysanthemum synthetic seeds, while maintaining genetic stability, although prolonged exposure negatively affected plant growth metrics.

Keywords: antioxidant capacity, *Chrysanthemum* × *morifolium* /Ramat./ Hemsl., molecular markers, nanotechnology, polyphenols, SCoT

Introduction

Chrysanthemum (*Chrysanthemum* × *morifolium* /Ramat./ Hemsl.) is one of the most popular ornamental plants worldwide, contributing significantly to the horticultural and floricultural industries.[1] The global cut flower market, which includes chrysanthemums, is projected to grow from $36.4 billion in 2022 to $45.5 billion by 2027, indicating a rising demand for ornamentals.[2] Chrysanthemum is mainly propagated vegetatively, as seed production faces several challenges that can hinder successful germination and plant development. The widespread distribution of S alleles (responsible for sporophytic self-

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incompatibility) among greenhouse cultivars leads to reduced compatibility and, consequently, lower seed set, which is impaired by the high heterozygosity of the species. Additionally, the long history of breeding, including mutation breeding, has resulted in inbreeding depression and a negative genetic load, further reducing the overall fertility of these plants.[3] To meet the growing demand for chrysanthemum plants, the development of synthetic seed technology has become increasingly important, as it ensures efficient propagation and genetic preservation of elite cultivars.

Synthetic seeds, also known as artificial seeds, manufactured seeds, or synseeds, are artificially encapsulated somatic embryos, vegetative buds, or other plant materials that can be used for sowing and regeneration of complete plants.[4] Synthetic seeds have several advantages that can significantly enhance plant propagation and conservation efforts. They facilitate easy handling, long-term storage, and transportation due to their small size, while also ensuring genetic uniformity since they are derived from vegetative explants that are genetically identical. This technology allows for large-scale propagation of elite plant genotypes, making it particularly useful for commercial applications in floriculture. Additionally, synthetic seeds can be directly sown in the field, avoiding the rooting and acclimatization phases typically required for tissue-cultured plants, which can streamline the production process and reduce costs.[5] Kulus and Zalewska[6] and Hung and Dung[7] developed the basics of synseeds production in chrysanthemum under both aseptic and non-aseptic conditions for direct transfer to commercial greenhouses. Further, innovative treatments, such as air surface dielectric barrier discharge (SDBD) plasma, have been explored to enhance the regrowth of chrysanthemum synthetic seeds. This technique has shown the potential to improve the overall viability and performance of the seeds, demonstrating the versatility of synthetic seed technology in chrysanthemum propagation.[8] The efficiency of synthetic seed production can be further optimized by incorporating various additives, such as nanoparticles (NPs) and plant growth regulators (PGRs) into the encapsulation matrix.

Iron (Fe) is an essential micronutrient for plant growth and development, playing a critical role in various biochemical processes, including chlorophyll synthesis, respiration, and DNA synthesis.[9] Despite its abundance in the soil, iron often exists in forms that are not readily available to plants, especially in alkaline conditions where it forms insoluble complexes.[10] Micronutrients in nanoscale size are more accessible to plants than their larger counterparts due to their unique physicochemical properties, better penetration, and easier transport within the plant.[11] Iron oxide nanoparticles (Fe_xO_y NPs) include magnetite (Fe_3O_4), hematite (α-Fe_2O_3), and maghemite (γ-Fe_2O_3). Studies using confocal laser scanning microscopy have shown that these NPs enter through the epidermis and move through the cortex to the endodermis, facilitating their distribution within the plant system. Root applications of Fe_3O_4 nanoparticles increased biomass by 23–37%, while foliar applications resulted in a 5–9% increase in maize (*Zea mays* L.) plants.[12] Similarly, Fe_2O_3 NPs have been shown to improve root length, plant height, and overall biomass in peanut (*Arachis hypogaea* L.) plants.[13] Iron oxide nanoparticles not only supply iron but also enhance the availability of other essential nutrients such as phosphorus (P) and potassium (K). For example, studies have shown that Fe_3O_4 can lead to increased leaf concentrations of these nutrients, thereby improving overall plant health and productivity in wheat (*Triticum aestivum* L.).[14]

Indole-3-acetic acid (IAA), a naturally occurring auxin, has been used to further enhance the performance of plants both in vitro and in vivo. Supplementing MS[15] medium with IAA at a concentration of 2 $mg \cdot L^{-1}$ can enhance root length and number in chrysanthemum microshoots. This is particularly effective when combined with other auxins like indole butyric acid (IBA) and cytokinins, which can synergistically improve root induction and overall plantlet development.[16] In field experiments, IAA has been applied as a foliar spray to enhance the growth of plants. Concentrations ranging from 10 to 100 $mg \cdot L^{-1}$ have been used effectively to improve plant height, biomass, and chlorophyll content.[17,18] When applied to the soil or as a root dip, IAA can stimulate root growth, which is vital for nutrient uptake and overall plant vigor. Research indicates that exogenous application of IAA can significantly improve root length and density, contributing to better establishment and quality of chrysanthemum plants in various environmental conditions.[19]

However, the effects of IAA in combination with different forms of iron oxide nanoparticles, such as pure Fe_3O_4 NPs or citrate-stabilized Fe_3O_4 (Fe_3O_4CA NPs), on the production and performance of chrysanthemum synthetic seeds have not been yet investigated. Citrate stabilization can improve the stability and dispersibility of nanoparticles, as well as serve better as carriers of PGRs, potentially enhancing their efficacy in synthetic seed applications.[20]

This study aimed to evaluate the effects of pure Fe_3O_4 NPs, Fe_3O_4CA NPs, and indole-3-acetic acid on the germination, growth, and ex vitro development of chrysanthemum synthetic seeds. The effect of the studied compounds

on the genetic and metabolic stability of plants is also considered. The results of this research will contribute to the optimization of synthetic seed technology for chrysanthemum and potentially other ornamental species, ultimately improving their propagation efficiency and commercial potential.

Materials and Methods

Nanoparticles were produced at the Institute of Fundamental Technological Research, Polish Academy of Sciences. The plant experiments were conducted in the Laboratory of Horticulture and Greenhouse at the Faculty of Agriculture and Biotechnology, Bydgoszcz University of Science and Technology (42°9′6.768″ N, 18°0′29.27195″ E), from July 2023 to September 2024.

Synthesis of Iron Oxide Nanoparticles and Modification with Citrate

Iron oxide nanoparticles were synthesized using the wet co-precipitation method from a solution containing Fe^{3+} and Fe^{2+} ions, following the procedure described by Thanh et al.[21] The synthesis involved dissolving iron chloride salts in water, adjusting the pH with ammonia solution, and washing the obtained nanoparticles to remove excess chlorides. Approximately 440 mg of Fe_3O_4 nanoparticles were suspended in water for plant application.

Citric acid (CA) was dissolved in distilled water, and NaOH was added to adjust the pH to 6.5. The citrate solution was then added to the obtained Fe_3O_4 nanoparticles. The suspension was stirred magnetically at 600 rpm for 10 min, followed by 200 rpm at 60 °C overnight. After 1 h of stirring without heating, the suspension was centrifuged with acetone to separate the Fe_3O_4CA NPs. The washing was repeated three times with an acetone-water mixture, and the Fe_3O_4CA NPs were suspended in water. The solution was warmed to 50 °C for 1 h to remove acetone traces and diluted to the desired volume.

Culture Medium and Physical Conditions in the Growth Room

The MS medium, supplemented with 3% sucrose and solidified with 0.8% agar, was used for the initial propagation of plants. The pH was adjusted to 5.8 after all components were added, before autoclaving at 105 kPa and 121 °C for 20 min. A volume of 40 mL of the medium was poured into 350-mL glass jars, which were sealed with plastic caps. All chemical compounds were provided by Chempur, Piekary Śląskie, Poland, except for agar (Biocorp, Warsaw, Poland).

