



RESEARCH ARTICLE

Nano-enabled enhancement of reproductive efficiency and metabolic signaling in broccoli (*Brassica oleracea* var. *italica*) under *in vitro* conditions

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Abstract

This study investigated the comparative effects of zinc oxide nanoparticles (ZnO-NPs), silver nitrate (AgNO₃), salicylic acid (SA) and methyl jasmonate (MeJA) on *in vitro* reproductive development of broccoli (*Brassica oleracea* var. *italica*) under controlled conditions. Murashige and Skoog (MS) media were supplemented with each treatment at 0, 10, 20 and 30 mg L⁻¹ concentrations and explants were cultured under uniform photoperiod and temperature. An integrative statistical framework comprising analysis of variance (ANOVA), canonical correlation analysis (CCA), principal component analysis (PCA) and heatmap visualisation was employed to interpret morphogenic, reproductive and physiological responses. Among all treatments, ZnO-NPs at 20 mg L⁻¹ exhibited superior bioefficacy, significantly enhancing shoot elongation (5.5 cm, $p < 0.05$), pollen viability (92 %), stomatal conductance (0.38 mol H₂O m⁻² s⁻¹) and photosystem II (PSII) efficiency (Fv/Fm = 0.84), while reducing flower abortion (5.2 %) and shedding (12.5 %). Antioxidant enzyme activities (SOD = 15.2, CAT = 10.4, POD = 18.3 U g⁻¹ fresh weight (FW)) increased significantly, correlating with reduced lipid peroxidation (malondialdehyde (MDA) = 4.2 nmol g⁻¹ FW). Multivariate analyses distinctly separated ZnO-NP from other treatments, confirming its integrative regulatory influence. These results highlight the potential of ZnO-NPs as an effective nanoelicitor for improving reproductive physiology and antioxidative balance in broccoli under controlled *in vitro* conditions.

Keywords: antioxidant enzymes; broccoli; reproductive physiology; tissue culture; zinc oxide nanoparticles

Introduction

Reproductive development in plants, encompassing floral organogenesis, gametophyte formation and fertilisation, is a highly regulated process influenced by genetic, physiological and environmental factors (1). The ability to regulate these processes under controlled conditions is central to understanding and improving crop reproductive efficiency. *In vitro* culture systems offer a powerful platform for dissecting reproductive and physiological responses in plants, allowing precise control over environmental and nutritional variables. Broccoli (*Brassica oleracea* var. *italica*), a nutritionally valuable cruciferous crop and is particularly suitable for such studies due to its sensitivity to fluctuations in growth regulators, nutrient balance and oxidative status during reproductive transition (2). Also, zinc oxide nanoparticles (ZnO-NPs) and silver nitrate (AgNO₃) have gained attention for their roles in influencing hormone signaling, antioxidant metabolism and reproductive processes (3). Zinc oxide nanoparticles, in particular, are involved in Zn-mediated activation of auxin biosynthesis and enzymatic systems that enhance meristematic activity, cell division and oxidative stress management (4). Similarly, AgNO₃ acts as an ethylene inhibitor, extending floral longevity and improving pollen viability, while elicitors such as salicylic acid (SA) and methyl jasmonate (MeJA)

are known to regulate stress-related signaling cascades that influence reproductive success (5, 6). Despite these advances, there remains a lack of comparative studies evaluating how nanoparticles (NPs) and classical elicitors jointly affect reproductive and physiological performance under *in vitro* conditions. Most previous work has focused on stress mitigation or vegetative growth responses, while little is known about how such nanoelicitors can optimise reproductive outcomes such as pollen viability, flower retention and antioxidant balance under non-stress and controlled environments. Moreover, mechanistic insight into how NPs modulate antioxidant and hormonal pathways during floral development remains incomplete. Therefore, the present study aimed to investigate the comparative effects of ZnO-NPs, AgNO₃, SA and MeJA on the morphogenic and reproductive performance of broccoli under controlled *in vitro* conditions. In addition, physiological and biochemical parameters such as photosynthetic efficiency, stomatal conductance and antioxidant enzyme activities were assessed to elucidate treatment-induced responses. Multivariate approaches, including principal component analysis (PCA), canonical correlation analysis (CCA) and heatmap analysis, were subsequently applied to integrate the datasets and determine the most effective elicitor and concentration for reproductive enhancement. We hypothesised that ZnO-NPs, at optimised concentrations, would enhance

reproductive efficiency by promoting pollen viability and floral retention through improved redox balance and photosynthetic performance. The findings are expected to aid in developing NP based approaches for precision modulation of reproductive development in high-value horticultural crops.

Materials and Methods

Experimental overview

The experiment was conducted in the Plant Tissue Culture Laboratory, Division of Plant Physiology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu (SKUAST-J). The study aimed to assess the influence of ZnO-NPs, AgNO₃, SA and MeJA on reproductive and physiological responses of *Brassica oleracea* var. *italica* (broccoli) under controlled *in vitro* conditions.

Preparation and characterisation of nanoparticles

Commercially synthesised ZnO-NPs (Sigma-Aldrich, USA; ≥ 99.9 % purity) were used in this study. The physicochemical characteristics of the NPs were obtained from the manufacturer's specifications. According to the supplier's data sheet, the ZnO-NPs had an average particle size of 25–35 nm with predominantly spherical morphology and a polydispersity index (PDI) of 0.22, indicating good dispersion characteristics. Before incorporation into the culture medium (MS media), ZnO-NPs were suspended in sterile distilled water and subjected to sonication at 40 kHz for 30 min to obtain a uniform dispersion and reduce particle aggregation. The stability of dispersions was visually confirmed by the absence of sedimentation for at least 48 hr. No chitosan or copper oxide nanoparticle (CuO-NP) treatments were included in this experiment and all references to these materials have been removed for consistency.

Media preparation and treatment composition

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 3 % (w/v) sucrose and 0.7 % (w/v) agar was used as the basal medium. Zinc oxide nanoparticles, AgNO₃, SA and MeJA were added to the molten medium at concentrations of 0 (control), 10, 20 and 30 mg L⁻¹ following the protocol of a previous study (7). The pH was adjusted to 5.8 before autoclaving at 121 °C and 15 psi for 20 min. After cooling (~45 °C), NP suspensions were gently mixed to ensure uniform dispersion before solidification.

Plant material and surface sterilisation

A local broccoli genotype, SJBc-07 (developed by SKUAST-J and suited to subtropical conditions), was used. Explants were derived from *in vitro* grown leaves and petioles as shown in Fig. 1. Surface sterilisation involved washing with 1 % labolene detergent and 2–3 drops of tween-20 for 10 min, followed by disinfection with 0.1 % mercuric chloride (HgCl₂) for 2 min under laminar airflow (LAF). The explants were then rinsed 4–5 times with sterile distilled water and transferred to MS basal medium for germination and culture initiation.

In vitro culture conditions and experimental timeline

Cultures were maintained under a 16 hr photoperiod, light intensity of 40 μmol m⁻² s⁻¹ and temperature of 25 ± 2 °C. The experimental timeline is depicted in Table 1.

Floral buds were sampled at the pre-anthesis stage for pollen viability analysis, while leaves from the same plantlets were used for physiological and biochemical assays.

