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Fe_3O_4 nanoparticles and IAA auxin affect secondary metabolism over time without altering genetic stability in chrysanthemum

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This research studied the impact of bare iron oxide nanoparticles (Fe_3O_4 NPs), citrate-stabilized iron oxide nanoparticles (Fe_3O_4CA NPs), and indole-3-acetic acid (IAA) on the genetic stability and metabolic activity of *Chrysanthemum* × *morifolium* (Ramat.) Hemsl. plants obtained from synthetic seeds. For this purpose, axillary buds of chrysanthemum 'Richmond' were embedded in 3% calcium alginate supplemented with NPs and IAA, either singularly or in combination. Next, the synthetic seeds were stored at 4 °C in the dark (for eight weeks) on an agar-water medium and then transferred to room temperature for 30 or 60 days. Next, the germinated seeds were transplanted to the greenhouse until the plants were fully flowering. The total polyphenol content (TPC) was determined in the leaves and inflorescences of the plants. Moreover, the content of anthocyanins was measured in the inflorescences. RAPD markers were used to assess the genetic stability of plants. Most treatments stimulated the accumulation of polyphenols in the leaves of chrysanthemum by as much as 59% after 30 days, and up to 28% after 60 days. Fe_3O_4NPs and IAA + Fe_3O_4CA NPs stimulated the biosynthesis of polyphenols and anthocyanins in the inflorescences after 30 days of treatment (by 36% and 68%, respectively); however, a decline in the content of these compounds (22–33%) was reported after 60 days in most experimental objects, except for Fe_3O_4CA NPs and IAA + Fe_3O_4CA NPs. The inflorescences of plants treated with nanoparticles usually exhibited a larger diameter than the control, but only after a shorter exposure to the analyzed factors. In contrast, prolonged treatment resulted in an opposite effect. The genetic uniformity of the plants was confirmed as no polymorphism was detected in 2160 RAPD markers.

Keywords Axillary buds, Nanoparticle–plant interaction, Plant biotechnology, RAPD, Secondary metabolism regulation, Synthetic seeds

Ornamental plants are an important part of horticulture and our culture as a source of beauty and emotional enjoyment, while also contributing to the economy. As living standards continue to improve, the demand for these plants has grown consistently[1]. In order to meet the growing demands of the market, micropropagation, i.e. the reproduction of plants in vitro, is gaining interest among commercial plant producers[2]. According to Podwyszyńska et al. (2022), the global annual production of ornamental plants through in vitro cultures has increased from 800 million to 2 billion in the last decade[3]. Unfortunately, like any other technique, micropropagation also faces several challenges, e.g. contamination, plantlet browning, in vitro rooting difficulty, somaclonal variations, hyperhydricity, shoot tip necrosis, problems with acclimatization[4]. Some of these problems can be overcome, by the application of nanotechnology.

Nanoparticles (NPs) are a promising tool useful in horticulture, particularly for the production of ornamental plants in vitro. This is due to their unique physiochemical properties, versatility, and the possibility

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of direct interaction with the plant cell[5]. For example, silver nanoparticles (Ag NPs), applied at 20–100 ppm to non-meristematic explants (leaves and internodes), increased genetic variation and mutation occurrence in chrysanthemum [*Chrysanthemum* × *morifolium* (Ramat.) Hemsl.] plants regenerated through adventitious organogenesis. By those means, it was possible to create novel cultivars of this species with distinctive flower colours and shapes[6,7]. Kumar et al. (2024) highlight that various nanomaterials modulate plant hormone pathways (including auxin, cytokinin, gibberellin, ethylene, salicylic acid, jasmonic acid, and brassinosteroids), influencing growth, plasticity, and nutrient uptake[8]. Gold nanoparticles (50–100 ppm Au NPs) significantly improved micropropagation rates and post-acclimatization plant quality in bleeding heart [*Lamprocapnos spectabilis* (L.) Fukuhara] compared to the control and traditional plant growth regulators (PGRs)[9,10]. Zinc oxide nanoparticles (25–75 ppm ZnO NPs), on the other hand, elevated phenolic content (*p*-coumaric, chlorogenic, and caffeic acid) in the bulblets of lilium (*Lilium candidum* L.)[11]. Likewise, ZnO NPs treatment increased the levels of anthocyanins, chlorophyll, and flavonoids, as well as root length, leaf length, leaf number, and bulb count in the explants of *Lilium ledebourii* Bioss[12]. The results obtained by Krzepiłko et al. indicated that high concentrations of ZnO NPs (30–40 ppm) stimulated the propagation of stevia (*Stevia rebaudiana* Bertoni) shoots[13]. On the other hand, NPs negatively affected shoot length, root number and length, as well as the fresh weight of the plantlets[13]. Nanomaterials also play a significant role in improving plants' resistance to environmental stress, such as pathogens or drought, by influencing the synthesis of plant hormones and nutrient absorption[14]. In contrast with the previous reports, Ag NPs inhibited rhizogenesis in chrysanthemum and gerbera (*Gerbera* × *jamesonii* H. Bol), as well as adventitious organogenesis in chrysanthemum. Gold nanoparticles (Au NPs) were less toxic and improved the efficiency of Cape Primrose (*Streptocarpus* × *hybridus* Voss.) micropropagation[15]. A newly designed nanohybrid technology that conjugates indole acetic acid (IAA) with nanoparticles of iron (Fe) and manganese (Mn) micronutrients was developed to combat heavy metal stress in strawberry (*Fragaria* spp. 'Fertona')[16]. Despite their undeniable promise, further studies are needed to fully explore the potential of NPs and their application in the large-scale production of ornamental plants. Notably, little is known about the use of NPs in the production of synthetic seeds.