The cultures were maintained in a growth room at 24°C ± 1°C, under a 16-hour photoperiod with a photosynthetic photon flux density (PPFD) of approximately 30.0 $\mu mol \cdot m^{-2} \cdot s^{-1}$, provided by standard cool daylight TLD 54/36W fluorescent lamps (Koninklijke Philips Electronics N.V., Eindhoven, The Netherlands).

Biological Material and Multiplication of Plants

In vitro-derived plants of chrysanthemum (*Chrysanthemum* × *morifolium* /Ramat./ Hemsl.)' Richmond' were used as the source of explants. The plants were obtained from the gene bank of the Laboratory of Horticulture, Bydgoszcz University of Science and Technology, Poland. The plants were identified and authenticated by Prof. Marek Jerzy. A voucher specimen (no. 1594) was deposited in the Herbarium of the Department of Botany, Ecology and Landscape Architecture, Bydgoszcz University of Science and Technology, Poland. No additional approvals were required to conduct research with plant material.

Donor plants, measuring 10–12 cm in length, were cloned via the single-node method in MS medium without plant growth regulators to obtain the required amount of plant material. For this purpose, shoots were segmented into nodal pieces and cultured on fresh medium (six explants per jar) for eight weeks.

Preparation, Storage, and in vitro Germination of Synthetic Seeds

Nodal segments (3–4 mm in length) with a single axillary bud were excised and immersed for 10 min in 3% (*w/v*) sodium alginate. The alginate solution was prepared on MS medium salts, without $CaCl_2$, with the addition of 3% sucrose, indole-3-acetic acid (IAA; 1 $mg \cdot L^{-1}$) and/or iron oxide nanoparticles (pure Fe_3O_4 NPs or stabilized with citrate Fe_3O_4CA NPs; 7.7 $mg \cdot L^{-1}$). IAA was obtained from Sigma-Aldrich, Darmstadt, Germany. Before bead formation, alginate solutions with NPs was placed for 30 min in the Elmasonic S80(H) Ultrasonic Cleaner (37 kHz, 150 W; Elma Schmidbauer GmbH, Singen,

Germany) for proper nanoparticle dispersion. Subsequently, the beads (4–5 mm in diameter) were hardened in a 0.1 M $CaCl_2$ solution for 30 min. The encapsulated explants were then rinsed thrice with distilled sterile water. A control group without IAA or NPs was also included. The synthetic seeds were inoculated onto a water-agar medium in a 90-mm Petri plate sealed with parafilm (10 explants per plate). Each culture plate was considered as a single repetition. The experiment was repeated at least three times. The cultures were maintained in the initial growth room for 30 or 60 days. The share of germinating/growing synthetic seeds was evaluated after these two culture periods. Moreover, the length of shoots, rooting effectiveness (share of rooted shoots and number of roots per shoot), and length of the longest root were measured in all plants.

Ex vitro Growth and Planimetric Measurements of Leaves

The initially germinated synthetic seeds, after 30 or 60 days of culture, were sown in a greenhouse, in a mixture of peat and perlite (2:1), provided by Hartmann (Poznań, Poland), in multi-pots. The acclimatization efficiency, ie survival of chrysanthemum plants ex vitro, was measured two weeks after transferring the plants to the greenhouse. After three months, the plants were transplanted into plastic pots filled with the same substrate and grown for another four months.

By applying the WinFolia 2016b and XLFolia 2016a software (Regent Instruments, Quebec, Canada) and a Perfection V800 Photo scanner (EPSON, Amsterdam Zuidoost, The Netherlands), the morphometric analysis of leaves randomly selected from various parts of the stem was performed, including their area (cm^2), perimeter (cm), length (cm), width (cm), as well as the aspect (width to length) ratio and shape coefficient. The form coefficient is a numerical value that grades the leaf shape between circular (shortest perimeter for a given area) and filiform (longest perimeter for a given area).

Biochemical Activity of Plants During Glasshouse Cultivation

Preparation of Plant Material

Fresh plant leaves were placed in zip-lock bags, frozen, and stored at −20 °C for 24 hours in an AFG 6402 E-B freezer (Whirlpool, Milan, Italy). The frozen material was then subjected to lyophilization (Alpha 1–4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) until a constant weight was achieved (moisture content <2%). The lyophilized samples were ground into a fine powder (particle size 0.3–0.5 mm) using an Ultra-Centrifuge Retsch ZM 100 mill (Retsch, Haan, Germany). The ground samples were stored in dark conditions in air-tight bags within desiccators until further analysis.

Extraction

A 0.5 g sample was mixed with 30 mL of a methanol-water mixture (70:30 *v/v*), followed by shaking for 1 hour using a KS 130 basic shaker (IKA, Staufen im Breisgau, Germany). To separate the supernatant from the sediment, the samples were centrifuged for 15 min at 3000 rpm at +4°C in a ROTINA 420R centrifuge (Hettich, Tuttlingen, Germany). The resulting solution was decanted into a 100 mL volumetric flask. This procedure was repeated thrice. Next, the flask was filled with the methanol-water mixture and mixed to obtain the final extract used for analyses.

Total Polyphenol Analysis

The total polyphenol content (TPC) was determined using the method described by Keutgen and Pawelzik[22] with the tungsten-molybdenum reagent, Folin-Ciocalteu. This method measures the absorbance of the complex formed between polyphenols and the Folin-Ciocalteu reagent. Absorbance was measured using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) at a wavelength of 735.5 nm. Results were calculated based on a calibration curve prepared for gallic acid equivalent (GAE) and expressed in $mg \cdot g^{-1}$ dry weight.

Analysis of Antioxidant Capacity - Ferric Reducing Antioxidant Power (FRAP)

The antioxidant capacity was determined using the FRAP method as developed by Benzie and Strain.[23] Immediately before analysis, a working FRAP solution was prepared by mixing 250 mL of acetate buffer at pH 3.6, 25 mL of a TPTZ (2,4,6-tripyridyl-s-triazine) solution (10 mm in 40 mm HCl), and 25 mL of a hexahydrate iron (III) chloride solution (20 mm). The solution was incubated at 37 °C before measurements were taken. A 0.1 mL sample of the extract was combined with 1 mL of the working FRAP solution and incubated in a water bath at 37 °C for 4 min. After incubation, the

mixture was cooled to room temperature, and absorbance was measured immediately using a UV-1800 spectrophotometer at a wavelength of 593 nm. Each sample was measured four times, and results were expressed in mg TEAC g^{-1} dry weight.

Analysis of Antioxidant Capacity – [2,2′-Azino-Bis(3-Ethylbenzothiazoline-6-Sulfonic Acid)] Assay (ABTS)

The antioxidant capacity was determined using the ABTS radical cation method according to Re et al.[24] The ABTS radical cation was generated chemically by incubating a mixture of 7 mm ABTS solution and 2.45 mm $K_2S_2O_8$ in a 1:0.5 ratio in the dark for 12 hours. Immediately before measurement, the ABTS radical cation solution was diluted with phosphate buffer (PBS) at pH 7.4 until an absorbance of 0.700 (±0.020) was achieved at a wavelength of 734 nm. Subsequently, 0.1 mL of the extract or standard solution was mixed with 3.9 mL of the ABTS working solution and kept in the dark at room temperature for 6 min. Next, absorbance was measured at 734 nm using a SHIMADZU UV-1800 spectrophotometer. The rate of ABTS radical cation reduction for each sample was calculated using the equation:

$$\text{Inhibition rate} = (\text{A\_ref} - \text{A\_sample})/\text{A\_ref} \times 100\%$$

where A_ref and A_sample are the absorbance values for the standard sample and extract, respectively. Each measurement was performed four times.