Experimental design and replication structure

The experiment followed a completely randomised design (CRD) with 4 treatments (ZnO-NPs, AgNO₃, SA, MeJA) × 3 concentrations (10, 20, 30 mg L⁻¹) + control, each with 3 replications and 10 explants per replicate (n = 30 per treatment). Thus, n = 24 shown

Table 1. Experimental timeline

Stage	Duration	Description
Seed germination	0–7 days	MS basal medium without treatment
Shoot initiation	8–21 days	Initial morphogenic response
Floral induction and treatment	22–35 days	Application of ZnO-NPs, AgNO ₃ , SA, MeJA (10–30 mg L ⁻¹)
Data collection	36–56 days	Pollen viability, flower retention and physiological assays

ZnO-NPs: zinc oxide nanoparticles; AgNO₃: silver nitrate; SA: salicylic acid; MeJA: methyl jasmonate.



Fig. 1. Sequential stages of *in vitro* regeneration in broccoli (*Brassica oleracea* var. *italica*), showing callus induction, shoot initiation, root development and establishment of complete plantlets under controlled culture conditions.

in Fig. 2 captions refers to the total number of observations per treatment group used for statistical analysis.

Reproductive and physiological measurements

Pollen viability

Assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) staining (8). Viable pollen stained dark blue are shown in Fig. 3 and expressed as:

$$\text{Viability (\%)} = \frac{\text{Viable pollen}}{\text{Total pollen observed}} \times 100$$

Flower abortion and shedding

Aborted flowers and shed blossoms were counted at full bloom and percentages were calculated following previous studies (9, 10).

Photosynthetic efficiency

Assessed by the Fv/Fm ratio after 30 min dark adaptation using a Licor 6400 portable photosynthesis system (11).

Lipid peroxidation (malondialdehyde)

Estimated following a earlier experiment via the thiobarbituric acid method (12).

Antioxidant enzyme activities

Superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) activities were determined using standard protocols (13–15) and expressed in U g⁻¹ FW.

Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA) in SPSS v.27.0. Means were compared using Tukey's honestly significant difference (HSD) test at $p < 0.05$. Each figure includes error bars (SE $\pm$) and alphabetical letters (a, b, c) to denote significant differences. Critical difference (CD), standard error of mean (SE_m) and standard error of difference (SEd) were computed accordingly.

Multivariate analyses were conducted in Python

Canonical correlation analysis (CCA) was performed using the Statsmodels package to examine associations between different sets of variables. Principal component analysis (PCA) was carried out using the Scikit-learn library to evaluate sample clustering patterns and trait contributions. Heatmaps and radar plots were

generated using Seaborn and Plotly, respectively, after normalising the data to a 0–1 scale. Prior to visualisation, the normalised dataset was assessed for homogeneity to minimise bias arising from differences in parameter ranges. All NP handling procedures followed SKUAST-J biosafety guidelines. Waste NP suspensions were collected in sealed containers for centralised disposal in compliance with the Institutional Biosafety Committee (IBC) norms. No genetically modified organisms were used.

Results

This section presents the observed effects of different elicitor treatments. Zinc oxide nanoparticles, AgNO₃, SA and MeJA on *in vitro* reproductive development and physiological performance of *B. oleracea* under controlled culture conditions were done. All results are expressed as mean $\pm$ SE (n = 24). Treatments with different letters (a, b, c) are significantly different at $p < 0.05$ according to Tukey's HSD test.

In vitro regeneration and shoot growth

Shoot elongation showed significant variation across treatments (Fig. 4). The maximum shoot length was recorded in plants treated with ZnO-NPs at 20 mg L⁻¹ (5.5 $\pm$ 0.2 cm, a, $p < 0.05$). Treatments with ZnO-NPs at 10 mg L⁻¹ (4.0 $\pm$ 0.1 cm, b) and 30 mg L⁻¹ (4.3 $\pm$ 0.2 cm, b) produced intermediate shoot lengths and did not differ significantly from each other. The control exhibited the shortest shoots (3.2 $\pm$ 0.1 cm, c). Silver nitrate, SA and MeJA also enhanced shoot elongation relative to the control, although their effects were less pronounced than those observed with ZnO-NPs at 20 mg L⁻¹. The results indicate a concentration-dependent response, with 20 mg L⁻¹ being the optimum for growth promotion.

Pollen viability

Pollen viability varied significantly among treatments (Fig. 5). Zinc oxide nanoparticle (20 mg L⁻¹) recorded the highest viability (92 $\pm$ 1.4 %, a, $p < 0.05$), followed by MeJA (85 $\pm$ 1.8 %, b) AgNO₃ and SA (80 $\pm$ 2.0 %, b). The control displayed 72 $\pm$ 1.9 % viable pollen (c). At 30 mg L⁻¹, ZnO-NPs declined to 65 $\pm$ 1.6 %, suggesting potential toxicity at higher concentrations. These results confirm that ZnO-NPs at moderate concentration significantly enhanced pollen performance under non-stress conditions.



Fig. 2. Developmental stages of broccoli grown under *in vitro* conditions, from vegetative growth to flowering.



Fig. 3. Microscopic observation of viable and non-viable pollens.

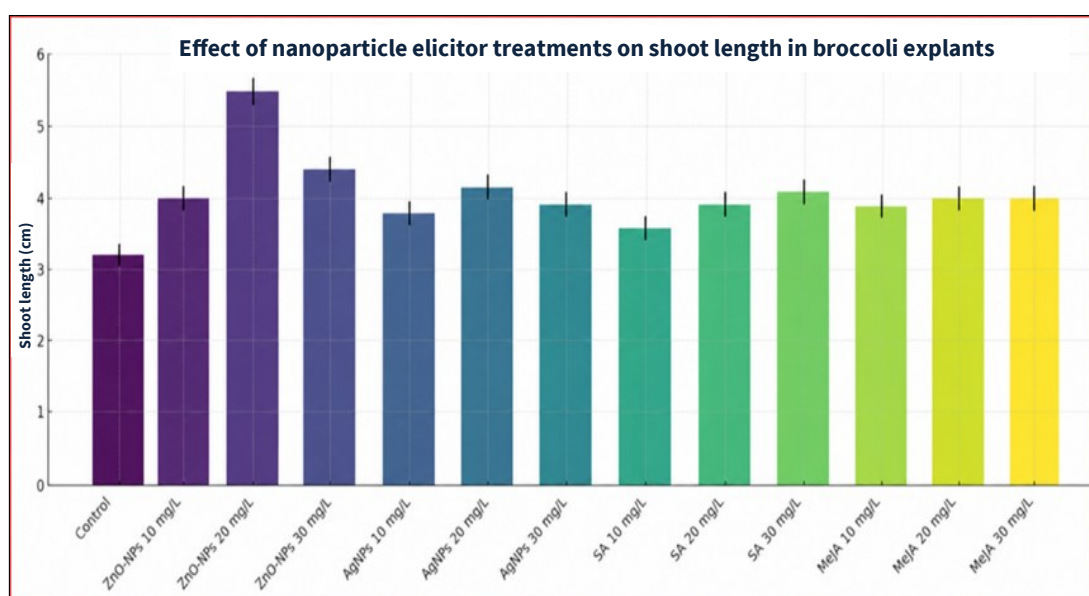


Fig. 4. Effect of various nanoparticle (ZnO-NPs, AgNO₃) and elicitor (SA, MeJA) treatments at 10, 20 and 30 mg L⁻¹ on shoot length in broccoli explants. Zinc oxide nanoparticle (ZnO-NPs) at 20 mg L⁻¹ significantly enhanced shoot length compared to control and other treatments. Error bars represent mean $\pm$ standard error (SE) (n = 24).