The synthetic seed technology, developed primarily by Murashige in 1977, is a highly useful technique in the propagation of seedless species, such as chrysanthemum[17]. In this approach, somatic embryos (usually) or other vegetative parts; such as shoot tips, cell aggregates, auxiliary buds, or any other micropropagules; are artificially encapsulated, sown, and converted into a plant under in vitro or in vivo conditions[18]. Sodium alginate is the most common type of hydrogel coating used in the synthetic seed technology. It is water-soluble; however, when sodium ions are replaced by calcium ions, ionic cross-binding occurs between calcium and the alginate polymer chains, leading to the formation of an insoluble gel[19]. This artificial coating mimics natural endosperm, by supplying carbon sources, minerals, and vitamins to support the early development of the encapsulated explants. Moreover, sodium alginate is considered a potential elicitor that improves plants' tolerance to environmental stress, such as drought or salinity[20]. Despite the growing interest in synthetic seed technology for mass propagation and conservation of plant genetic resources, the role of nutritional composition within the encapsulation matrix is still poorly explored. Optimization of alginate matrix composition is essential for synthetic seed technology, as inadequate or imbalanced nutrition can reduce germination rate or plant vigour[21]. Most studies focus on the use of basal Murashige and Skoog (MS) medium[22] with traditional plant growth regulators (PGRs) or carbon sources[23]. Moreover, there is little research on nutrient interactions with novel additives, such as nanoparticles (NPs), particularly in a time-dependent manner[24]. Supplementation of the artificial alginate matrix with NPs alone or as the carriers of PGRs can improve the performance of synthetic seeds. Iron oxide nanoparticles (Fe_3O_4 NPs) are particularly useful as iron is an essential micronutrient for plants, significantly affecting crop yield[25]. As a central atom in iron–sulphur (FeS) proteins, it plays a crucial role in electron transport during respiration and photosynthesis. Iron is a key component of cytochromes and is essential for chlorophyll biosynthesis by activating enzymes, such as *cis*-aconitase and glutamyl-tRNA reductase[26]. Mitochondria are also abundant in iron-dependent enzymes, with respiratory complexes I and II containing multiple FeS clusters. Iron-containing proteins can also be found in other organelles, i.e. peroxisomes and the endoplasmic reticulum house haem proteins, whereas mono- and di-iron enzymes are found in all cell compartments[27]. Finally, in response to pathogenic fungal attacks, plants redistribute iron to the apoplast, where its controlled accumulation affects both intracellular and extracellular defence mechanisms, supporting the overall health of plants[28]. Its deficiency can severely affect plant growth and productivity[29]. Plants take up iron oxide nanoparticles primarily via root tips through diffusion and membrane-bound carriers, then transport them through apoplastic and symplastic routes to the shoot. From there, it is redistributed to actively growing tissues, with the xylem and phloem serving as the main systems for long-distance iron transport[30]. In foliar applications, iron oxide NPs can also enter leaves through stomata or cuticle pores and adhere to epidermal surfaces, although this process is less effective than in roots[31]. Fe_3O_4 NPs are the most efficient NPs type, compared to γ-Fe_2O_3 and α-Fe_2O_3 and bulk Fe_3O_4. Once inside the cells, Fe_3O_4 NPs may dissociate, releasing Fe ions that enhance photosynthetic gene expression and antioxidant enzyme activity, resulting in improved plant growth, as observed in field-grown maize[31]. Kulus et al. reported that the addition of Fe_3O_4 NPs into the alginate matrix can increase the germination rate of synthetic seeds and the acclimatization efficiency of chrysanthemum plants, however, their effect on the content of metabolites, particularly in the inflorescences, and the development of generative organs remains unknown[24]. The aims of this study included (1) to investigate the time-dependent effects of Fe_3O_4 NPs and IAA on the metabolism of germinated synthetic seeds; (2) to verify the genetic stability of chrysanthemum plants under nanoparticle treatment; (3) to optimize the regulatory conditions for ornamental traits (inflorescence diameter).

Results

Morphology studies of NPs

The morphology results obtained with Transmission Electron Microscope (TEM) confirmed the spherical shape of bare Fe_3O_4 NPs sized below 20 nm (Fig. 1A). As for the Fe_3O_4CA NPs presented in Fig. 1B, a thin organic layer of citrate on the surface of NPs can be distinguished. The Dynamic Light Scattering (DLS) analysis shown in Fig. 1C revealed that stabilized Fe_3O_4CA NPs exhibit a monodisperse distribution with a lower hydrodynamic diameter of about 90 nm. The presence of a citrate coating effectively improved dispersion in aqueous media, whereas bare NPs Fe_3O_4 had a broader hydrodynamic diameter due to the lack of citrates, resulting in agglomeration. The Zeta potential of Fe_3O_4CA NPs showed a narrow peak centred around −20 mV, indicating a negatively charged surface in the measured conditions (pH 7, 0.01 M NaCl at room temperature).

Morphometric analysis of inflorescences

It was found that after 30 days of in vitro culture, plants treated with iron oxide nanoparticles (both bare and stabilized) and a combination of Fe_3O_4CA NPs and IAA produced inflorescences of greater diameter (7.6–8.5 cm) than the untreated control (6.6 cm) (Fig. 2). In contrast, after 60 days, plants treated with Fe_3O_4 NPs alone or in combination with IAA had smaller inflorescences (6.1 cm) than the control (7.6 cm). Representative inflorescences of chrysanthemum 'Richmond' after various treatments are shown in Fig. 3. The inflorescences maintain a generally stable colour range across all treatments and time points, consistent with the typical purple colour for this cultivar. Minor variations in petal intensity are observed but remain within the expected phenotypic range.

Biochemical array of leaves and inflorescences

The repeatability of the biochemical methods was assessed using quality control samples analysed in triplicate. Precision was evaluated based on intra-day repeatability and expressed as %RSD (relative standard deviation). The mean RSD values were around 4–11%, indicating acceptable precision of the protocols.

Most IAA- and NP-treated plants had an increased TPC value compared to the control, regardless of treatment duration (Fig. 4). The highest content of polyphenols in the leaves of chrysanthemum plants grown for 30 days was found after incorporating a combination of IAA and Fe_3O_4CA NPs. As for the plants grown for 60 days, the highest TPC value was detected after the sole application of IAA. In contrast, the lowest TPC value was found in the control and Fe_3O_4CA NPs treatment.

As for the inflorescences of plants treated for 30 days, the highest concentration of polyphenols was reported after applying Fe_3O_4 NPs and IAA + Fe_3O_4CA NPs. No significant differences were found between the other treatments and the control ($p > 0.05$) (Fig. 5). After 60 days, inflorescences of plants treated with IAA + Fe_3O_4CA NPs and the control had the highest TPC, whereas the Fe_3O_4 NPs treatment—the lowest.