Determination of Pigments

The relative content of flavonoids, anthocyanins, chlorophyll content index (CCI), and Leaf Soil-Plant Analysis Development (SPAD) value in the leaves was measured in each plant using an MPM-100 multi-pigment meter (Opti-Sciences Inc, Hudson, NH, USA).

Genetic Stability Analysis

The genetic fidelity of 36 ex vitro-grown shoots (six plants from each of the six experimental treatments) was assessed using start codon targeted (SCoT) polymorphism. Total genomic DNA was isolated from fresh leaves using a Genomic Mini AX Plant Spin kit (A&A Biotechnology, Gdańsk Poland). In this analysis, only the plants cultured for 60 days were included, with the assumption that if no genetic variation is found in these samples then mutation occurrence after 30 days of exposition is even less probable.

Five primers were used for the PCR reaction. Each 25 µL reaction mixture included 2 mm $MgCl_2$ in the reaction buffer, 1 mm dNTP mix, 1 µM of a single primer, 0.05 $U \cdot \mu L^{-1}$ Taq DNA polymerase, 0.8 $ng \cdot \mu L^{-1}$ template DNA (20 ng), and molecular-grade water to reach the final volume. The amplification was carried out in a C1000 Touch thermal cycler (Bio-Rad, Hercules, CA, USA) with the following program: an initial DNA denaturation at 94 °C for 4 min; followed by 35 cycles of 94 °C for 1 min for denaturation, 50 °C for 1 min for annealing, and 72 °C for 2 min for extension. The final cycle included an additional extension step at 72 °C for 10 min.

The amplified DNA fragments were separated on a 1.5% agarose gel (EURx, Gdańsk, Poland) in TBE buffer at 110 V for 60 min (Biometra P25, Jena, Germany) and visualized by staining with ethidium bromide. Gel images were captured using a GelDoc XR+ Gel Documentation System (Bio-Rad) UV transilluminator with Image Lab 4.1 software.

The banding patterns were documented as a binary matrix, with "1" indicating the presence and "0" the absence of a specific fragment. For each primer tested, the total number of bands, monomorphic, polymorphic (present in the electrophoretic profile of more than one individual), and specific *loci* (present in the electrophoretic profile of a single individual) were counted.

Experimental Design and Statistical Analysis

Six experimental combinations were included (alginate + IAA, alginate + Fe_3O_4 NPs, alginate + Fe_3O_4CA NPs, alginate + IAA + Fe_3O_4 NPs, alginate + IAA + Fe_3O_4CA NPs, alginate only /control/) for each in vitro culture duration (30 or 60 days), independently. The experiment was performed in a completely randomized design.

The obtained results underwent statistical analysis through one-way ANOVA, and mean comparisons were conducted using Fisher's Test ($p \leq 0.05$) with Statistica 12.0 (StatSoft, Poland). Letters in tables and graphs point homogenous groups. As for the data based on counts expressed as percentages, with a binomial distribution, the arcsine transformation was used.

Results

Effect of Iron Oxide Nanoparticles and IAA on the Germination and in vitro Growth of Chrysanthemum Synthetic Seeds

The highest germination capacity of synthetic seeds after 30 and 60 days of in vitro culture was found in the samples treated with IAA (83.33–92%) alone or Fe_3O_4CA NPs (91.67–92.18%). At the same time, 56.67–64.18% of control synthetic seeds germinated (Table 1). A decrease (in a range of 5.48–19.76%) in the viability of seeds in the second month of culture was observed in most experimental objects, except for those where Fe_3O_4CA NPs were applied alone or in combination with IAA (these two treatments remained stable throughout the experiment). Simultaneous application of IAA and Fe_3O_4CA NPs resulted in the development of longer shoots (6.33 mm) compared to the control (4.67 mm) after 30 days of culture. However, longer exposition to Fe_3O_4CA NPs, regardless of IAA presence, had a deleterious effect on shoot elongation (6.21–6.44 mm after 60 days) compared with all other treatments and the control (9.67–11.63 mm) (Table 1). None of the treatments affected the rhizogenesis efficiency (the share of shoots rooted and the number of roots per shoot) compared with the control (Figure 1), although the combination of IAA and Fe_3O_4CA NPs (37.69–71.11% rooted shoots) was more effective than IAA alone (25.14–32.14%), regardless of culture duration (Table 1). On the other hand, IAA stimulated the development of longer roots. All NPs treatments improved the acclimatization efficiency of 30 day-old plants (45.02–57.33%) compared with the untreated control (22.51%). This effect, however, was no longer observed with older (60-day-old) microshoots, in the case of which only Fe_3O_4 NPs improved the acclimatization efficiency (100%) compared to all other experimental objects (55.56–69.44%). In general, the ex vitro survival rate of older plants was higher.

Effect of Iron Oxide Nanoparticles and IAA on the Leaf Growth of ex vitro-Grown Plants

Supplementation of alginate coating with Fe_3O_4CA NPs for 30 days enhanced the mean area (23.2 cm^2), perimeter (29.4 cm), and length (9.2 cm) of chrysanthemum leaves compared with the control (15.0 cm^2, 22.9 cm, 6.8 cm, respectively) (Figure 2). None of the IAA or NPs treated plants differed in terms of leaf width or their aspects ratio from the control. On the other hand, the application of Fe_3O_4CA NPs (with or without the addition of IAA) resulted in a significantly lower form coefficient (0.32–0.33) than in the control plants (0.38) (Figure 2).

None of the studied factors affected the size of leaves (area, perimeter, length, and width) or their aspect ratio in the plants cultured for 60 days (Figure 3). However, the plants from the treatment IAA + Fe_3O_4 NPs had an increased form coefficient (0.36) compared to the control (0.32), which means that their shape was altered.

Table 1 Effect of Indole-3-Acetic Acid (IAA) and Iron Nanoparticles in Pure (Fe_3O_4 NPs) or Stabilized Form (Fe_3O_4CA NPs), Applied Singularly or in Combination, on the Germination Efficiency of Synthetic Seeds, Shoot Length, Rooting Efficiency, and Acclimatization Efficiency of Chrysanthemum 'Richmond' After 30 and 60 days of in vitro Culture

Treatment	Germination and Growth (%)		Shoot Length (mm)		Rooting (%)		Acclimatization (%)	
	30 days	60 days	30 days	60 days	30 days	60 days	30 days	60 days
Control	64.18 b	56.67 c	4.67±0.46 b	9.67±0.79 a	31.01 ab	61.11 a	22.51 c	69.44 b
IAA	92.00 a	83.33 ab	4.60±0.51 b	10.80±1.11 a	25.14 b	32.14 b	37.52 bc	55.56 b
Fe_3O_4 NPs	81.78 ab	76.30 a-c	6.07±0.63 ab	11.63±0.92 a	34.69 ab	65.28 a	57.53 a	100 a
Fe_3O_4CA NPs	92.18 a	91.67 a	6.03±041 ab	6.21±0.54 b	35.18 ab	44.90 ab	49.33 ab	66.67 b
IAA + Fe_3O_4 NPs	81.33 ab	61.57 bc	4.63±0.51 b	10.71±1.06 a	27.76 ab	56.67 ab	45.02 ab	55.56 b
IAA + Fe_3O_4CA NPs	66.55 b	65.96 bc	6.33±0.55 a	6.44±0.42 b	37.69 a	71.11 a	50.00 ab	58.33 b

Notes: Means ± standard errors marked in columns with the same letter do not differ significantly according to Fisher's test at $p \leq 0.05$.

