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HORMETIC ROLE OF ZINC OXIDE (ZnO) NANOPARTICLES IN IMPROVING *IN VITRO* GROWTH AND DEVELOPMENT OF CAVENDISH BANANA (*MUSA* SP. CV. GRAND NAIN)

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ABSTRACT

This study investigates the hormetic effects of zinc oxide nanoparticles (ZnO NPs) on the micropropagation of Cavendish banana (*Musa sp.* cv. Grand Nain). *In vitro* cultures were established by supplementing Murashige and Skoog (MS) medium with four concentrations of ZnO NPs (0, 100, 200, 300, and 400 ppm) to evaluate their impact on key growth stages: initiation, multiplication, shooting, and rooting. A comprehensive assessment of morphological, physiological, and biochemical parameters was conducted to determine the optimal concentration for enhancing propagation efficiency. Results demonstrated a biphasic, dose-dependent hormetic response. The 300 ppm ZnO NPs treatment consistently yielded superior outcomes across all growth stages. Morphologically, this concentration significantly reduced the time to bud break and root initiation while enhancing shoot proliferation, number of shoots per explant, increased shoot and root length, and higher total biomass accumulation compared to the control and lower doses. Physiologically, plantlets treated with 300 ppm exhibited significantly higher chlorophyll content, relative water content, and fresh and dry weights of shoots and roots, indicating improved photosynthetic capacity and hydration status. Biochemically, the hormetic effect was evident in the antioxidant defense system as 300 ppm treatment induced the highest activity of key antioxidant enzymes, superoxide dismutase and peroxidase, alongside a significant increase in total soluble protein content and overall antioxidant potential. Conversely, a reduction in total phenolic content at this optimal dose suggested a strategic reallocation of metabolic resources from secondary defense to primary growth processes. The study concludes that 300 ppm ZnO NPs represent the optimal hormetic dose, effectively priming plant defenses and stimulating robust growth. This nano-elicitation strategy significantly enhances the efficiency and quality of Grand Nain banana micropropagation, offering a promising tool for commercial production.

Keywords **:** *Musa*, Hormesis, Micropropagation, Nanoparticle, Zinc Oxide.

Introduction

Banana (*Musa* sp.) is one of the most important fruit crops worldwide, providing essential nutrition and income for millions in tropical and subtropical areas. Nevertheless, banana farming encounters major obstacles, such as vulnerability to various abiotic and biotic stresses, postharvest losses (30-40%), and traditional propagation challenges that restrict the swift production of disease-free planting materials (Singh *et al*., 2025). The limitations of conventional propagation methods, such as their slow multiplication rate and the risk of pathogen transmission, have resulted in the increased use of tissue culture techniques for the large-scale production of consistent, disease-free banana plantlets. Nevertheless, micropropagation systems

encounter challenges, particularly inadequate growth conditions and microbial contamination that may hinder efficiency and scalability (Permadi *et al*., 2023).

The rise of nanotechnology has opened new frontiers in agricultural science, offering innovative solutions to persistent challenges in crop production and protection. Among various nanomaterials, ZnO NPs have attracted significant attention due to antimicrobial activity, nutrient delivery capabilities, and ability to modulate physiological pathways in plants at very low concentrations (Nan *et al*., 2024; Mohamad *et al*., 2019). These nanoparticles exhibit a remarkable phenomenon known as hormesis – a dose-response relationship characterized by low-dose stimulation and high-dose inhibition, which positions them as particularly valuable tools for enhancing plant growth and development while suppressing pathogens (Gunasena *et al*., 2024).

Zinc is a critical cofactor for enzymes involved in auxin synthesis, protein formation, and carbohydrate metabolism. The nanoparticle form of zinc enhances bioavailability and reactivity compared to traditional zinc salts, potentially leading to more efficient uptake and action (Hanif *et al*., 2024). Preliminary studies suggest that ZnO NPs can profoundly influence key stages of *in vitro* culture from initiation and multiplication to shooting and rooting by modulating oxidative stress, enhancing antioxidant defense systems, altering gene expression, and improving nutrient uptake (Jan *et al*., 2025). However, these effects are profoundly concentration-dependent, with optimal levels promoting growth while excessive amounts can induce phytotoxicity.