Plants treated for 30 days with Fe_3O_4 NPs and IAA + Fe_3O_4CA NPs had a higher concentration of anthocyanins in their inflorescences than the chrysanthemums from other treatments (Fig. 6). The results were somewhat

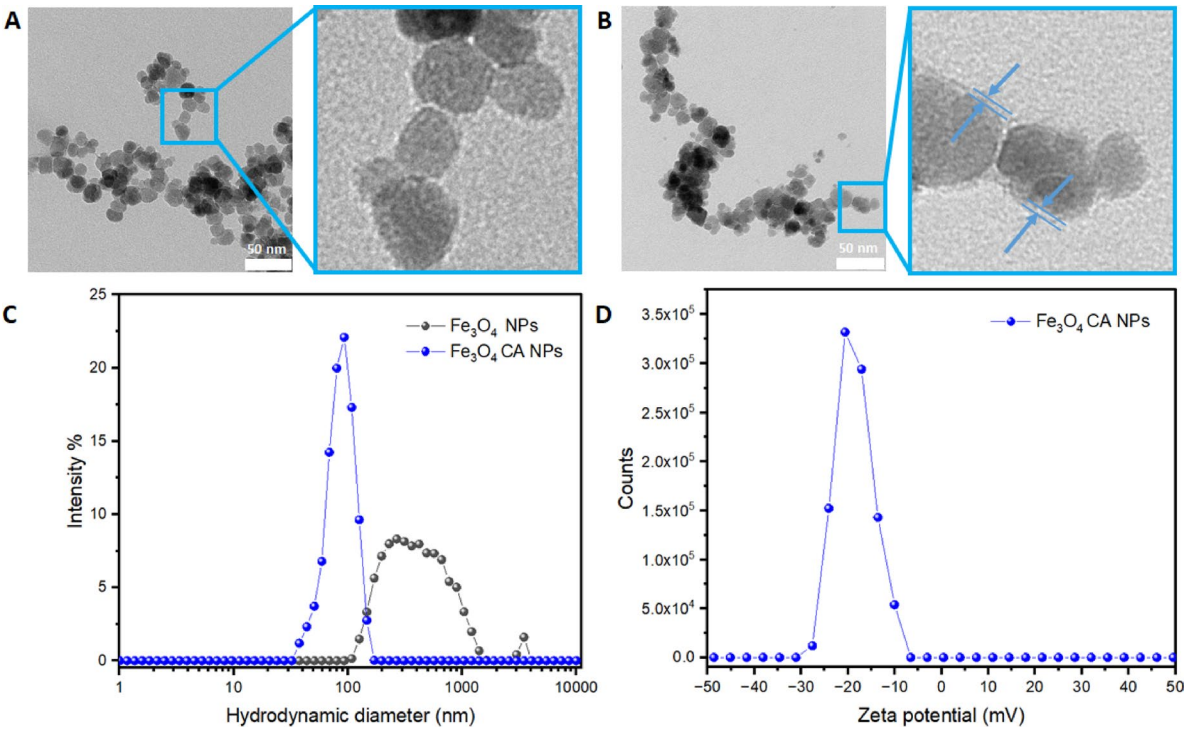

Fig. 1. TEM images of Fe_3O_4 NPs (**A**), Fe_3O_4CA NPs (**B**), hydrodynamic size distribution for both NPs (**C**), and Zeta potential for Fe_3O_4CA NPs (**D**).

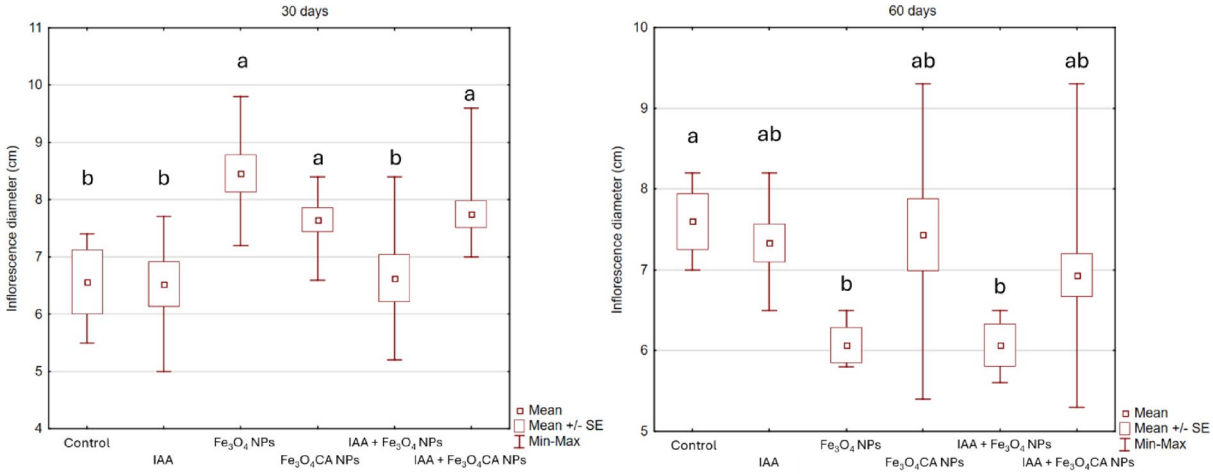

Fig. 2. Effect of indole-3-acetic acid (IAA) and iron oxide nanoparticles in bare (Fe_3O_4 NPs) or citrate-stabilized form (Fe_3O_4CA NPs), applied singularly or in combination, on the inflorescence diameter of *Chrysanthemum* × *morifolium* 'Richmond' after 30 and 60 days of treatment. Box-and-whisker plots with the same letters (a, b) are not significantly different ($p < 0.05$) according to one-way ANOVA followed by Duncan's multiple range test ($n = 98$).