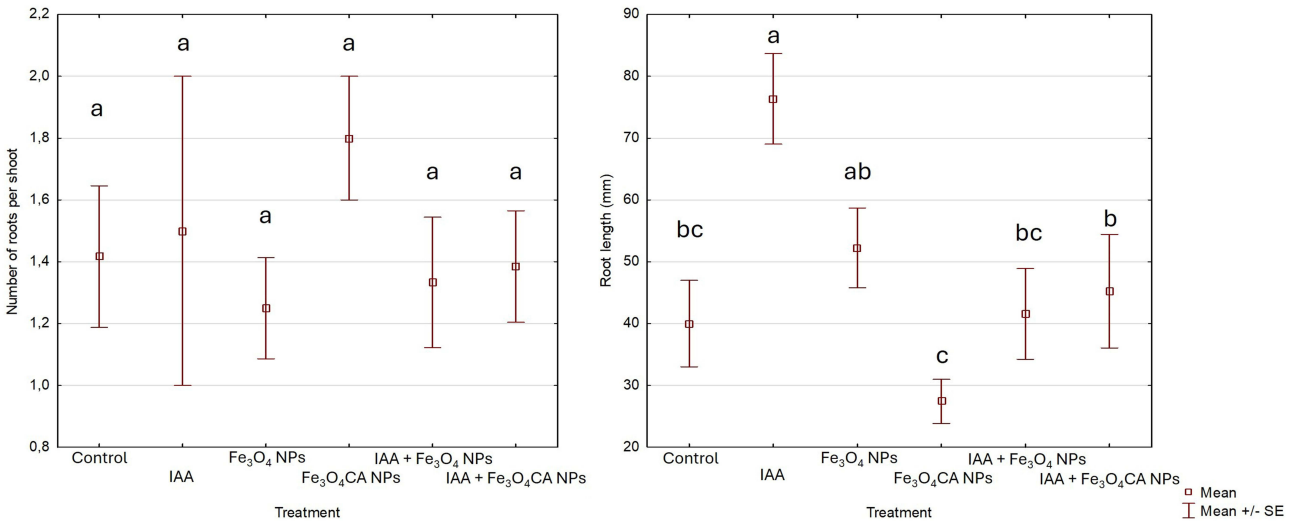

Figure 1 Effect of indole-3-acetic acid (IAA) and iron nanoparticles in pure (Fe_3O_4 NPs) or stabilized form (Fe_3O_4CA NPs), applied singularly or in combination, on the root number per shoot and length of the longest root in chrysanthemum 'Richmond' after 30 days of in vitro culture. Means ± standard errors (SE) marked with the same letter do not differ significantly according to Fisher's test at $p \leq 0.05$.

Effect of Iron Oxide Nanoparticles and IAA on the Metabolic Activity of ex vitro-Grown Plants

The duration of in vitro culture significantly influenced the effects of IAA and iron oxide nanoparticles on the biochemical activity of the glasshouse-grown plants (Figure 4). After 30 days of exposition, the plants treated with Fe_3O_4 NPs, Fe_3O_4CA NPs, and IAA + Fe_3O_4CA NPs contained significantly more flavonoids (0.63–0.66) than the non-treated control (0.42). On the other hand, Fe_3O_4 NPs, Fe_3O_4CA NPs, and IAA + Fe_3O_4 NPs stimulated the biosynthesis of anthocyanins. None of the studied factors enhanced or decreased chlorophyll production or the SPAD index value compared to the control plants (Figure 4).

As for the plants cultured for 60 days before acclimatization, the highest content of flavonoids was found in the plants treated with Fe_3O_4CA NPs (0.76). Conversely, supplementation of alginate matrix with Fe_3O_4 NPs, alone or in combination with IAA, resulted in a significantly lower relative content of these pigments (0.38–0.42) than in the control (0.61). Longer exposition to Fe_3O_4 NPs and Fe_3O_4CA NPs resulted also in a lower concentration of anthocyanins, while the treatments Fe_3O_4 NPs, Fe_3O_4CA NPs, and IAA + Fe_3O_4 NPs downregulated the synthesis of chlorophyll and SPAD value (Figure 4).

All plants treated with IAA and/or NPs had an increased total polyphenol content (25.5–37 $mg \cdot g^{-1}$ DW) compared with the control (23.3–25.0 $mg \cdot g^{-1}$ DW), except for the treatment with Fe_3O_4CA NPs for 60 days (18.0 $mg \cdot g^{-1}$ DW). The highest content of polyphenols was found after the simultaneous application of IAA and Fe_3O_4CA NPs, regardless of culture duration (Table 2). Likewise, supplementation of alginate matrix with the studied compounds increased the plants' antioxidant capacity determined with the FRAP method, with the highest values obtained after the combined use of IAA + Fe_3O_4 NPs and IAA + Fe_3O_4CA NPs (after 30 days of culture) or IAA + Fe_3O_4 NPs (after 60 days) (Table 2). In the ABTS assay, the stable and colored ABTS radicals interact with antioxidants, leading to a decrease in color intensity. The higher the EC50, the lower the antioxidant ability, and vice versa. In the present study, the samples had the ABTS quenching EC50 value of 0.35 $mg \cdot mL^{-1}$ to 0.89 $mg \cdot mL^{-1}$ after 30 days and between 0.19 $mg \cdot mL^{-1}$ and 0.76 $mg \cdot mL^{-1}$ for 60 days. According to this method, after 30 days of culture, the highest antioxidant activity was found in the IAA + Fe_3O_4CA NPs-treated plants (0.35 $mg \cdot mL^{-1}$) and the lowest in control (0.89 $mg \cdot mL^{-1}$). As for the 60-day treatment, the highest and lowest values were found in the treatments: IAA + Fe_3O_4 NPs (0.19 $mg \cdot mL^{-1}$), and Fe_3O_4CA NPs (0.76 $mg \cdot mL^{-1}$), respectively (Table 2).