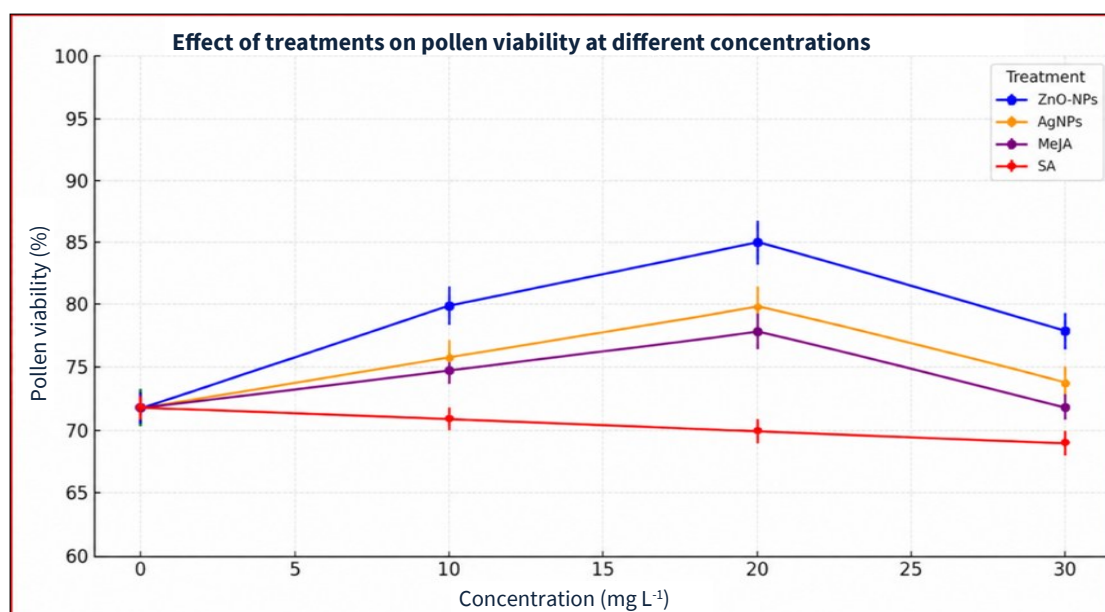


Fig. 5. Impact of ZnO-NPs, AgNO₃, SA and MeJA at 0, 10, 20 and 30 mg L⁻¹ concentrations on pollen viability (%) in broccoli. Zinc oxide nanoparticles at 20 mg L⁻¹ led to the highest pollen viability (92 %), while higher concentrations reduced efficacy across treatments. Error bars represent mean $\pm$ standard error (SE) (n = 24).

Flower abortion and shedding

Zinc oxide nanoparticles at 20 mg L⁻¹ showed the lowest flower abortion rate (5.2 ± 0.3 %, a), significantly lower than the control (10.5 ± 0.4 %, c) and AgNO₃ (7.5 ± 0.5 %, b) (Fig. 6). Similarly, flower shedding percentage was minimum in ZnO-NPs (12.5 ± 0.4 %, a) followed by AgNO₃ (14.2 ± 0.5 %, b), while the control exhibited the highest shedding (22.1 ± 0.6 %, c) (Fig. 6). The reduction in both parameters under ZnO-NPs treatment indicates improved flower retention and reproductive stability.

Photosynthetic efficiency (Fv/Fm ratio)

The maximum quantum yield of photosystem II (PSII) (Fv/Fm) differed significantly among treatments (Fig. 7). Zinc oxide nanoparticles (20 mg L⁻¹) achieved the highest ratio (0.84 ± 0.01 , a), compared to MeJA (0.80 ± 0.01 , b), SA (0.78 ± 0.01 , b) and control (0.72 ± 0.02 , c). Elevated Fv/Fm values confirm improved photosynthetic efficiency and photochemical performance under ZnO-NP exposure at optimal concentration.

Lipid peroxidation (Malondialdehyde content)

Lipid peroxidation, expressed as malondialdehyde (MDA) content, was significantly lower in ZnO-NPs (20 mg L⁻¹) with 4.2 ± 0.2 nmol g⁻¹ FW (a) compared to the control (6.5 ± 0.2 nmol g⁻¹ FW, c) (Fig. 8). Salicylic acid and MeJA treatments also reduced MDA levels moderately (4.9 – 5.4 nmol g⁻¹ FW, b). The results indicate that ZnO-NPs effectively minimised oxidative membrane damage, reflecting an enhanced antioxidant defence system.

Antioxidant enzyme activities (SOD, CAT, POD)

Enzyme activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) were assessed to evaluate the antioxidant defence mechanisms in response to treatments at 10, 20 and 30 mg L⁻¹ concentrations of NPs and elicitors. Superoxide dismutase activity was highest in plants treated with 20 mg L⁻¹ ZnO-NPs (15.2 U g⁻¹ FW), followed by MeJA (12.1 U g⁻¹ FW) and SA (13.0 U g⁻¹ FW). At 10 mg L⁻¹, ZnO-NPs still showed notable SOD activity (~ 13.4 U g⁻¹ FW), but it decreased to ~ 11.5 U g⁻¹ FW at 30 mg L⁻¹, indicating a concentration-dependent response. The

control group exhibited the lowest SOD activity (9.8 U g⁻¹ FW), confirming the beneficial effect of ZnO-NPs in enhancing antioxidant defence. Catalase activity was also highest in plants treated with 20 mg L⁻¹ ZnO-NPs (10.4 U g⁻¹ FW), followed by MeJA (9.1 U g⁻¹ FW) and SA (8.5 U g⁻¹ FW). At 10 mg L⁻¹, ZnO-NPs showed a slight decrease in CAT activity (~ 9.1 U g⁻¹ FW), while 30 mg L⁻¹ treatment exhibited a further reduction (~ 8.8 U g⁻¹ FW). The control group had the lowest CAT activity (6.7 U g⁻¹ FW). Similarly, POD activity was highest in plants treated with 20 mg L⁻¹ ZnO-NPs (18.3 U g⁻¹ FW), followed by MeJA (16.2 U g⁻¹ FW) and SA (15.4 U g⁻¹ FW). At 10 mg L⁻¹, ZnO-NPs maintained relatively high POD activity (~ 16.7 U g⁻¹ FW), but this decreased at 30 mg L⁻¹ (~ 15.8 U g⁻¹ FW). Control plants had the lowest POD activity (13.2 U g⁻¹ FW). These results indicate that ZnO-NPs, especially at 20 mg L⁻¹, significantly enhanced the activities of SOD, CAT and POD, providing strong antioxidant defence. Other treatments, such as MeJA and SA, showed intermediate effects, while the control group exhibited the least antioxidant activity, highlighting the role of NPs in boosting plant resilience against oxidative stress. The inclusion of error bars highlights the variability of the data (Fig. 9).

Canonical correlation analysis (CCA)

The CCA biplot (Fig. 10) displayed clear separation of ZnO-NPs (20 mg L⁻¹) from all other treatments, indicated strong multivariate influence. Each treatment point represents the centroid of three replicates. Zinc oxide nanoparticles clustered with positive loadings of pollen viability, photosynthetic efficiency and antioxidant enzymes, whereas the control grouped negatively with MDA and flower abortion. This multivariate alignment validates the integrative physiological advantage of ZnO-NPs.

Heatmap visualisation

The heatmap illustrated responses across treatments (Fig. 11). Zinc oxide nanoparticles 20 mg L⁻¹ showed the highest normalised scores for shoot length, Fv/Fm, SOD and pollen viability and the lowest for MDA and flower abortion. Data were normalised using Z-score scaling, ensuring comparability among parameters with different measurement units.