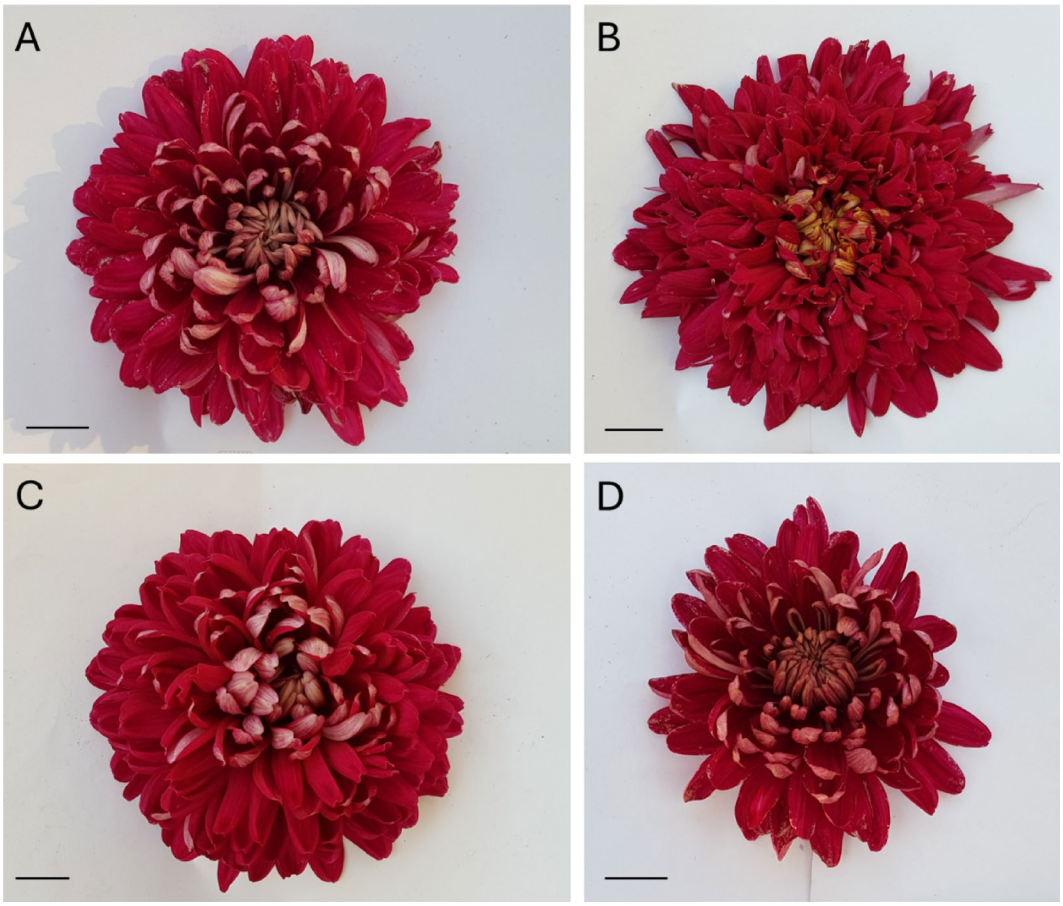

Fig. 3. Example inflorescences of *Chrysanthemum* × *morifolium* 'Richmond': control after 30 days of in vitro culture (**A**), treatment with Fe_3O_4 NPs for 30 days (**B**), control after 60 days of in vitro culture (**C**), treatment with Fe_3O_4 NPs for 60 days (**D**). Scale bar: 1 cm.

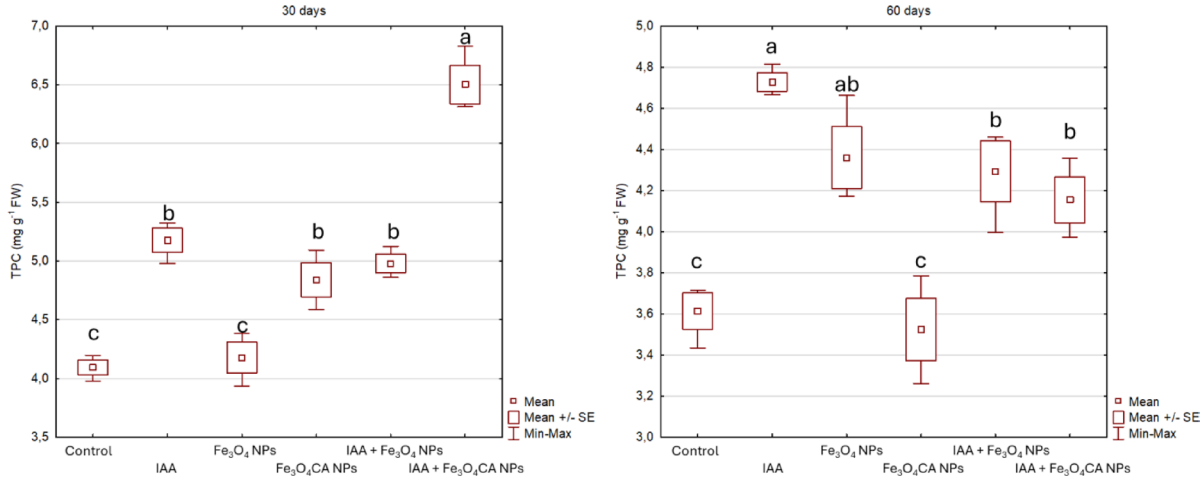

Fig. 4. Effect of indole-3-acetic acid (IAA) and iron oxide nanoparticles in bare (Fe_3O_4 NPs) or citrate-stabilized form (Fe_3O_4CA NPs), applied singularly or in combination, on the content of polyphenols in the leaves of *Chrysanthemum* × *morifolium* 'Richmond' after 30 and 60 days of treatment. Box-and-whisker plots with the same letters (a–c) are not significantly different ($p < 0.05$) according to one-way ANOVA followed by Duncan's multiple range test (three biological replicates).