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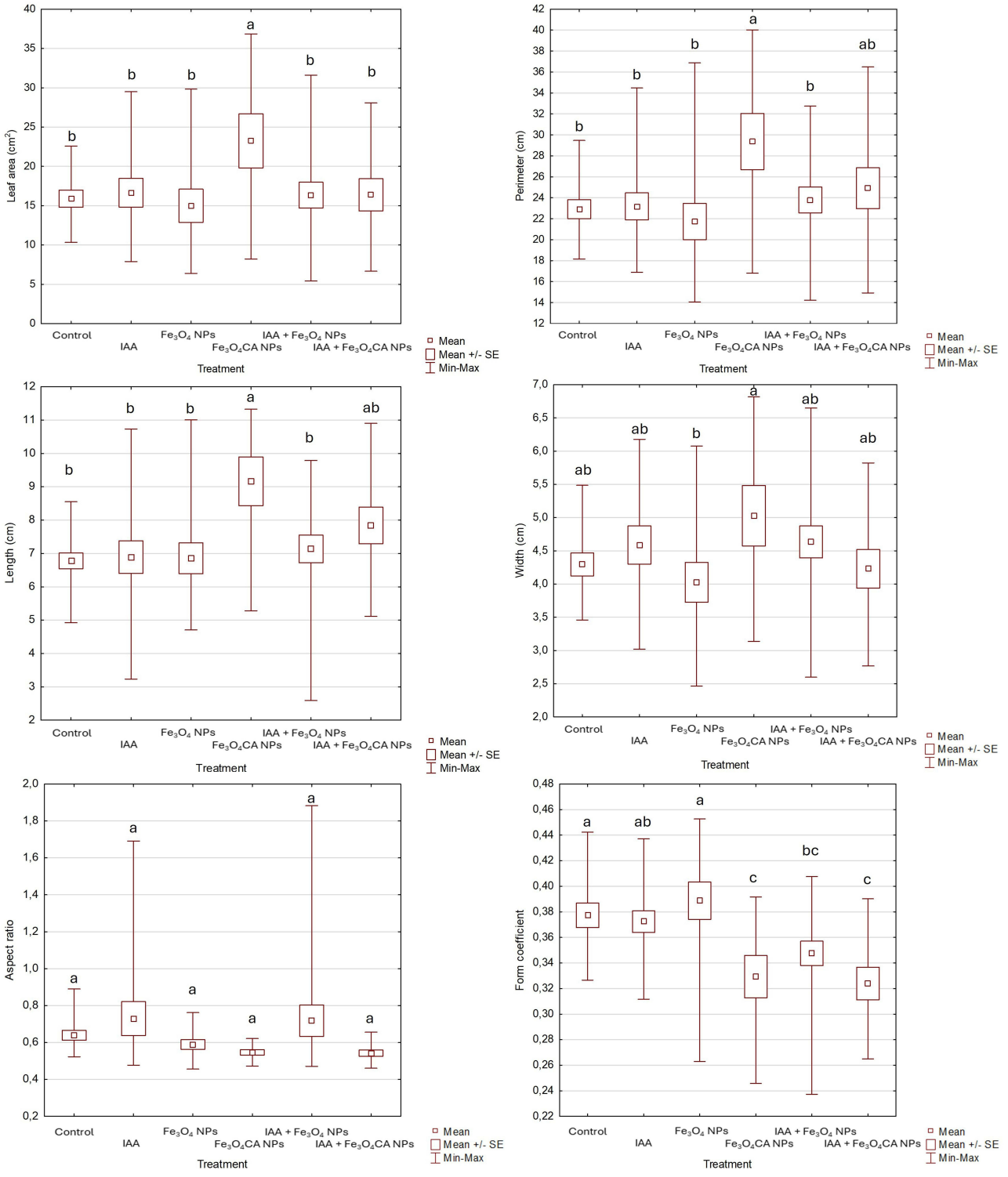

Figure 2 Effect of indole-3-acetic acid (IAA) and iron nanoparticles in pure (Fe_3O_4 NPs) or stabilized form (Fe_3O_4CA NPs), applied singularly or in combination, on the biometrical parameters of leaves in glasshouse-grownchrysanthemum'Richmond', acclimatized after 30 days of in vitro culture. Means ± standard errors (SE) marked with the same letter do not differ significantly according to Fisher's test at $p \leq 0.05$.

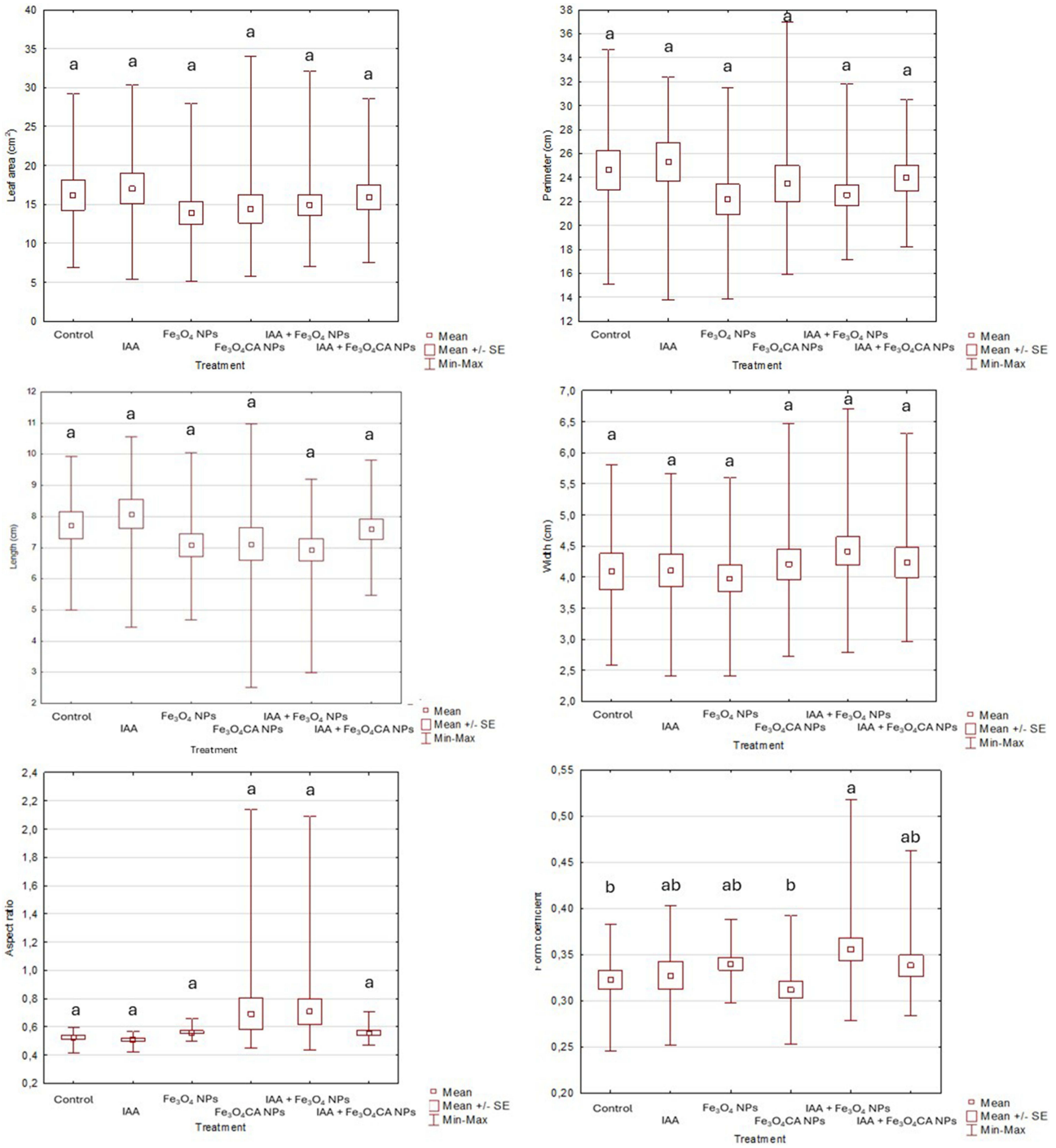

Figure 3 Effect of indole-3-acetic acid (IAA) and iron nanoparticles in pure (Fe_3O_4 NPs) or stabilized form (Fe_3O_4CA NPs), applied singularly or in combination, on the biometrical parameters of leaves in glasshouse-grown chrysanthemum 'Richmond', acclimatized after 60 days of in vitro culture. Means ± standard errors (SE) marked with the same letter do not differ significantly according to Fisher's test at $p \leq 0.05$.