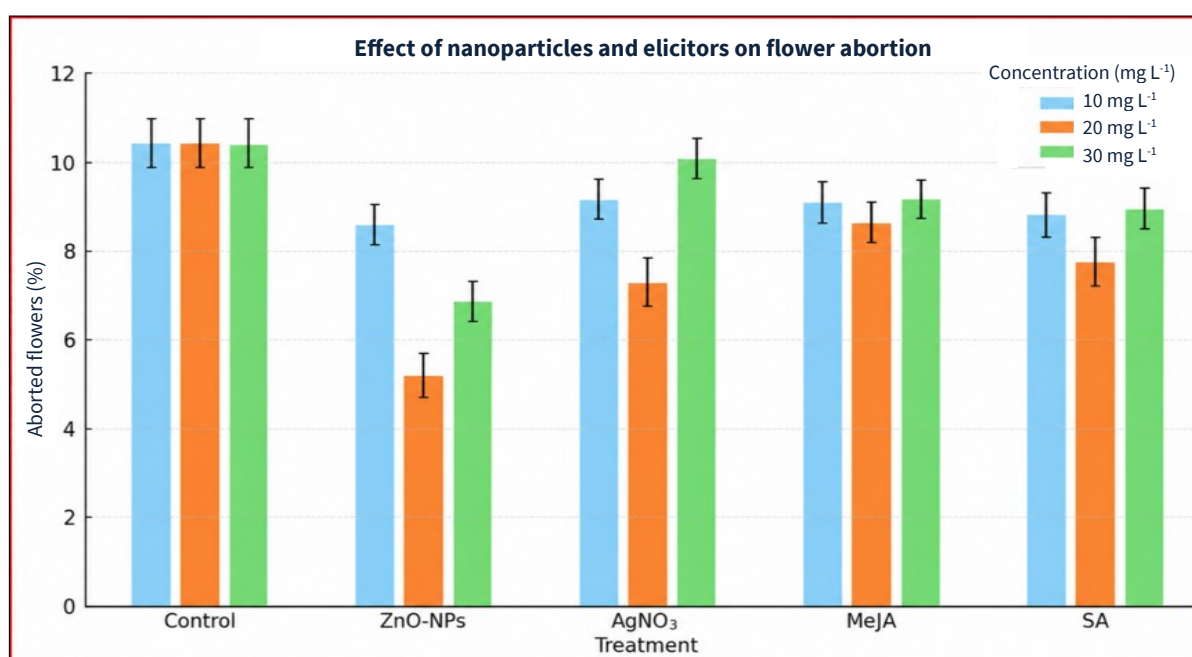


Fig. 6. Effect of ZnO-NPs, AgNO₃, SA and MeJA at 10, 20 and 30 mg L⁻¹ on flower abortion and flower shedding (%) in broccoli. Zinc oxide nanoparticles at 20 mg L⁻¹ significantly reduced flower abortion to 5.2 %, representing the lowest value among all treatments. Error bars indicate mean $\pm$ standard error (SE) (n = 24).

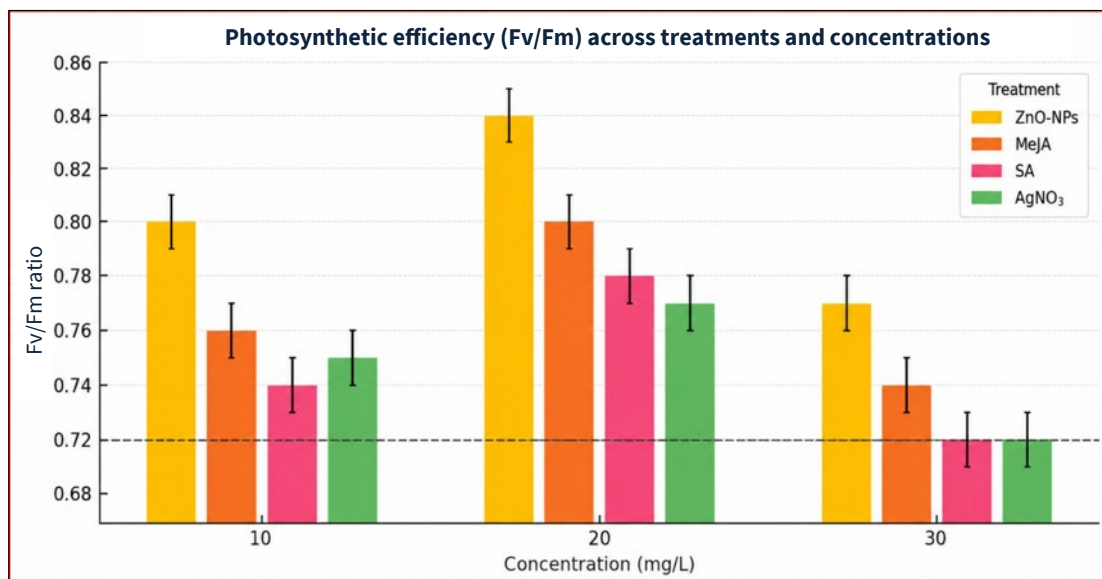


Fig. 7. Graph illustrating photosynthetic efficiency (Fv/Fm) under different treatments and concentrations. Error bars represent mean $\pm$ standard error (SE) (n = 24).

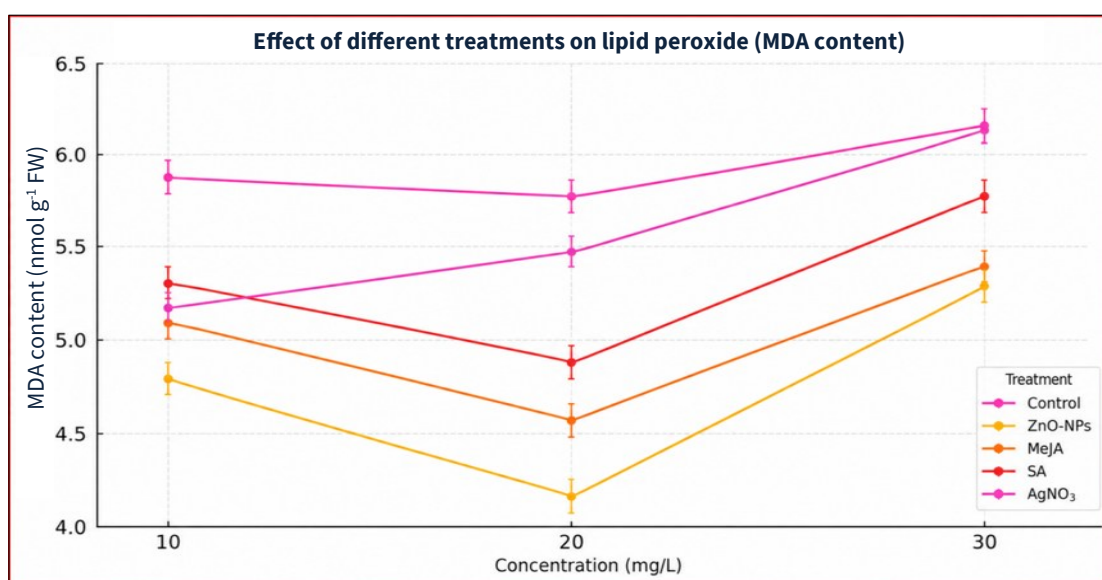


Fig. 8. Graphical representation of lipid peroxidation (MDA content) under different treatments and concentrations. Error bars represent mean $\pm$ standard error (SE) (n = 24).