Another significant mechanism involves the modulation of oxidative stress levels within plant tissues. ZnO NPs can induce the production of reactive oxygen species (ROS) in plant cells, which at moderate levels acts as signaling molecules that trigger defense responses and enhance antioxidant systems (Zaeem *et al*., 2020). This elicitation effect leads to increased production of antioxidant enzymes (such as superoxide dismutase, catalase, and ascorbate peroxidase) and non-enzymatic antioxidants (including phenolics, flavonoids, and carotenoids), which collectively enhance the plant's stress tolerance and may contribute to improved growth and development under in vitro conditions (Zaeem *et al*., 2020; Salih *et al*., 2021). However, excessive ROS production beyond the plant's capacity to neutralize can lead to oxidative damage, explaining the biphasic response observed with ZnO NP treatments. Moreover, ZnO NPs also play an important role in phytohormonal crosstalk, especially the auxin–cytokinin balance, which determines morphogenic outcomes such as organogenesis (Krzepiłko *et al*., 2024; Ghareb *et al*., 2025). This balance is critical for directing *in vitro* development toward specific pathways like somatic embryogenesis or adventitious organ formation. ZnO NPs also interact with stress-responsive hormones like abscisic acid (ABA) and ethylene, potentially enhancing stress tolerance while modulating growth under in vitro conditions (Tripathi *et al*., 2022).

Despite the contemporary research on the applications of nanoparticles in agriculture, significant knowledge gaps exist regarding the specific use of ZnO NPs in banana micropropagation. Present investigation was carried out to gain insight into the concentration thresholds that separate positive from negative impacts, particularly in relation to micropropagation of banana and culture conditions. This research work aims to address this knowledge gap by systematic investigation of the hormetic effects of ZnO NPs on micropropagation efficiency, biochemical attributes and growth of banana under in vitro condition, which potentially establishes a new paradigm for nanoparticle-assisted tissue culture protocols.

Materials and Methods

Procurement of Plant Material and Explant Preparation

The sword suckers were obtained from healthy-looking, disease-free, 14-month-old field-grown banana (cv. Grand Nain) from farmer's field of Asarawalkala village of Prayagraj district of Uttar Pradesh, India. Suckers were thoroughly washed in tap water, roots and leaf sheaths were removed, and basal portion of the corm was excised and trimmed to a size of 15 × 15 × 20 mm. The explants were kept under running tap water for 60 minutes, then soaked in Tween-20 for 30 minutes with constant shaking and were shaken continuously. Further explants were then washed with distilled water to remove the detergent particles. The explants were treated with bavistin (2%) for 30 min followed by distilled water wash. Explants were transferred to laminar-airflow chamber" for further sterilization process. Inside l laminar-airflow chamber, explants were treated with 70% ethanol for 2 min, followed by washing with sterile distilled water for three times. After that the explants were treated with 0.1% $HgCl_2$ for 5 min followed by three 5-minute rinses each with sterile distilled water.

Preparation of ZnONPs suspension

ZnO NPs with particle size >30 nm and <50 nm were purchased from Sigma Aldrich, USA. The details of the properties of ZnONPs supplied by the manufacturer are shown in Table 1.

Table 1 : Properties and characteristics of the ZnONPs nanoparticles

Properties	Specification
Purity	99.0(%)
Average particle size	<30 nm
Morphology/ Shape	Spherical
Color	Milky white
Specific Surface Area	70 m^2/g
Crystal Phase	Single crystal
True Density	5.5 g/cm^3

The ZnO NPs powder was mixed with deionized water (2.5 mg/ml) and the solution was sonicated at an ultrasonic water bath (collagen) for 30 min at 35°C to disperse the nanoparticles properly. To prepare four concentrations of ZnO NPs (100, 200, 300, and 400 ppm), the pre-calculated amount of ZnONPs was added to the deionized water. For adequate dispersion of nanoparticles, the prepared suspensions were sonicated in an ultrasonic water bath for 30 min at 35°C.