https://doi.org/10.2147/NSA.S503868

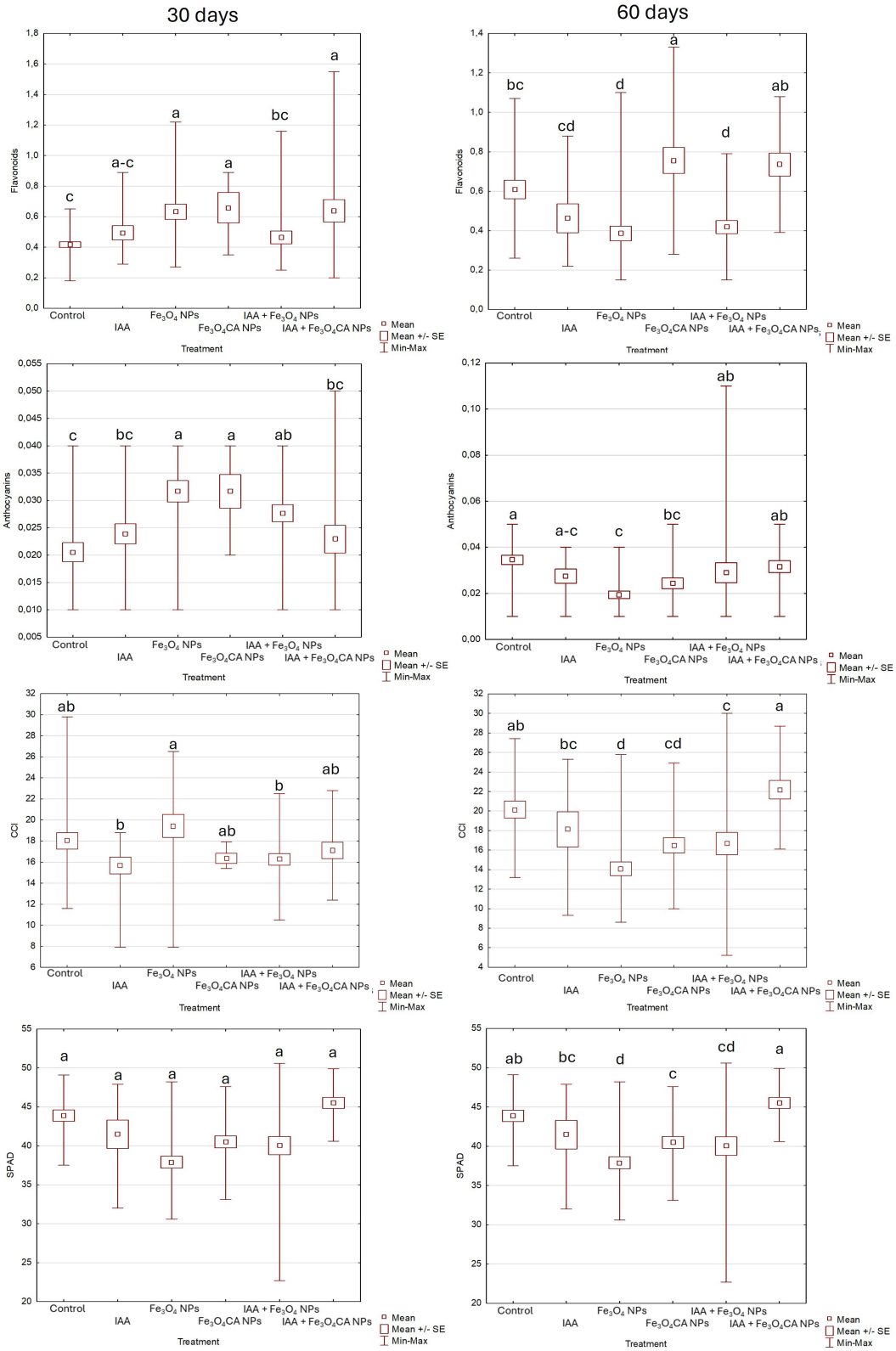

Figure 4 Effect of indole-3-acetic acid (IAA) and iron nanoparticles in pure (Fe_3O_4 NPs) or stabilized form (Fe_3O_4CA NPs), applied singularly or in combination, on the relative content of flavonoids, anthocyanins, chlorophyll (CCI) and SPAD value in the leaves of glasshouse-grownchrysanthemum'Richmond', acclimatized after 30 and 60 days of in vitro culture. Means ± standard errors (SE) marked with the same letter do not differ significantly according to Fisher's test at $p \leq 0.05$.

Table 2 Effect of Indole-3-Acetic Acid (IAA) and Iron Nanoparticles in Pure (Fe_3O_4 NPs) or Stabilized Form (Fe_3O_4CA NPs), Applied Singularly or in Combination, on the Content of Total Polyphenols (TPC) and Antioxidant Capacity According to FRAP and ABTS Methods in Chrysanthemum 'Richmond' After 30 and 60 days of in vitro Culture

Treatment	Total Polyphenols (mg g^{-1} DW)		FRAP (mg TEAC g^{-1} DW)		ABTS (mg mL^{-1})	
	30 days	60 days	30 days	60 days	30 days	60 days
Control	23.3±0.18 e	25.0±0.21 d	128.5±0.39 d	136.9±1.46 e	0.89±0.01 a	0.58±0.03 b
IAA	26.6±0.15 c	25.5±0.14 c	165.4±0.59 b	142.3±0.92 d	0.43±0.05 c	0.51±0.01 c
Fe_3O_4 NPs	25.8±0.07 d	29.0±0.11 b	164.9±0.33 b	168.0±0.50 b	0.84±0.03 a	0.31±0.01 d
Fe_3O_4CA NPs	25.7±0.08 d	18.0±0.30 e	157.0±1.33 c	126.8±2.86 f	0.69±0.01 b	0.76±0.02 a
IAA + Fe_3O_4 NPs	32.2±0.14 b	31.3±0.15 b	215.8±0.85 a	190.2±0.76 a	0.42±0.02 c	0.19±0.01 e
IAA + Fe_3O_4CA NPs	37.0±0.11 a	28.7±0.08 a	216.8±0.55 a	155.6±0.53 c	0.35±0.03 c	0.34±0.01 d

Notes: Means ± standard errors marked in columns with the same letter do not differ significantly according to Fisher's test at $p \leq 0.05$.

Effect of Iron Oxide Nanoparticles and IAA on the Genetic Fidelity of Chrysanthemum

A total of 720 scorable bands were detected by five SCoT primers in 36 'Richmond' plants (Table 3). Primers S4 generated the highest number of bands (7 per sample), while primer S3 produced only one amplicon per sample. The band profiles of individual plants did not differ among each other, ie no polymorphic genotypes were detected (Figure 5).

Discussion

The utilization of iron oxide nanoparticles and indole-3-acetic acid in the production of chrysanthemum synthetic seeds represents a significant advancement in plant biotechnology, particularly in the context of enhancing germination rates and improving growth parameters. The findings of this study can be placed within the broader context of current research in plant propagation and nanobiotechnology.

Germination Capacity and Explant Viability

The results indicate that the highest germination capacity of synthetic seeds was achieved with treatments of IAA or Fe_3O_4CA NPs, with rates reaching between 83.33% and 92.18% after 30 and 60 days of in vitro culture. In contrast, the control group demonstrated a significantly lower germination rate of 56.67% to 64.18%, depending on culture duration. This finding aligns with previous studies that have shown the positive effects of IAA and zinc oxide nanoparticles (ZnO NPs) on seed germination and early plant development in *Arabidopsis* and *Allium cepa* L., respectively.[25,26] Nanoparticles can enhance seed germination

Table 3 PCR Products Obtained from Chrysanthemum 'Richmond' with a Start Codon Targeted Polymorphism (SCoT) Marker System

No.	Primer Sequence 5′ → 3′	No. of bands	No. of *loci*				Total Polymorphic *loci* [%]	No. of Polymorphic Plants	No. of Genotypes	PIC
			∑	mono.	poly.	spec.				
S1	CAACAATGGCTACCACCG	108	3	3	0	0	0	0	1	0
S2	CAACAATGGCTACCACCT	108	3	3	0	0	0	0	1	0
S3	CAACAATGGCTACCACGT	36	1	1	0	0	0	0	1	0
S4	ACGACATGGCGACCAACG	252	7	7	0	0	0	0	1	0
S5	ACGACATGGCGACCATCG	216	6	6	0	0	0	0	1	0
∑		720	20	20	0	0	–	0	–	–
Mean from a single primer		144	4	4	0	0	0	–	–	–

Abbreviations: mono., monomorphic; poly., polymorphic; spec., specific; PIC, polymorphism information content.