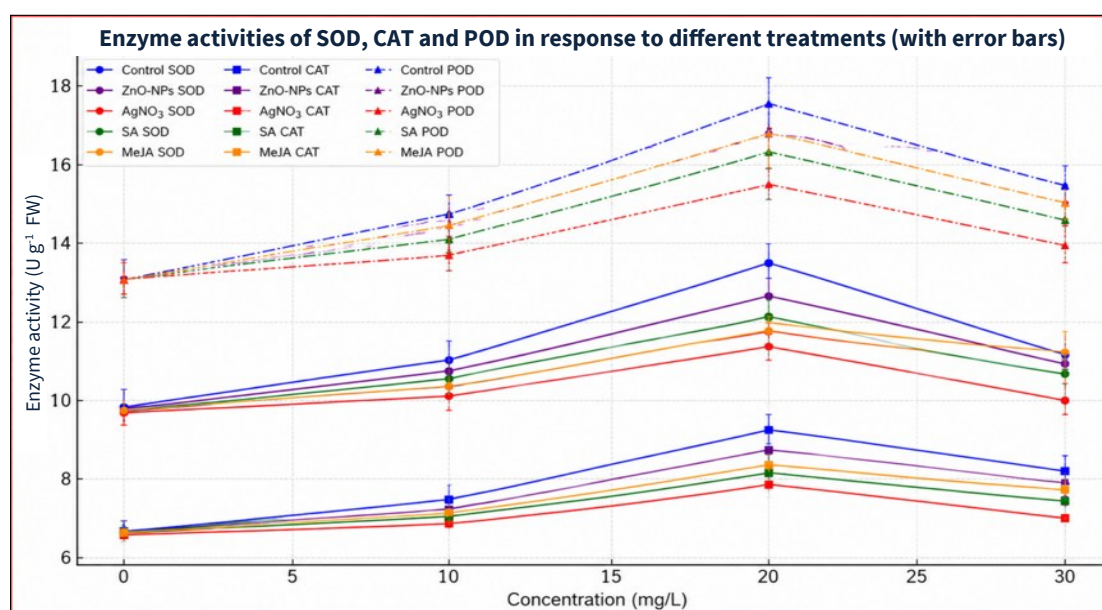


Fig. 9. Enzyme activities (SOD, CAT and POD) at different concentrations and treatments. The plot clearly shows the trends for each enzyme across the 10, 20 and 30 mg L⁻¹ concentrations, highlighting the peak activity at 20 mg L⁻¹ for all three enzymes and the decreased activity at higher concentrations. Error bars represent mean $\pm$ standard error (SE) (n = 24).

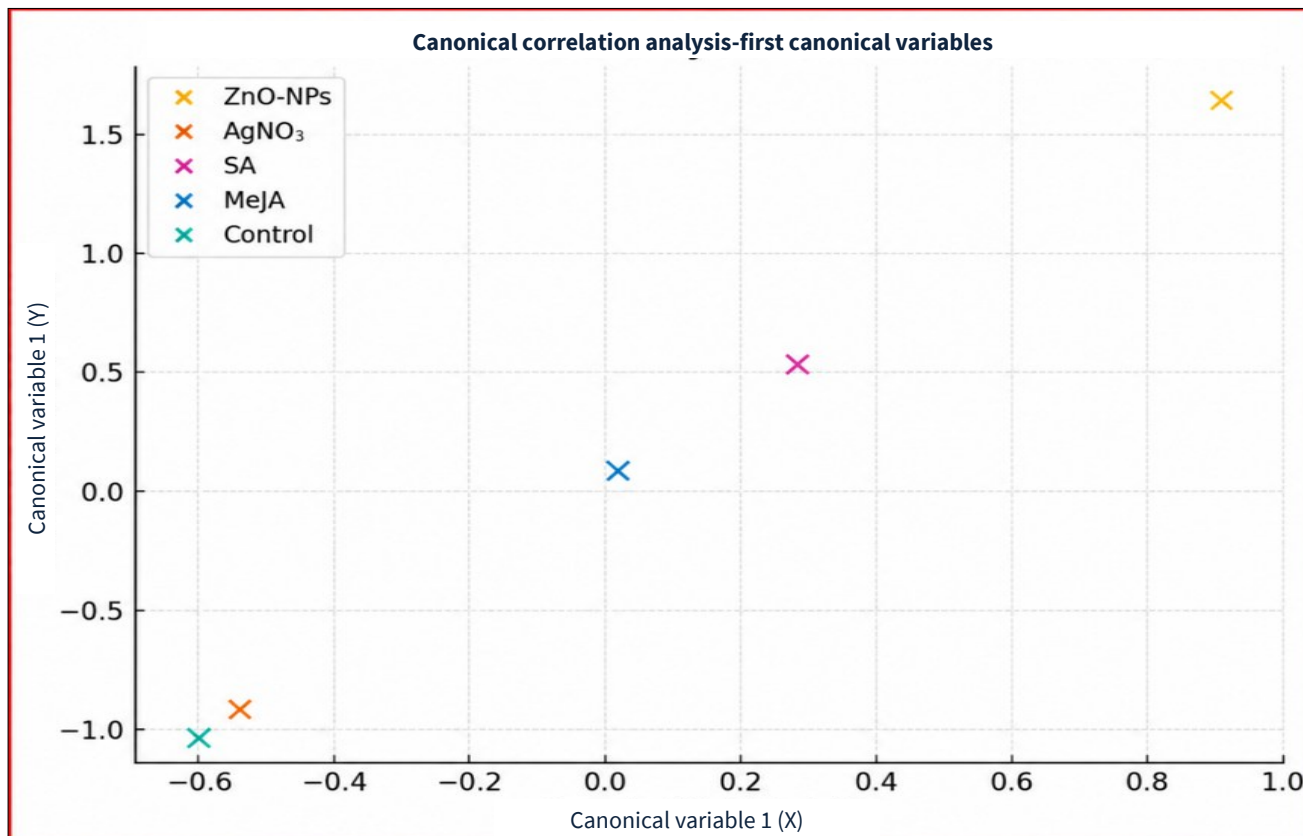


Fig. 10. Canonical correlation analysis (CCA) biplot illustrating the association between treatments and the first pair of canonical variables derived from physiological and biochemical response data in *Brassica oleracea*. Each point represents the canonical scores for one treatment group, plotted on the first canonical variate pair (X and Y).