Medium and culture condition

Murashige and Skoog (MS) medium was used as the basal medium and 3% (w/v) Sucrose was supplemented into the medium. The optimized concentrations of phytohormones for different growth stages of banana at the Centre for Tissue Culture Technology, SHUATS, Prayagraj, India were used in this study. Different concentration of phytohormones for initiation (BAP-5.0 mg/l; IAA-1.0 mg/l), multiplication (BAP-5.0 mg/l; Kinetin-1.0 mg/l), shooting (BAP-5.0 mg/l) and rooting (IAA-1.0 mg/l) were used in MS basal medium. Further f four different concentrations (100, 200, 300, and 400 ppm) were added to the medium prior to the pH adjustment. The pH of the medium was adjusted to 5.8 with 0.1 M HCl or 0.1 M NaOH followed by addition of 0.8% (w/v) agar. A total of 30 ml of medium was poured into sterilized bottles and allowed for solidification. The culture bottles were autoclaved at 121ºC, 15 psi for 15 minutes and cooled before inoculation. The sterilized explants derived from suckers were cultured on MS medium containing the appropriate hormonal conditions according to the desired growth condition and the cultured bottles kept in growth room at 25±2ºC under dark for one week, then transferred to a 16 hr light:8 hr dark photoperiod and dark cycle. Later, cultures were subcultured at 2–3 week intervals for up to six cycles for multiplication using a modified media composition that contains 3.0 mg/l BAP, 25.0mg of adenine sulphate and 30.0 mg/l ascorbate. Further, newly formed shoots were excised individually and transferred to rooting media consisting of half-strength MS salts and IBA (1.0 mg L^{-1}) with activated charcoal. The observations on rooting behaviour of *in vitro* regenerated shoots were made after 4 weeks of culture. Completely randomized block design (CRD) was used for the analysis of data.

Morphological Assessment

Observation on morphological parameters in all the four developmental stages (Initiation, multiplication, shoots induction and rooting) were manually recorded after 3 weeks of first sub-culture. The morphological parameters like number of days to respond for initiation, and total biomass were recorded. Under multiplication stage, number of days to multiplication, and number of multiplied shoots. Moreover, under shoot development stage shoot length, and number of leaves were recorded. Simultaneously to investigate the root developmental root days to root initiation, root fresh weight, root dry weight, root length, and number of roots were recorded. Simultaneously after successful root establishment fresh and dry weights of saplings were measured to study impact of ZnO NPs in banana micro-propagation under *in vitro* condition. Number of days for initiation, number of leaves, and number of roots were manually recorded. To measure total biomass, the cultures were taken out from culture bottles and rinsed with sterilized distilled water to remove surface adhered gelling agent followed by wipe off the residual water with tissue paper and weighed.

Physiological Assessment

The physiological assessments including total fresh biomass, total dry biomass and relative water content (RWC) were measured in all the four developmental stages. To determine RWC plant tissues were collected and wiped with tissue paper and fresh weight was recorded separately by using a precise balance. Further the samples were separately immersed in the distilled water until weight plateau was observed. After that samples were taken out and blot dried with tissue paper to remove excess water and turgid weight (TW) was measured. To measure dry weight (DW) samples were dried in an oven at 85°C for 24 h and allow them to cool in a desiccator and weighed. RWC was calculated by using the formula given below:

$$RWC(\%) = \frac{FW - DW}{TW - DW} \times 100$$

During the shooting stage, total chlorophyll content was also measured according to the method described by Arnon *et al.* (1976). To measure total chlorophyll content 1.0 g of leaf tissue from all treatments was collected and ground to a fine pulp with 20 ml of 80% acetone using a mortar and pestle. The homogenate was centrifuged at 5,000 rpm for 5 minutes and supernatant was transferred to a 100 ml volumetric flask. The absorbance of the solution was recorded at 645 nm and 663 nm, by using 80% acetone as the blank. The total chlorophyll was calculated as

$$\text{Total Chlorophyll} = \frac{20.2\,XA_{645} + 8.02XA_{663}}{1000 \times W} \times V$$

Biochemical Assessment

The biochemical assessment was carried out in terms of total antioxidant capacity, total phenolic content, soluble protein, enzyme activity of peroxidase (POX), and superoxide dismutase (SOD), in all the four developmental stages. Effect of ZnONPs on the total antioxidant capacity of in vitro grown banana plants was carried out by the method described by Brand-Williams *et al.* (1995). To measure total antioxidant capacity 1.0g of fresh leaves tissue from each treatment were ground in liquid nitrogen and 10.0 mL of 80% methanol was added to the powder in a 50 ml centrifuge tube followed by vortex and shake for 30-60 min at room temperature in the dark. The sample mixture was centrifuged at the >10,000 × g for 15 minutes and supernatant was collected. 4.5ml of freshly prepared solution of DPPH (0.1mM) was added to 500 µl of supernatant mixed through vortexing and incubate the reaction in the dark at room temperature for 30 min. After incubation, the absorbance at 517 nm was measured against a blank of pure methanol and ascorbic acid as standard. Calculation of % DPPH radical scavenging activity was done according to formula given below:

DPPH Radical Scavenging Activity (%)

$$= [(A_{Control} - A_{Sample}) / A_{Control}] \text{ X } 100$$

Final results were expressed in µmol ascorbic acid equivalents (AA) per gram of plant fresh weight (µmol AA /g FW).