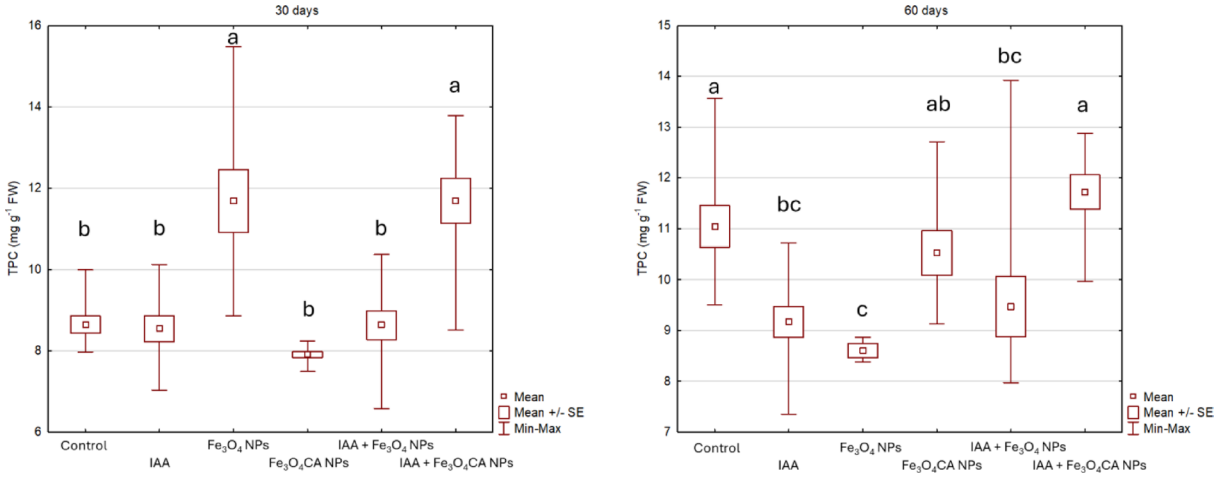

Fig. 5. Effect of indole-3-acetic acid (IAA) and iron oxide nanoparticles in bare (Fe_3O_4 NPs) or citrate-stabilized form (Fe_3O_4CA NPs), applied singularly or in combination, on the content of polyphenols in the inflorescences of *Chrysanthemum* × *morifolium* 'Richmond' after 30 and 60 days of treatment. Box-and-whisker plots with the same letters (a–c) are not significantly different ($p < 0.05$) according to one-way ANOVA followed by Duncan's multiple range test (nine biological replicates).

different after 60 days of exposure, i.e. the highest content was found in the untreated control, whereas the lowest – after the application of Fe_3O_4 NPs.

Genetic stability of plants

A total of 2160 scorable bands were detected by eight RAPD primers in 72 'Richmond' plants (Table 1). Primer R3 generated the highest number of bands (7 per sample), while primer R5 produced only one amplicon per sample. The band profiles of individual plants did not differ among each other, i.e. no polymorphic genotypes were detected by any of the primers (Fig. 7).

Discussion

The integration of nanoparticles (NPs) and plant growth regulators has emerged as a promising strategy in plant biotechnology[32]. Our study confirmed the application of engineered NPs as PGR carriers in the management of synthetic seed practices. Nonetheless, the observed time-dependent effects of iron oxide nanoparticles on inflorescence diameter in chrysanthemum plants highlight complex interactions between NPs treatments and plant developmental stages.

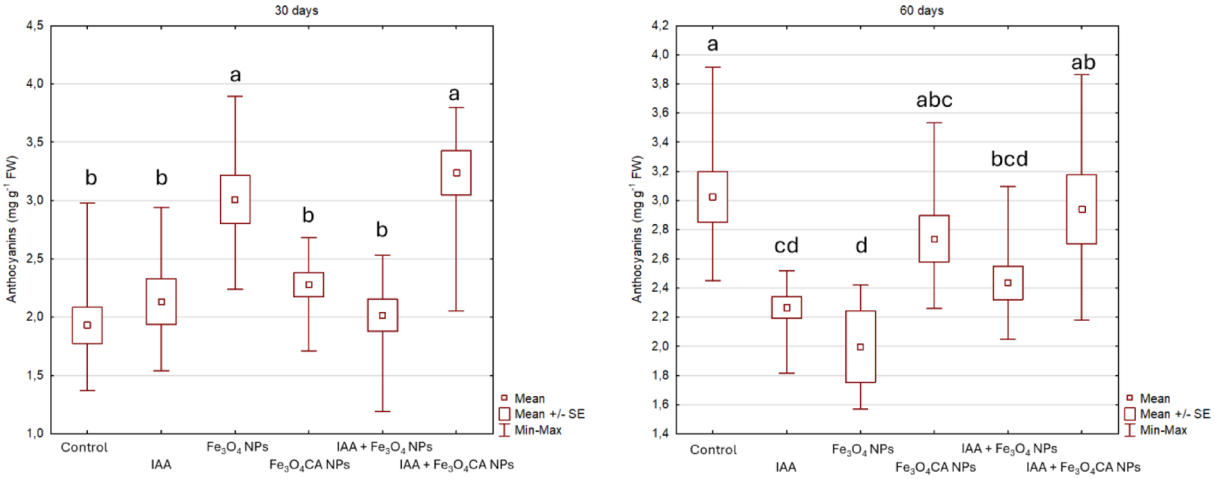

Fig. 6. Effect of indole-3-acetic acid (IAA) and iron oxide nanoparticles in bare (Fe_3O_4 NPs) or citrate-stabilized form (Fe_3O_4CA NPs), applied singularly or in combination, on the content of anthocyanins in the inflorescences of *Chrysanthemum* × *morifolium* 'Richmond' after 30 and 60 days of treatment. Box-and-whisker plots with the same letters (a–d) are not significantly different ($p < 0.05$) according to one-way ANOVA followed by Duncan's multiple range test (nine biological replicates).

No.	Primer sequence 5′–3′	No. of bands	No. of loci				Total polymorphic loci (%)	No. of polymorphic plants	No. of genotypes	PIC
			Σ	mono.	poly.	spec.				
R1	GGG AAT TCG G	144	2	2	0	0	0	0	1	0
R2	GAC CGC TTG T	360	5	5	0	0	0	0	1	0
R3	GGA CTG GAG T	504	7	7	0	0	0	0	1	0
R4	GCT GCC TCA GC	432	6	6	0	0	0	0	1	0
R5	TAC CCA GGA GCG	72	1	1	0	0	0	0	1	0
R6	CAA TCG CCG T	288	4	4	0	0	0	0	1	0
R7	GGT GAC GCA G	216	3	3	0	0	0	0	1	0
R8	CCC AGT CAC T	144	2	2	0	0	0	0	1	0
Σ		2160	30	30	0	0	–	0	–	–
Mean from a single primer		270	3.75	3.75	0	0	0	–	–	–

Table 1. PCR products from *Chrysanthemum* × *morifolium* 'Richmond' obtained with a randomly amplified polymorphic DNA (RAPD) marker system. *mono.* monomorphic, *poly.* polymorphic, *spec.* specific, *PIC* polymorphism information content.