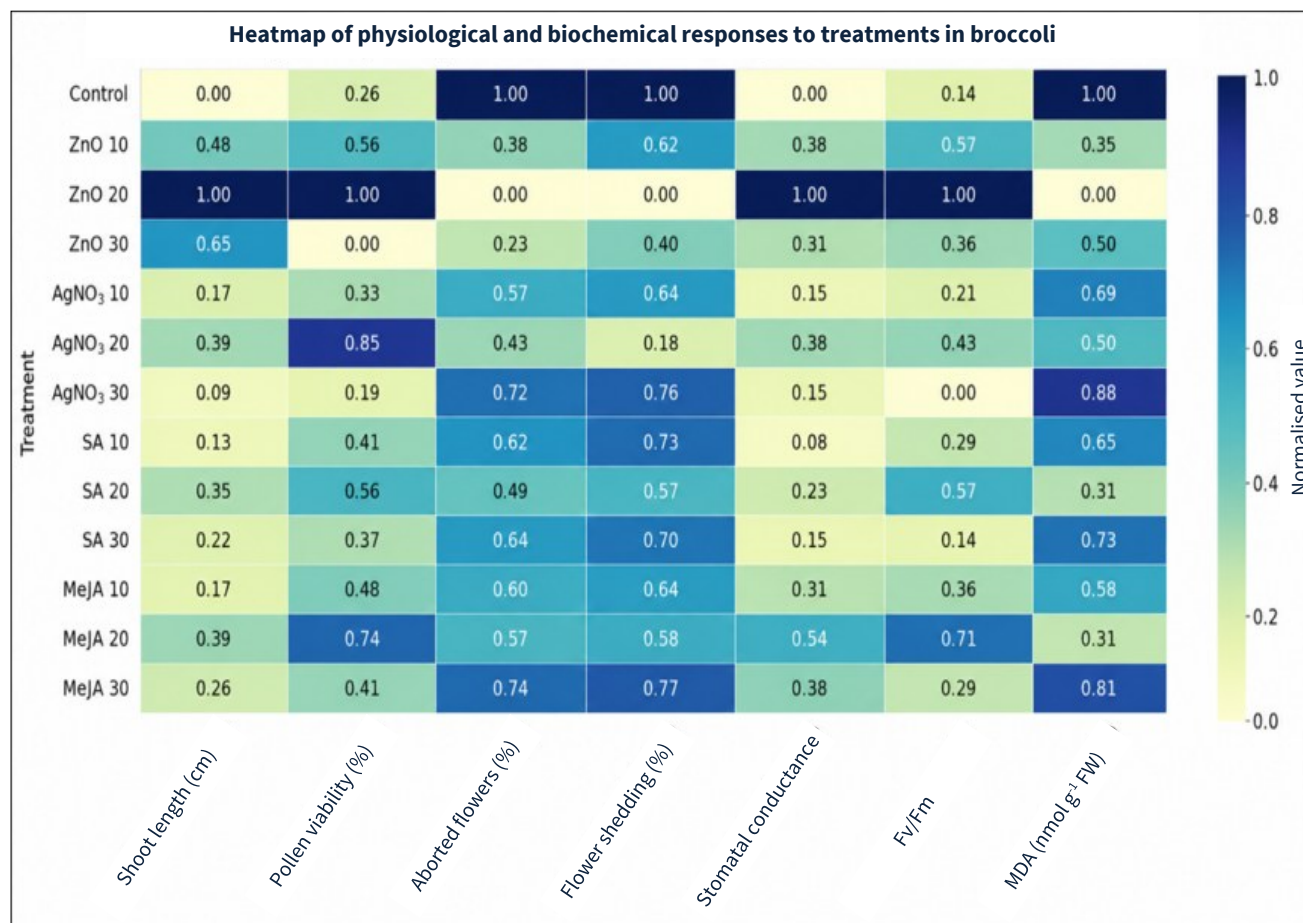


Fig. 11. Heatmap displaying normalised physiological and biochemical responses of broccoli to various treatments, including ZnO-NPs, AgNO₃, SA and MeJA at 10, 20 and 30 mg L⁻¹ concentrations. Darker colours represent higher normalised values. Parameters include shoot length, pollen viability, flower abortion and shedding, photosynthetic efficiency (Fv/Fm) and lipid peroxidation (MDA content).

Radar plot and principal component analysis

Principal component analysis (PCA) further explained 91.2 % of total variance (first principal component (PC1) = 83.1 %, second principal component (PC2) = 8.1 %). Zinc oxide nanoparticles (20 mg L⁻¹) contributed most positively to PC1, correlating with improved physiological and reproductive attributes. Control and AgNO₃ treatments clustered negatively, indicating weaker overall performance (Fig. 12).

The enhanced PCA biplot illustrates the multivariate relationships among physiological and biochemical parameters in broccoli plants under different treatments, highlighting the variability and grouping patterns (Fig. 13). The first principal component (PC1) explains 83.1 % of the total variance, while PC2 contributes an additional 8.1 %, together accounting for over 91 % of the datasets total variability. Red vectors represent the loadings of individual response traits. Shoot length, stomatal conductance,

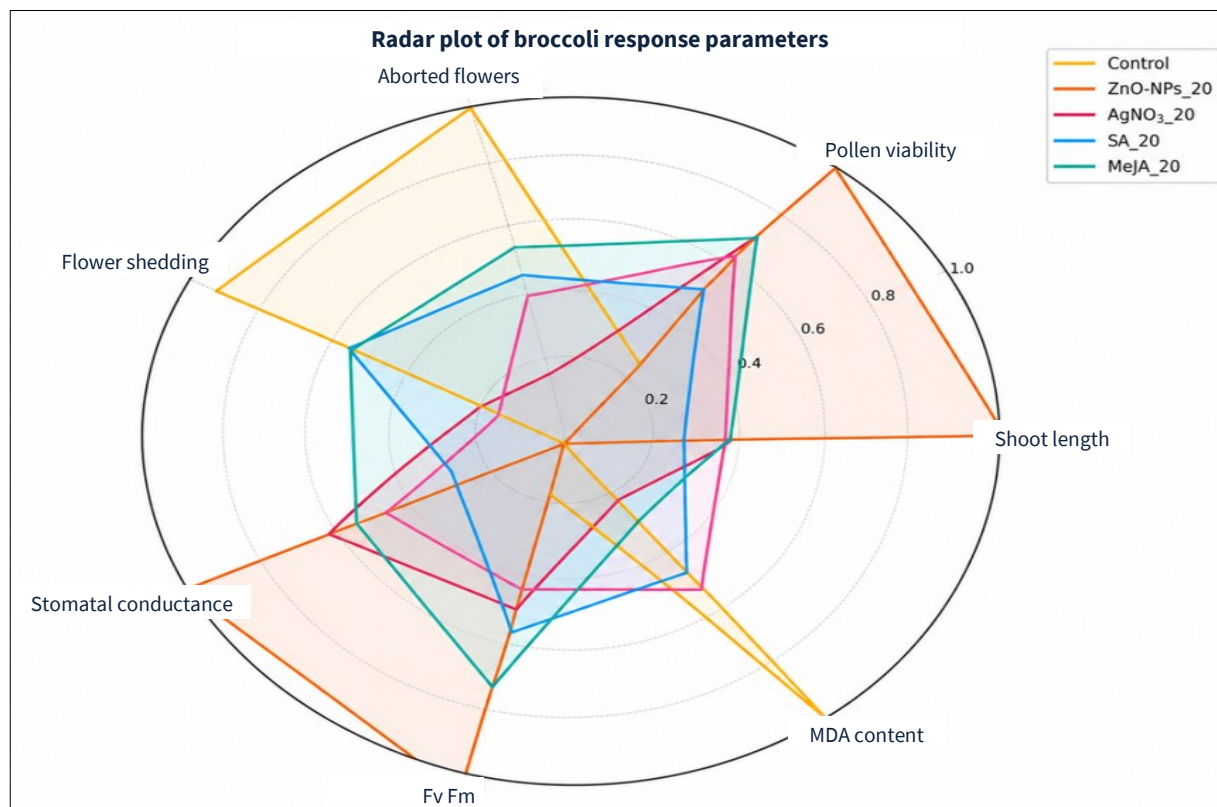


Fig. 12. Radar plot visualisation confirmed that ZnO-NPs (20 mg L⁻¹) exhibited the most balanced and superior profile across all seven response parameters, followed by MeJA and SA.