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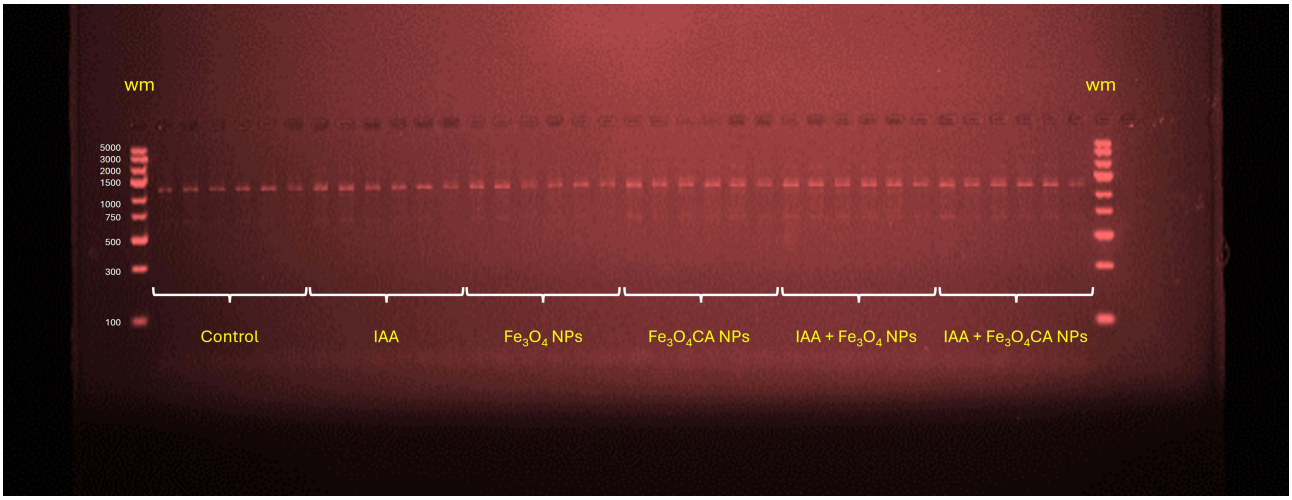

Figure 5 Example SCoT band profiles of chrysanthemum 'Richmond' received with primer S2 as a result of auxin and/or nanoparticle treatments. The outermost lanes (wm) serve as DNA base pair (bp) weight markers (GeneRuler Express DNA Ladder from Thermo Scientific, Waltham, MA, USA), while inner lines represent control plants, plants treated with indole-3-acetic acid (IAA), iron nanoparticles in pure (Fe_3O_4 NPs) or stabilized form (Fe_3O_4CA NPs), applied singularly or in combination.

by improving water absorption and nutrient uptake. Their small size allows them to penetrate the alginate matrix more effectively, facilitating the initiation of germination processes.[27] The stability of germination rates in treatments involving Fe_3O_4CA NPs, which did not exhibit a decrease in viability over time, suggests that these nanoparticles may play a role in maintaining cellular integrity and promoting metabolic activity during the critical germination phase.[28] The reason why citrate-stabilized NPs were more effective than pure Fe_3O_4 NPs could be explained by the enhanced colloidal stability of iron nanoparticles in aqueous solutions. Citrate ions adsorb onto the surface of the NPs, providing electrostatic repulsion that prevents aggregation.[29] In contrast, non-stabilized nanoparticles tend to agglomerate, reducing their effective concentration and limiting their availability for uptake by the explants. Moreover, citrate stabilization has been shown to reduce the cytotoxic effects of nanoparticles on cells.[30] Interestingly, in the present study, when nanoparticles and IAA were applied together, their individual effects did not synergistically enhance germination. This could be due to hormonal imbalance, oxidative stress, or physical interference with seed germination processes.[26]

Shoot Elongation and in vitro Rooting

The present study also revealed that the simultaneous application of IAA and Fe_3O_4CA NPs resulted in longer shoots (6.33 mm) compared to the control (4.67 mm) after 30 days. However, prolonged exposure to Fe_3O_4CA NPs, regardless of IAA treatment, negatively affected shoot elongation after 60 days, indicating a potential threshold beyond which the benefits of nanoparticle treatment may diminish.[31] This phenomenon could be attributed to the accumulation of nanoparticles in plant tissues, which might interfere with physiological processes essential for growth as described by Ruttkay-Nedecky et al.[32] The observed decrease in shoot length with prolonged Fe_3O_4CA NP exposure highlights the need for careful optimization of nanoparticle concentrations and exposure durations to maximize growth benefits while minimizing potential toxic effects.

Interestingly, none of the treatments significantly enhanced rhizogenesis compared to the control. However, the combination of IAA and Fe_3O_4CA NPs improved the percentage of rooted shoots (37.69% to 71.11%) compared to IAA alone (25.14% to 32.14%). This suggests that while the treatments may not directly influence root formation, they can enhance the overall rooting efficiency when combined. Previous studies have indicated that the application of auxins, particularly IAA, plays a crucial role in root induction,[16] and the presence of nanoparticles may facilitate nutrient uptake, thereby indirectly supporting root development.[33]

Acclimatization and ex vitro Growth

The acclimatization efficiency of 30-day-old plants was significantly improved with all NP treatments (45.02% to 57.33%) compared to the untreated control (22.51%). However, this effect diminished in older microshoots, with only

Fe_3O_4 NPs maintaining a 100% acclimatization rate. This finding underscores the potential of nanoparticles in enhancing the condition of young plants during the acclimatization process, probably due to improved nutrient and moisture retention capabilities, as well as controlled release of microelements, in this case - iron.[34,35] The higher survival rates of older plants in ex vitro conditions further suggest that while younger plants benefit from NP treatments, older plants may have developed sufficient physiological adaptations to survive self-sufficiently.

The application of Fe_3O_4CA NPs for 30 days significantly enhanced leaf area, perimeter, and length, indicating a positive effect on leaf development. However, the lower form coefficient observed in NP-treated plants suggests alterations in leaf shape, which could have implications for the overall plant morphology. One should keep in mind that broad, flat leaves have a larger surface area exposed to the sun, allowing them to absorb more light and CO_2 than narrow or curled leaves.[36,37] The lack of significant differences in leaf width and aspect ratio between treated and control plants suggests that while NP treatments can enhance certain growth parameters, they may not uniformly affect all morphological traits. On the other hand, the lack of NP or IAA effect on leaf development after 60 days of treatment further corresponds with the results observed during the in vitro growth phase, highlighting the time-limited outcome of the studied factors (these compounds were probably absorbed by the plants during the first month of culture). Studies indicate that iron nanoparticles can penetrate and accumulate in plant cells, leading to improved biomass and metabolic activity.[38] However, excessive accumulation may pose risks, as prolonged exposure can alter physiological responses and potentially lead to toxicity.[39] Additionally, environmental concerns arise from the leaching of these NPs into soil and water systems, where they may affect ecosystem health and plant interactions.[40] To avoid such unwanted events, a low concentration of NPs (7.7 $mg \cdot L^{-1}$) was used in the present study.