After 30 days of in vitro culture, iron oxide nanoparticles and their combination with IAA significantly increased inflorescence diameter (7.6–8.5 cm vs. 6.6 cm in the control). A similar phenomenon was reported by Srivastava et al. (2022) in calendula (*Calendula officinalis* L.)[33]. This is likely due to enhanced nutrient uptake, increased photosynthesis and respiration efficiencies, as well as improved water utilization, stimulated by nanoparticles, described by other researchers[34]. Similar to our study, Fe_3O_4 NPs enhanced plant growth of wheat (*Triticum aestivum* L.) through improved iron and phosphorous uptake and enhanced content of photosynthetic pigments in the leaves. This effect, however, depended on the light intensity during plant growth and the age of the plants[35]. Au NPs (10 mg L^{-1}) promoted the antioxidant activities of ascorbate peroxidase, catalase, and guaiacol peroxidase, which in turn resulted in higher fresh and dry biomass, as well as leaf area in wheat[36]. Silicon NPs stimulated crop development in tomato (*Solanum lycopersicum* L.) through increased availability and accumulation of macronutrients (nitrogen, potassium, calcium, and sulphur) and micronutrients (iron and manganese), as well as improved drought resistance by optimized water usage efficiency and reduced water loss during transpiration[37]. Zinc oxide NPs, on the other hand, enhanced growth and nutrient uptake in maize[38].

As in the case of biometrical analysis, most of the treatments in the present study generally increased the content of total polyphenols in the leaves and inflorescences of chrysanthemum, with effects depending on the duration of exposure. After 30 days, most treatments stimulated polyphenol and anthocyanin accumulation, especially Fe_3O_4 NPs and IAA combined with Fe_3O_4CA NPs. These compounds play crucial roles in plant defense mechanisms but are also appreciated by the phytopharmaceutical industry, therefore their increase is highly beneficial[39,40]. Similarly, Fe_2O_3 NPs in combination with endophytes boosted the levels of phenolic compounds, flavonoids, and essential oils in thyme (*Thymus vulgaris* L.) and *Dracocephalum kotschyi* Boiss[41,42]. However, in the present experiment, after a longer exposition of 60 days, a decline in these compounds was observed in most

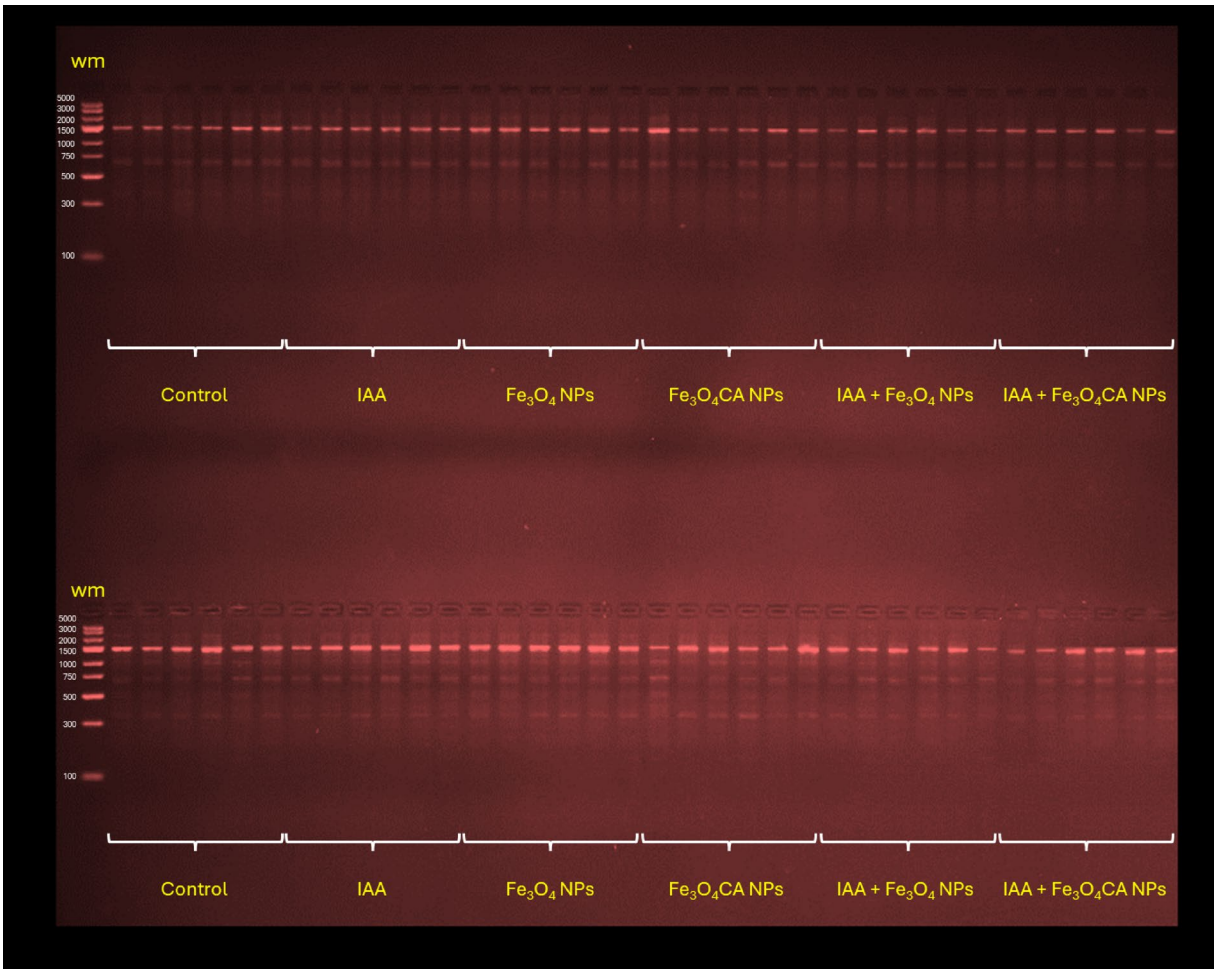

Fig. 7. Example RAPD band profiles of *Chrysanthemum* × *morifolium* 'Richmond' (cultured for 30 days) received with primer R1 (upper line) and R2 (bottom line) as a result of auxin and/or nanoparticle treatments. The outermost lanes (wm) serve as DNA base pair (bp) weight markers, while inner lines represent control plants, plants treated with indole-3acetic acid (IAA), iron oxide nanoparticles (Fe_3O_4 NPs), and/or iron oxide nanoparticles stabilized with citrate (Fe_3O_4CA NPs).