Total phenolic content (TPC) was estimated according to Vinothani *et al.* (2017). The leaves and root tissue of all the treatments were separately ground in 2 ml of pre-chilled solution of acetone: water (1:1, v/v). Further 100 µl of extract was mixed with 1 ml of Folin Ciocalteu (FC) reagent and incubate at room temperature for 5 min. Thereafter, 1.0 ml of Na_2CO_3 solution (7.5 % w/v) was added to the extract and mixed thoroughly and kept at room temperature for 10 min. The absorbance of solution was measured at 760 nm. The TPC was calculated by preparing a standard plot of gallic acid and expressed as mg/g gallic acid equivalent (GAE).

To measure total water-soluble protein 1.0 gm of fresh shoot tissue of banana was boiled in 10 ml distilled water for two hours. The mixture was cooled and centrifuged at 15000g for 10 min at 4ºC. The supernatants were collected and transferred into clean tube. Water-soluble protein was determined as described by Lowery *et al.* (1951).

To determine the activities of peroxidase (POX) and superoxide dismutase (SOD), 1.0g plant tissue was homogenized in 50mM phosphate buffer (pH=7) and 1% (w/v) polyvinylpyrrolidone. Homogenized mixtures were centrifuged at 15000g for 10 min at 4°C and supernatant was collected, which was further used to determine POX activity according to MacAdam *et al.* (1992) and SOD activity according to the method described by Kwon *et al.* (2002).

Results

Effect of ZnONPs on *in vitro* initiation of Banana

In the present investigation, the application of ZnO NPs at varying concentrations exerted a significant influence on the growth parameters of the *in vitro* cultures of banana. The data particularly showed a clear concentration-dependent response to ZnO NPs in morphology, physiology, and biochemistry (Fig. 1a-e, Table 2). The MS medium supplemented with 300 ppm ZnO NPs found highly responsive among all the treatments as initiation was evident within 13 days after inoculation which was 7 days earlier than control explants (Fig. 1a-b). However, at 400 ppm of ZnO NPs, it was increased to 22 days, and it was 3 days higher than that of control group (Fig. 1c). Under 100 ppm and 200 ppm ZnO NPs, initiation occurred after 17 and 15 days, respectively of inoculation. Total biomass of the cultures was significantly affected by ZnO NPs supplementation in the initiation medium ($p < 0.05$). The highest biomass was observed in the cultures grown in medium supplemented with 300 ppm ZnO NPs (Fig. 1d). This value was significantly higher than that of the control group (0 ppm) and all other treatments. Cultures with 100 ppm and 200 ppm of ZnO NPs showed a moderate increase in biomass in contrast with the control. Conversely, increment in biomass was lowest at 400 ppm ZnO NPs, which indicated a potential stress-induced response at

elevated ZnO NP levels. A similar trend was also recorded in terms of the RWC (%). The RWC was markedly influenced by ZnO NPs treatments. RWC values were not significantly different among the control, 100 ppm, 200 ppm, and 300 ppm of ZnO treatments. NPs treatment and it was recorded above 91%. However, a toxic effect was observed in the culture initiated in MS medium supplemented with 400 ppm ZnO NPs, as RWC was recorded 46% (Fig. 1e).

The application of ZnO NPs elicited significant and concentration-dependent alterations in the biochemical profile of the initiation cultures. The application of ZnO NPs elicited a significant ($p < 0.05$) and concentration-dependent modulation in the activity of key antioxidant enzymes, peroxidase (POX) and superoxide dismutase (SOD) in 21-day-old *in vitro* banana cultures under initiation stage. Cultures cultivated on MS medium supplemented with 300 ppm ZnO NPs exhibited the highest specific activity for both POD and SOD (Table 2). The POX activity at this concentration was 0.34 U/mg protein, which was 61.9%, 41.6%, and 21.4% higher than that of control, 100 ppm and 200 ppm treatment, respectively. Similarly, SOD activity peaked at 300 ppm, reaching 67.0 U/mg protein, a value 63.4%, 42.5% and 28.8% higher than the control group, 100 ppm and 200 ppm treatment, respectively. Notably, the enhancement of antioxidant enzyme activity was not sustained at the highest tested concentration of ZnO NP. The 400 ppm ZnO NP treatment resulted in a substantial decline in the activity of both enzymes. The POX and SOD activities at 400 ppm were declined by 33.3%, and 53.65% from the control treatment. The activity of these two enzymes was also significantly lower than those recorded at the 300ppm level. This indicates a shift from an elicitation response at 300 ppm to a potential stress effect at 400 ppm.