Metabolic Activity

The study on the effects of iron oxide nanoparticles and indole-3-acetic acid on the metabolic activity of ex-vitro-*grown* plants revealed significant insights into the biochemical responses influenced by these treatments, particularly concerning flavonoid and anthocyanin biosynthesis.

The duration of in vitro culture before acclimatization played a crucial role in how plants responded to nanoparticles and IAA. After 30 days of treatment, plants exposed to Fe_3O_4 NPs, Fe_3O_4CA NPs, and the combination of IAA with NPs exhibited a significant increase in flavonoid and anthocyanin content compared to the control group. This enhancement suggests that these treatments may stimulate secondary metabolite production, which is essential for plant defense mechanisms and stress responses. However, longer exposure (60 days) had different effects. The highest flavonoid concentration was observed in plants treated with Fe_3O_4CA NPs, indicating that this particular treatment may be more effective over extended periods. Conversely, when Fe_3O_4 NPs were used alone or in combination with IAA, the relative content of flavonoids decreased, highlighting a potential inhibitory effect on flavonoid synthesis with prolonged exposure. The results also indicate that longer exposure to Fe_3O_4 NPs and Fe_3O_4CA NPs leads to a reduction in anthocyanin concentrations. This decline may further suggest an adverse effect of prolonged nanoparticle exposure on pigment biosynthesis. Similar to our research, in the study by Tymoszuk and Kulus,[41] the content of primary and secondary metabolites (chlorophyll *a*, chlorophyll *b*, total chlorophylls, carotenoids, anthocyanins, and phenolic compounds) in leaf explants of chrysanthemum 'Richmond' differed depending on silver nanoparticles (Ag NPs) treatment and age of culture. Tymoszuk[42] described a varied response of tomato (*Solanum lycopersicum* L.), radish (*Raphanus sativus* L.), and kale (*Brassica oleracea* L.) seedlings to Ag NPs treatment, with some of them resulting either in a decrease or increase in chlorophyll, carotenoid, and anthocyanin levels, depending on the species. Surprisingly, in the present study, none of the treatments significantly altered chlorophyll production or the SPAD index value compared to control plants after 30 days of culture. This finding indicates that while iron oxide nanoparticles and IAA can enhance certain metabolic pathways related to secondary metabolites like flavonoids and anthocyanins, they do not affect the overall photosynthetic capacity.

Most of the scientific reports on chrysanthemums focus on the study of flowers. Meanwhile, it turns out that leaves are also a valuable source of many polyphenolic compounds and have antioxidant capacity. Moreover, Uranishi et al[43] and Doan et al[44] showed a higher content of polyphenolic compounds and antioxidant capacity in leaf extracts than in flower extracts. In the present study, most of the studied experimental treatments increased the total polyphenol content (TCP) in plants. Polyphenols are secondary metabolites produced as part of plant defense

mechanisms.[45] A strong positive correlation of R^2 = 0.94 was obtained between total polyphenol content and antioxidant capacity as measured by the FRAP method. At the same time, a moderately high negative correlation of R^2 = 0.78 was found between total polyphenol content and EC50 values from the ABTS test, suggesting that as polyphenol content increases, the EC50 value decreases, reflecting better antioxidant capacity. An increase in TPC and FRAP values suggests that the plant's secondary metabolism has been upregulated, possibly due to the synergistic effects between IAA and iron oxide nanoparticles. Auxins, including IAA, have been previously described as secondary metabolism enhancers.[46,47] Krzepiłko et al[48] observed that like in our study, ZnO NPs stimulated increased total phenolic content in some stevia (*Stevia rebaudiana* Bertoni) cultivars. As potent antioxidants, polyphenols can neutralize reactive oxygen species (ROS) and free radicals.[49] Chrysanthemums are edible and have been used for medicinal purposes for thousands of years.[50] The observed here increase in TPC can enhance the nutritional value and potential health-promoting properties of the plants.[51,52] On the other hand, the accumulation of NPs in edible plant parts raises concerns about their transfer through the food chain and should be further monitored.[53] In the past decade, several studies have highlighted the presence of exogenous particles in the human body. The health risks posed by magnetic Fe_xO_y NPs have gained increasing attention due to their detection in human blood and brain tissues, particularly regarding their potential link to neurodegenerative diseases such as Alzheimer's disease.[54] In the present study, however, an ornamental/floricultural 'Richmond' cultivar has been used that has little use in herbology.

Different antioxidant assays measure distinct aspects of antioxidant capacity. The contrasting results observed between the two antioxidant capacity assays used (FRAP and BTBS) indicate that while certain treatments enhance antioxidant capacity through specific pathways (as seen in the FRAP results), they may not universally increase all forms of antioxidant activity measured by different assays. The FRAP method assesses the ability to reduce ferric ions,[23] while the ABTS assay evaluates radical scavenging ability.[24] Apparently, IAA and NPs treatments enhance one type of antioxidant activity without affecting another in the same way. Our results underscore the importance of evaluating antioxidant capacity through multiple methods to gain a comprehensive understanding of how different treatments affect plant quality.

Genetic Fidelity

The present study investigated the effects of iron oxide nanoparticles and indole-3-acetic acid on the genetic fidelity of chrysanthemum. Our finding suggests that the application of nanoparticles and auxin did not induce significant genetic variation in the treated plants, which is crucial in the context of plant biotechnology and reproduction. The absence of polymorphic genotypes in the tested chrysanthemum plants indicates a high level of genetic stability. Previous studies, however, have shown that certain nanoparticles, such as Ag NPs, can induce genetic variation and mutations if applied at higher concentrations.[41,55] The results from this study suggest that Fe_3O_4 NPs may not possess the same mutagenic potential as Ag NPs or other nanoparticles. This is consistent with the findings of Kulus et al,[56] which suggested that while some nanoparticles can induce genetic changes, others may promote stability, depending on their physical parameters and concentration. The lack of polymorphism observed here could be beneficial for maintaining the desired traits in chrysanthemum multiplication, as genetic fidelity is essential for cultivar consistency. Nonetheless, future studies should also consider employing a wider range of molecular markers, such as single nucleotide polymorphism (SNP) analysis or sequence-related amplified polymorphism (SRAP), to detect potential minor genetic changes that may not be captured by SCoT markers.[57]

Conclusion

The findings of this study highlight the beneficial effects of iron oxide nanoparticles and IAA on the germination and growth of chrysanthemum synthetic seeds. These treatments not only enhance germination rates but also improve shoot elongation and acclimatization efficiency. However, the duration of treatment is critical; while short-term exposure enhances flavonoid and anthocyanin production, longer exposition can lead to decreased synthesis of these pigments. The potential adverse effects of prolonged NP treatment on shoot growth and metabolite production necessitate further investigation into optimal application strategies. Importantly, the use of iron oxide nanoparticles in chrysanthemum

cultivation does not disturb its genetic stability, as evidenced by the absence of polymorphic genotypes in the treated plants. Elevated content of total polyphenols in plants indicates improved antioxidant capacity, potential health benefits for consumers, and the plants' ability to respond to various environmental stresses. Therefore, our findings emphasize the potential for using alginate matrices as delivery systems for enhancing antioxidant properties in chrysanthemum, which could have implications for horticultural practices aimed at improving crop quality and stress resistance. The insights gained from this research contribute to the growing knowledge of the application of nanomaterials in plant propagation and underscore the potential for developing more efficient propagation systems for ornamental plants. Further research will focus on the application of other nanoparticle types and studying their effect in various chrysanthemum cultivars. The search for NPs that promote root development in chrysanthemum is particularly desirable for more efficient propagation.

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Disclosure

The authors have no relevant financial or non-financial interests to disclose.

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