cases. This negative effect of prolonged treatments on both the development of plants and their phytochemical composition could be related to NPs accumulation; iron toxicity or oxidative stress, disrupting metabolic pathways[43]. Excessive iron content can catalyse the Fenton reaction, producing highly reactive hydroxyl radicals (•OH) that induce oxidative stress. These reactive oxygen species (ROS) can damage lipids, proteins, and DNA, including enzymes involved in phenylpropanoid biosynthesis – the primary pathway responsible for polyphenol production[44]. At the same time, iron overload may disturb IAA homeostasis, affecting auxin signalling and gene expression, including members of the auxin/induced-3-acetic acid (Aux/IAA) family[45]. These genes are essential for auxin-regulated transcriptional repression that affects plant growth and resistance to abiotic stresses[46]. Altered Aux/IAA expression under iron-induced stress may shift metabolic priorities toward cell survival and stress mitigation, rather than secondary metabolism. Moreover, IAA-NPs interactions might overstimulate early growth, reducing resources for later reproductive stages[47]. The observed differences in the effect of Fe_3O_4 NPs and Fe_3O_4CA NPs, such as sustained polyphenol content after 60 days in plants treated with Fe_3O_4CA NPs, can be explained by surface modification (citrate coating) of nanoparticles. Citrate-coated NPs have increased colloidal stability and higher aqueous solubility, which enhances their bioavailability and facilitates more controlled cellular uptake[48,49]. The negatively charged citrate layer reduces aggregation and may limit uncontrolled ROS generation by minimizing rapid iron ion release, consequently reducing oxidative stress as observed in soybean (*Glycine max* L.) or alfalfa (*Medicago sativa* L.) plants[50]. Răcuciu et al. (2022) confirmed a positive influence of Fe_3O_4 NPs coated with aspartic acid on the performance of maize (*Zea mays* L.) seedlings[51]. On the other hand, no effect of Fe_3O_4 NPs on the primary photochemical processes was observed in wheat, regardless of the experimental factors studied; NPs concentration and light intensity[35]. This highlights the species-dependent reaction of plants to similar treatments.

Stability verification of the in vitro-derived material is of paramount importance. RAPD markers are a valuable tool for identifying genetic variation within chrysanthemum, due to their simplicity, low cost, and high efficiency corroborated by numerous studies[6,52,53]. The performed here analyses confirmed the genetic and

phenotypic homogeneity of plants, regardless of IAA or NPs treatment. Our results correspond with Sun et al.[54], who claimed that Fe_3O_4 NPs had no evident phytotoxicity even at the level of 1000 mg L^{-1} in mung bean (*Vigna radiata* (L.) R. Wilczek). This is extremely important since nanoparticles are known to induce genotoxic effects on plants if used at suboptimal concentrations[55]. Research indicates that nanoparticles, such as Ag NPs and ZnO NPs, can penetrate plant cells and generate reactive oxygen species that contribute to oxidative stress and subsequent DNA damage. For example, exposure to Ag NPs resulted in increased DNA fragmentation and chromosomal aberrations in faba bean (*Vicia faba* L.) and tobacco (*Nicotiana tabacum* L.)[56,57]. In a study by Fouda et al. (2021), Fe_3O_4 NPs showed the lowest genetic toxicity in *Salvadora persica* L. callus cultures, causing only minimal changes in genomic template stability (GTS) compared to ZnO and SiO_2 nanoparticles[58]. The extent of genotoxicity, however, is influenced by several factors, i.e. nanoparticle size, concentration, and exposure duration. Apparently, iron oxide nanoparticles applied at low concentrations (7.7 mg L^{-1}) do not cause adverse effects on the DNA and can be safely used in synthetic seed technology. Likewise, plant growth regulators (e.g. auxins) may stimulate the development of callus and occurrence of somaclonal variation, reported also in chrysanthemum[59]. In the present study, no indirect regeneration happened, therefore stability was maintained. This is crucial for the commercial propagation of plants. Nevertheless, it should be highlighted that RAPDs are dominant markers, which may miss recessive mutations. In future studies it is recommended to supplement SSR or SNP marker verification. Propagating genetically and phenotypically stable ornamental plants ensures predictable, high-quality products, efficient production, intellectual property protection, and meeting consumer demands[60].

In summary, this study provides a promising nano-regulation strategy for the large-scale production of synthetic seeds in ornamental plants. By using iron oxide nanoparticles in tissue culture systems of chrysanthemum, it is possible to achieve sustained-release effects that enhance PGR stability and bioavailability, reduce the frequency of media supplementation, and lower overall chemical reagent consumption. Our findings open new pathways in commercial plant biotechnology.

Materials and methods

Nanoparticles utilized in the experiment were synthesized (using the chemical co-precipitation method), modified (citrate-coated), and characterized at the Institute of Fundamental Technological Research, Polish Academy of Sciences, as described by Kulus et al.[24]. The experiment on plants was performed in the Laboratory of Horticulture and Landscape Architecture, and the Department of Agronomy and Food Technology, Bydgoszcz University of Science and Technology, Poland.

Characterization of nanoparticles

The morphology of nanoparticles was studied using Transmission Electron Microscopy Libra 120 (Carl Zeiss, Stuttgart, Germany). The aqueous suspensions of NPs were placed onto the copper mesh with a Formvar layer and dried overnight. The Zeta potential was studied using Zetasizer Nano Sizer (Malvern Instruments, Malvern, UK).