The application of ZnO NPs significantly influenced the antioxidant capacity of 21-day-old banana cultures under initiation stage, as assessed by the DPPH radical scavenging assay. A clear concentration-dependent effect was observed, with the optimal response distinct from both the control and other treatment levels. Cultures grown on MS medium supplemented with 300 ppm ZnO NPs exhibited the highest antioxidant activity, and significantly outperformed all other treatments ($p < 0.05$) (Table 2). The antioxidant capacity in the 300 ppm ZnO NPs treatment was measured as 83.3 µM AA/g FW, which was 34.0 % higher than that of the control group. The lower concentrations of ZnO NPs also induced a positive impact; however the values were lower than that of 300 ppm. The antioxidant capacity in cultures treated with 100 ppm and 200 ppm was recorded as significantly greater than the control but remained statistically lower than the peak value achieved at 300 ppm. The antioxidant capacity in the 400 ppm treatment declined to 21.0 µM AA/g FW. This suggests a potential toxicological threshold beyond which the NPs may induce excessive stress, impairing the plant's antioxidant response system.

The outcome of the present study revealed that 300 ppm ZnO NPs enhanced protein yield by 41.3% compared to the control (Table 2). Simultaneously at 400 ppm, accumulation of soluble protein was lowest among all the treatment, which was 39.4% lower than that of control. In contrast to the other parameters measured, the total phenolic content displayed a different response pattern: the 100 ppm and 200 ppm treatments showed a moderate decrease in TPC compared to the control, and the 300 ppm ZnO NPs resulted in the greatest reduction. The phenolic content at 300 ppm was 48.1% lower than that of the control (Table 2). However, at 400 ppm of ZnO NPs the phenolic content was increased by 62.9% as compared to the control.

Table 2 : Impact of different concentration of ZnO NPs on the Biochemical parameters of *in vitro* grown banana cultures in initiation stage

	Initiation				
	Control	**100 PPM**	**200 PPM**	**300 PPM**	**400 PPM**
POX (U/mg Protein)	0.21±0.014	0.24±0.012	0.28±0.013	0.34±0.011	0.11±0.02
SOD (U/mg Protein)	41.0±2.0	45.0±2.2	52.4±2.4	64.2±3.0	19.1±1.2
TAP (µmol AA /g FW)	53.2±2.7	66.5±3.0	69.3±3.4	78.2±3.8	21.4±2.3
SP (mg/g FW)	10.4±0.41	11.9±1.4	12.6±0.7	14.7±0.8	6.3±0.28
TPC (mg/g GAE)	2.7±0.11	2.1±0.14	2.0±0.01	1.4±0.05	4.4±0.017
	Control	**100 PPM**	**200 PPM**	**300 PPM**	**400 PPM**

POX: Peroxidase; SOD: Superoxide dismutase; TAP: Total antioxidant Potential; SP: Soluble protein; TPC: Total Phenolic Content.

Effect of ZnONPs on *in vitro* multiplication of Banana

The concentration of ZnO NPs in the culture medium significantly ($p < 0.05$) governs the multiplication in the *in vitro* banana cultures. The cultures established on multiplication medium supplemented with 300 ppm ZnO NPs exhibited highest performance**,** demonstrating the most rapid and prolific shoot multiplication response (Fig. 2a-b). This treatment resulted in the shortest duration to the onset of multiplication**,** with visible shoot primordia emerging within 13 days post inoculation. This was significantly earlier than all other treatments as under control treatments the onset of multiplication was recorded after 18 days after inoculation (Fig. 2c). Simultaneously after 21 days of sub-culture, the same 300 ppm treatment yielded the highest number of shoots per explant (Fig. 2b and d). An average of 5.6 shoots per explant was recorded, which was 75% higher than the control group and significantly greater ($p < 0.05$) than the shoots produced at 100 ppm and 200 ppm. However, the 400 ppm ZnO NPs treatment showed inhibitory action on multiplication onset and resulted in a delayed onset of multiplication, and the lowest shoot count. The concentration of ZnO NPs in the culture medium also influenced on the accumulation of phenolic content in *in vitro* banana shoots. The 300 ppm ZnO NPs treatment, which previously demonstrated the highest multiplication rate, exhibited the lowest level of total phenolic content (Fig. 2e).