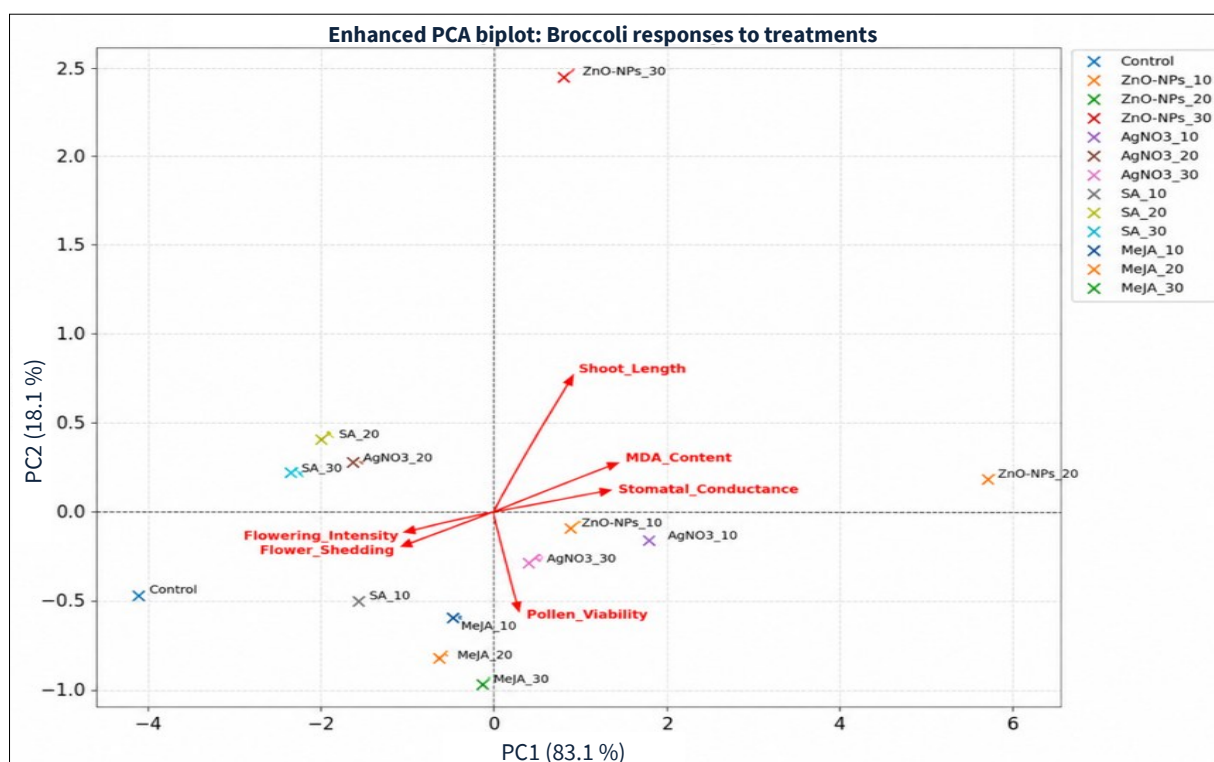


Fig. 13. Principal component analysis biplot visualising the distribution of broccoli responses to different treatments based on PCA (PC1 and PC2, explaining 91.2 % of total variance).

Fv/Fm, pollen viability, MDA content, flowering percentage and flower duration showing their contribution and direction of influence across the treatments. The plot clearly indicates that ZnO-NPs at 20 mg mL⁻¹ lies farthest along the PC1 axis in the positive direction, demonstrating the strongest positive association with critical growth and physiological traits such as shoot length, stomatal conductance and Fv/Fm, thereby signifying this treatment as the most effective. Zinc oxide nanoparticles at 30 mg mL⁻¹ is placed higher along PC2, suggesting some additional variance possibly due to stress-related responses. The control group clusters on the negative end of both axes, reflecting poor performance across most parameters. Other treatments like CuO-NPs 20 mg mL⁻¹, SA 20 mg mL⁻¹ and MeJA 20 mg mL⁻¹ show intermediate performance and cluster closer to the origin, indicating moderate influence on plant responses. Overall, this PCA biplot effectively discriminates treatments based on their impact on physiological and reproductive metrics in broccoli, reinforcing the superior efficacy of ZnO-NPs 20 ppm while revealing clear distinctions among other treatments.

Treatments are plotted alongside key physiological and biochemical traits represented as red vectors. The direction and length of vectors indicate the contribution and correlation of each trait to treatment variability.

Discussion

The present investigation demonstrates that ZnO-NPs, applied under controlled *in vitro* conditions, significantly improved reproductive development, pollen viability and physiological efficiency in *B. oleracea*. The observed improvements reflect the integration of NP induced modulation of hormonal signaling, antioxidant regulation and metabolic stability.

Enhanced reproductive and morphogenic response

Among all treatments, ZnO-NPs (20 mg L⁻¹) consistently produced the most favourable outcomes across reproductive parameters, including the highest pollen viability (92 %) and the lowest flower abortion (5.2 %). These effects align with earlier studies demonstrating that moderate ZnO-NP concentrations improve cell division and organogenesis in plants by promoting auxin synthesis and meristematic activity (16). Zinc acts as a structural and catalytic cofactor for enzymes involved in tryptophan-dependent auxin biosynthesis, explaining the observed enhancement in shoot elongation and floral organ development (17). At higher concentrations (30 mg L⁻¹), reduced performance suggests concentration-dependent toxicity, which observed that excessive NP accumulation can induce oxidative stress and inhibit growth (18). Thus, precise optimisation of NP dosage is essential for achieving beneficial rather than toxic outcomes.

Antioxidant defence and redox homeostasis

Improved antioxidant enzyme activities (SOD, CAT and POD) under ZnO-NPs indicate activation of a redox defence network that safeguards reproductive tissues from oxidative damage. Superoxide dismutase catalyses the conversion of superoxide radicals (O₂^{•-}) into hydrogen peroxide (H₂O₂), while CAT and POD further detoxify H₂O₂ into water and oxygen (12). The concurrent reduction in MDA content under ZnO-NPs (20 mg L⁻¹) confirms lower lipid peroxidation, consistent with the previous findings which reported that ZnO-NPs mitigate reactive oxygen species (ROS) accumulation by stabilising antioxidant enzyme systems (19).

This antioxidant strengthening likely underpins improved pollen viability and reduced flower abortion, as oxidative imbalance is known to impair microspore development and stigma receptivity (20). Hence, ZnO-NPs appear to enhance reproductive competence through reinforcement of redox homeostasis during floral differentiation.

Photosynthetic and physiological efficiency

The application of ZnO-NPs markedly improved photosynthetic efficiency and physiological performance in broccoli explants, as evidenced by the significant increase in stomatal conductance and the Fv/Fm ratio. These improvements indicate enhanced gas exchange and optimal photochemical energy conversion within the photosystems. Similar observations were reported, which demonstrated that ZnO-NPs stimulate stomatal aperture and increase chlorophyll biosynthesis, thereby facilitating higher carbon dioxide (CO₂) assimilation and electron transport efficiency in plant tissues (21). The increased Fv/Fm ratio in ZnO-NP-treated explants suggests improved integrity of PSII and a reduction in non-photochemical quenching losses. According to a previous study, Zn NPs can enhance photosynthetic pigment stability and improve light-harvesting capacity by stimulating the synthesis of chlorophyll-protein complexes (22). Furthermore, Zn plays a pivotal role in the structure and function of carbonic anhydrase and SOD enzymes, which regulate CO₂ fixation and protect chloroplasts from oxidative damage (23). The observed enhancement of these parameters in this study therefore reflects a coordinated physiological response aimed at maximising photosynthetic efficiency under NP influence. Enhanced PSII efficiency promotes the generation of assimilates that support floral organ differentiation, pollen maturation and ovule viability. The higher pollen viability and reduced floral abortion rates recorded in ZnO-NP treatments can thus be attributed to improved photosynthate translocation and energy availability for reproductive tissues. This aligns with the findings of an earlier study, which reported that nanonutrients enhance reproductive success through improved carbohydrate partitioning and hormonal balance (24). Moreover, the synergistic relationship between photosynthetic regulation and reproductive success observed here suggests that ZnO-NPs act as physiological enhancers even under non-stress, controlled culture conditions. By facilitating chloroplast stability, membrane integrity and redox balance, ZnO-NPs likely optimise both source (photosynthetic) and sink (reproductive) activities, thereby ensuring coordinated developmental progression. This integrative effect supports the view that low, optimised concentrations of ZnO-NPs can serve as efficient nanoelicitors for improving overall plant vigour and reproductive efficiency (25).