Preparation, storage, and conversion of synthetic seeds

Nodal segments (3–4 mm long) with a single axillary bud of chrysanthemum [*Chrysanthemum* × *morifolium* (Ramat.) Hemsl.] 'Richmond' were used in the experiment. The plants were obtained from our gene bank. Prof. Marek Jerzy undertook the formal identification of the plant material used. 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 approvals were needed to conduct research with this plant material. Explants were immersed for 10 min in 3% sodium alginate. The alginate solution was prepared on MS medium salts[22], without $CaCl_2$, with the addition of 3% sucrose, iron oxide nanoparticles (bare Fe_3O_4 NPs or stabilized with citrate Fe_3O_4CA NPs; 7.7 mg L^{-1}) and/or indole-3-acetic acid (IAA; 1 mg L^{-1}) obtained from Sigma-Aldrich, Darmstadt, Germany. The concentrations of NPs and IAA were chosen based on our previous studies[24]. Alginate solutions with NPs were placed for 30 min in the Elmasonic S80(H) Ultrasonic Cleaner (37 kHz, 150 W; Elma Schmidbauer GmbH, Singen, Germany) for proper nanoparticle dispersion. Next, the beads were hardened in a 0.1 M $CaCl_2$ solution for 30 min and rinsed thrice with distilled sterile water. A control group without NPs or IAA was also included. The obtained synthetic seeds were inoculated onto a water-agar medium in a 90-mm Petri dish sealed with parafilm. The cultures were stored in a refrigerator at 4 °C in the dark for two months and then transferred to the growth room for 30 or 60 days (16-h photoperiod, 22 °C).

Acclimatization and biochemical array of leaves and inflorescences

The initially germinated synthetic seeds were sown in a greenhouse, in a mixture of peat and perlite (2:1), provided by Hartmann (Poznań, Poland), in multi-pot trays. Next, the plants were transferred to pots (16 cm diameter) filled with the same substrate and brought to full flowering. At that time, the diameter of 98 inflorescences was measured with a ruler.

The determination of total polyphenol content (TPC) in the leaves was carried out in triplicates according to the method by Podsędek et al.[61] using a Dionex UltiMate 3000 set (4-component pump, autosampler, with sample chamber thermostated, diode array UV detector incl. 3D spectrum collection option, RI detector). The separation was carried out on a Kinetex C18 column (5 µm, 150 × 4.6 mm), using a gradient separation. A mixture of 6% acetic acid in 2 mmol L^{-1} acetate and 100% acetonitrile (Sigma Aldrich) was used as a solvent. The qualitative assessment was carried out based on retention times and the spectra of pure standards. For polyphenolic compounds for which external standards were not available, the content is presented as the equivalent of related substances; chlorogenic acid (Sigma Aldrich). The results were presented in mg g^{-1} fresh weight (FW).

Anthocyanins were extracted from 200 mg fresh weight (FW) of inflorescence samples using methanol containing 1% HCl, according to the Harborne (1984) method[62]. The same extract was used to analyze the total polyphenol content (TPC) with the Folin-Ciocalteau reagent, according to Waterhouse (2001)[63]. Absorbance was measured using a NanoPhotometer NP80 (Implen, München, Germany) at a wavelength of 530 nm (anthocyanins) and 765 nm (TPC). Nine replicates from each experimental treatment were included. Results were calculated based on a calibration curve prepared for gallic acid equivalent (GAE) and the molar extinction coefficient (ε) of cyanidin-3-O-glucoside (C3G), and expressed in mg g^{-1} FW.

Genetic stability analysis

The genetic fidelity of 72 ex vitro-grown shoots (six plants from each of the six experimental treatments and two treatment durations) was assessed using randomly amplified polymorphic DNA (RAPD). Total genomic DNA was isolated from fresh leaves using a Genomic Mini AX Plant Spin kit (A&A Biotechnology, Gdańsk, Poland) according to the manufacturer's protocol. The isolated DNA was stored at − 80 °C in a laboratory deep-freezer U101 Innova (Eppendorf, Hamburg, Germany).

Eight primers were used for the PCR reaction. A ready-to-use mix for PCR for GC-rich templates was used, containing Taq DNA polymerase, stabilizers, and PCR anti-inhibitors (A&A Biotechnology). The amplification was carried out in a C1000 Touch thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) with the program described by Miler et al.[52].

The amplified DNA fragments were separated on a 1.5% (*w/v*) DN- and RNase-free agarose gel in TBE buffer at 110 V for 60 min (Biometra P25, Jena, Germany) and visualized by staining with ethidium bromide (BrEt). Gel images were captured using a GelDoc XR + Gel Documentation System (Bio-Rad) UV transilluminator with Image Lab 4.1 software (Bio-Rad). The molecular weights of the fragments were estimated using a GeneRuler Express DNA Ladder (100–5000 bp) from Thermo Scientific, Waltham, MA, USA.

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.

Statistical analysis

A total of six experimental combinations were included, i.e. 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). The study was set in a completely randomized design.

The obtained results were statistically analysed through one-way ANOVA, and means were compared with Duncan's multiple range test ($p \leq 0.05$), using Statistica 12.0 software (StatSoft, Tulsa, OK, USA).

Conclusions

This study demonstrates that iron oxide nanoparticles and IAA can affect the metabolic activity and flower quality of *Chrysanthemum* × *morifolium* plants derived from synthetic seeds. The size of inflorescences was higher following shorter exposure to nanoparticles but decreased with extended treatment. Likewise, NPs and IAA generally increased the content of polyphenols and anthocyanins in the plants, especially after shorter application. Importantly, the RAPD analysis confirmed the genetic uniformity of plants, indicating that the studied factors did not cause mutations. Among the studied nanoparticle forms, citrate-stabilized iron oxide NPs in combination with IAA most effectively enhance phytochemical content and flower quality in chrysanthemum, presenting a promising nano-regulation approach. However, the duration of application is critical, as prolonged exposure may reduce these beneficial effects. Since only one chrysanthemum cultivar was tested and the transport pathway of nanoparticles in plants was not explored, further research is needed to better understand the mechanisms of NPs (inter)action and optimize treatment protocols for practical use in plant biotechnology. These should include comparative studies of multiple cultivars, transcriptomic analysis to reveal metabolic regulatory pathways, and development of nanoparticle sustained-release systems.

Data availability

Data that support the findings of this study are available from the public repository: https://doi.org/10.18150/TAS3FB.

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Author contributions

D.K., conceptualization, methodology, investigation, data analysis, draft preparation, visualization, supervision; A.T., methodology, investigation, draft revision and validation; M.C., investigation, text revision and editing; K.G., methodology, investigation; M.O., methodology, investigation, resources. All authors read and accepted the final version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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