The biochemical response of *in vitro*-grown banana cultures in multiplication stage to ZnO NPs was highly concentration-dependent. A beneficial effect was observed at an intermediate concentration, while a supra-optimal level induced significant metabolic stress. The activities of key antioxidant enzymes, peroxidase (POX) and superoxide dismutase (SOD), were significantly ($p < 0.05$) modulated by ZnO NP treatments (Table 3). POX activity increased in a dose-dependent manner up to 300 ppm ZnO NPs. The highest POX activity (0.36 ± 0.012 U/g FW) was recorded at 300 ppm, which was 56.2% higher than the control (0.23 ± 0.017U/g FW). This value was also significantly greater than activities at 100 ppm (0.26 ± 0.011 U/g FW) and 200 ppm (0.28 ± 0.013 U/g FW). Simultaneously a similar trend was also observed for SOD activity. The 300 ppm treatment resulted in highest SOD activity (71.0 ± 2.8 U/g FW), representing a 57.7% increase over the control value (45.0 ± 2.2 U/g FW). This activity was significantly higher than all other treatments (Table 3). In contrast, the application of 400 ppm ZnO NPs caused a sharp decline in the activities of both enzymes. POX and SOD activities at this concentration were recorded as 0.17 ± 0.01 and 20.0 ± 1.0 U/g FW protein, respectively. These values were significantly lower than those of the control and all other treatments (Table 3). The total antioxidant potential, as measured, followed the enzymatic data (Table 3). The antioxidant capacity was highest at 300 ppm ZnO NPs (77.2 ± 3.2 µmol AA/g FW), a value significantly higher than that of control (62.5 ± 2.8 µmol/g FW), 100 ppm, and 200 ppm treatments. The 400 ppm treatment resulted in the lowest antioxidant potential (23.5 ± 1.5 µmolAA/g FW), significantly reduced compared to all other groups. The soluble protein content followed a similar response pattern (Table 3). The concentration was highest in cultures supplemented with 300 ppm ZnO NPs (15.2 ± 0.8 mg/g FW), which was significantly higher than the control (12.2 ± 0.6 mg/g FW) and the lower concentration treatments. A significant reduction in soluble protein content (5.4 ± 0.22 mg/g FW) was observed at the 400 ppm, indicating a potential impairment of protein synthesis or enhanced proteolysis under high-concentration stress. The concentration of total phenolic content at 300 ppm ZnO NPs was measured as 1.9 ± 0.02 mg/g GAE, which was significantly lower than all other treatments, including the control ($p < 0.05$). The 400 ppm ZnO NP treatment, showed severe toxicity and inhibited growth, recorded the highest accumulation of total phenolic content (4.7 ± 0.3 mg/g GAE). This value was significantly greater than those of the control, 100 ppm, 200 ppm, and 300 ppm treatments ($p < 0.05$).

Table 3 : Impact of different concentration of ZnO NPs on the Biochemical parameters of *in vitro* grown banana cultures in multiplication stage

	Multiplication				
	Control	**100 PPM**	**200 PPM**	**300 PPM**	**400 PPM**
POX (U/g FW)	0.23±0.017	0.26±0.011	0.3±0.010	0.36±0.012	0.17±0.01
SOD (U/mg Protein)	45.0±2.2	50±2.0	56±2.2	71±2.8	20±1.0
TAP (µmol AA /g FW)	62.5±2.8	68.3±4.0	70.4±3.3	77.2±3.2	23.5±1.5
SP (mg/g FW)	12.2±0.6	13.4±0.5	13.8±0.51	15.2±0.8	5.4±0.22
TPC (mg/g GAE)	3.2±0.12	2.5±0.12	2.2±0.14	1.9±0.02	4.7±0.3