Pollen viability

The treatment with 20 mg L⁻¹ ZnO-NPs resulted in the highest pollen viability (92 %), which is significantly higher than that observed in the control group (72 %). This is an important finding, as pollen viability directly influences fertilisation success and overall reproductive success in plants. Previous studies have highlighted the positive effects of nanoparticles, including ZnO-NPs, on pollen germination and viability, likely due to their antioxidant properties and the modulation of cellular metabolic processes (26). The reduction in pollen viability at higher concentrations (30 mg L⁻¹) may indicate toxicity, which is consistent with the known dose-dependent nature of nanoparticle

toxicity (27). These results support the use of ZnO-NPs as an effective tool for improving pollen quality and reproductive outcomes in crops.

Aborted flower count and flower shedding

The reduction in aborted flowers (5.2 %) and flower shedding (12.5 %) in ZnO-NPs treated plants is a clear indication of improved floral development and flower retention under stress. This is particularly relevant in the context of plant reproductive health, where flower abortion and shedding are common responses to abiotic stress, such as drought or nutrient deficiencies. The reduction in flower abortion and shedding in ZnO-NPs treated plants is likely due to the enhanced physiological status, including improved water status, increased photosynthetic efficiency and reduced oxidative stress, all of which contribute to better flower development and retention (28, 29). Other treatments, such as MeJA and SA, also improved flower retention but to a lesser extent compared to ZnO-NPs, supporting the notion that NPs may offer more significant improvements in floral organogenesis compared to conventional elicitors.

Stomatal conductance and photosynthetic efficiency

The enhanced stomatal conductance ($0.38 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) and photosynthetic efficiency (Fv/Fm ratio of 0.84) in ZnO-NPs treated plants indicate better gas exchange and photosynthetic performance under controlled conditions. These findings align with previous studies that demonstrate the role of ZnO-NPs in promoting stomatal opening and facilitating better CO_2 uptake, leading to enhanced photosynthetic activity (30). The improvement in stomatal conductance and photosynthetic efficiency suggests that ZnO-NPs may promote more efficient water utilisation and overall physiological functioning in broccoli, critical factor for plant productivity, especially under stress conditions. In comparison, other treatments such as MeJA and SA also promoted stomatal conductance but were less effective than ZnO-NPs, highlighting the unique role of NPs in enhancing photosynthesis.

Lipid peroxidation and antioxidant enzyme activity

Lipid peroxidation, measured by MDA content, is a well-known indicator of oxidative stress. The lower MDA content in ZnO-NPs-treated plants ($4.2 \text{ nmol MDA g}^{-1} \text{ FW}$) compared to the control ($6.5 \text{ nmol MDA g}^{-1} \text{ FW}$) suggests a reduction in oxidative damage, likely due to the enhanced antioxidant defence mechanisms. This is further supported by the significantly higher activities of antioxidant enzymes (SOD, CAT and POD) in ZnO-NPs treated plants. These results are consistent with earlier findings, where ZnO-NPs were shown to activate plant antioxidant defence systems, thereby mitigating oxidative stress and improving plant stress tolerance (19, 31). The enhanced antioxidant activity could also contribute to the improved flower retention and pollen viability observed in this study.

Hormonal and redox regulation of nanoelicitor-induced reproductive development

The enhancement in reproductive traits under ZnO-NPs can be mechanistically linked to their interactions with plant hormonal pathways. Zinc participates in the activation of tryptophan aminotransferase and YUCCA-dependent enzymes in auxin biosynthesis, thereby promoting floral meristem initiation and pollen development (32). Concurrently, improved antioxidative protection likely stabilises ROS signaling, which acts as a secondary messenger in pollen tube growth and anther dehiscence. At supra-

optimal concentrations ($\geq 30 \text{ mg L}^{-1}$), ZnO-NPs may disrupt this delicate balance, generating excess ROS and interfering with hormonal homeostasis, leading to partial inhibition of reproductive processes. Beyond hormonal modulation, ZnO-NPs likely function as potent abiotic nanoelicitors that trigger a systemic signaling cascade. This process typically initiates with a transient 'oxidative burst' of ROS and calcium flux (Ca^{2+}) in the cytosol, which acts as a secondary messenger. These signals activate key transcription factor families, such as MYB, WRKY and bHLH, which are known to govern secondary metabolism in *Brassica* species (33). This genetic upregulation targets pivotal biosynthetic genes, including phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), reinforcing the phenylpropanoid pathway. Such molecular activation explains the observed enhancement in antioxidative balance and reproductive physiology, as these pathways produce flavonoids and phenolics that protect floral organs from oxidative attrition during development. This dual role underscores the fine threshold between beneficial and inhibitory nanoparticle effects.

Field-level applicability and environmental sustainability

From an applied perspective, the ability of ZnO-NPs to improve reproductive development under controlled conditions suggests potential utility in tissue culture and micropropagation protocols. However, scaling these findings to open-field applications requires consideration of environmental persistence, NP accumulation and biosafety. Studies indicate that ZnO-NPs degrade slowly in soil and may influence microbial activity (34). Therefore, controlled delivery systems and biodegradable NP formulations should be explored to minimise ecological impact. Economically, ZnO-NPs are relatively inexpensive compared to organic elicitors and small dosages ($10\text{--}20 \text{ mg L}^{-1}$) are sufficient to elicit strong physiological responses, making the approach feasible for commercial horticulture (35). Despite the documented benefits, the transition from *in vitro* success to *in vivo* application presents significant scalability and toxicity challenges. The high surface-to-volume ratio of ZnO-NPs increases their reactivity, which, at higher concentrations or under varying soil pH levels, can lead to phytotoxicity through chromosomal damage or the inhibition of nutrient uptake (36). Furthermore, scaling up to field conditions requires addressing the bioavailability of NPs in complex soil matrices where organic matter can cause aggregation, reducing efficacy. Long-term environmental effects, including the potential for NP leaching into groundwater and their impact on soil microbial diversity, remain critical areas that require rigorous validation before large-scale agricultural adoption.

Conclusion

This study highlights the beneficial positive role of zinc oxide nanoparticles (ZnO-NPs) in improving reproductive and physiological performance in broccoli (*Brassica oleracea*) under controlled *in vitro* conditions. An optimal concentration of 20 mg L^{-1} significantly enhanced key traits such as pollen viability, shoot growth and photosynthetic efficiency, while reducing oxidative stress and flower loss. These improvements are likely associated with better enhanced antioxidant activity and regulation of internal signaling pathways. Compared to conventional elicitors (SA and MeJA), ZnO-NPs showed greater effectiveness at moderate levels. However, further validation under field conditions and detailed molecular studies are needed before recommending their wider application.

Authors' contributions

MY contributed to the methodology, investigation, formal analysis, data curation, software development, validation, visualisation and preparation of the original draft manuscript. GC contributed to manuscript review and editing, validation and supervision. BKS was involved in data analysis, supervision and investigation. All authors read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

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During the preparation of this work the authors used Grammarly in order to improve grammar and for language editing. After using the tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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