Effect of ZnONPs on shoot attributes of banana grown under *in vitro* condition

The supplementation of the culture medium with ZnO NPs exerted a significant influence on all measured growth parameters of banana saplings under *in vitro* conditions. The morphological development of the micropropagated banana plants was significantly enhanced at moderate concentrations of ZnO NPs (Fig. 3 a, b). The treatment with 300 ppm ZnO NPs proved to be optimal, and showed maximum shoot length (11.7 ± 0.6 cm), highest number of leaves (5.8 ± 0.2), and chlorophyll content (1.73 ± 0.05), which were greater than those observed in the control and other treatments (Fig 3c-f). In comparison with control treatment the shoot length, number of leaves, and chlorophyll content in 300 ppm ZnO NPs were 46.2%, 50.0%, and 27.3% respectively higher. No significant difference was observed in the RWC in control, 100, 200 and 300 ppm treatments. In contrast, shoot length, number of leaves, relative water content, and chlorophyll content in the 400 ppm treatment were reduced by 7.2%, 30.0%, 8.7%, and 16.5%, respectively as compared to the control.

The biochemical profile of banana explants during shoot induction was significantly influenced by the supplementation of ZnO NPs to the culture medium. The concentration of 300 ppm consistently elicited the most positive effects on the measured parameters compared to both the control and lower concentrations of ZnO NPs. The activities of POX and SOD were markedly enhanced in cultures grown on medium containing 300 ppm ZnO NPs (Table 4). POX activity demonstrated a clear dose-response relationship up to the 300 ppm treatment. The highest enzymatic activity was observed in explants grown in the medium with 300 ppm ZnO NPs (0.47 ± 0.013 U/mg FW). This value was significantly greater ($p < 0.05$), representing a 95.8% increment as compared to the activity in the control group (0.24 ± 0.014 U/mg FW). Furthermore, POX activity at 300 ppm was significantly higher than activities recorded at both 100 ppm (0.27 ± 0.014 U/mg FW) and 200 ppm (0.47 ± 0.013 U/mg FW). However, In the culture medium with 400 ppm ZnO NPs, POX activity (0.2 ± 0.02 U/mg FW) was significantly lower than in all other treatments. Simultaneously a statistically identical trend was recorded for SOD activity. Explants cultivated on medium with 300 ppm ZnO NPs exhibited highest SOD activity (80.0 ± 4.0 U/mg FW). This activity level was significantly elevated, showing a 70.2% increase over the control value (47.0 ± 2.3 U/mg FW). The SOD activity at this concentration was also significantly higher to that measured at 100 ppm and 200 ppm ZnO NPs. However, at 400 ppm of ZnO NPs the activity of SOD was recorded as 23 ± 1.1 U/mg FW, which was significantly lowest among all the treatments (Table 4). The total antioxidant potential was highest in the 300 ppm ZnO NP treatment (78.6 ± 3.5 µmol AA/g FW) (Table 4). This antioxidant capacity was significantly higher ($p < 0.05$) than that of the control (65.4 ± 3.0 µmol AA /g FW) and significantly exceeded the values obtained for the 100 ppm and 200 ppm treatments. The soluble protein content followed a similar pattern of enhancement (Table 4). The maximum soluble protein concentration (18.6 ± 1.0 mg/g FW) was measured in explants from the 300 ppm ZnO NP treatment. This concentration was significantly greater than that of the control (6.0 ± 0.3 mg/g FW) and was also significantly higher than the values obtained at the 100 ppm and 200 ppm supplementation levels. However, at 400 ppm of ZnO NPs soluble protein concentration was decreased to 8.3 mg/g FW, which was significantly lower than that of 100, 200 and 300 ppm of ZnO NPs treatments.

Table 4 : Impact of different concentration of ZnO NPs on the Biochemical parameters of *in vitro* grown banana cultures in shooting stage

	Shooting				
	Control	**100 PPM**	**200 PPM**	**300 PPM**	**400 PPM**
POX (U/mg Protein)	0.24±0.014	0.27±0.014	0.32±0.012	0.47±0.013	0.2±0.02
SOD (U/mg Protein)	47.0±2.3	54.0±3.0	61.0±3.0	80.0±4.0	23±1.1
TAP (µmol AA /g FW)	65.4±3.0	71.6±3.2	74.3±3.6	78.6±3.5	26.3±1.2
TPC (mg/g GAE)	3.2±0.2	3.0±0.13	2.6±0.12	1.5±0.07	4.4±0.21
SP (mg/g FW)	6.0±0.3	17.2±0.8	17.7±0.9	18.6±1.0	8.3±0.4

Effect of ZnONPs on root attributes of banana grown under *in vitro* condition

The supplementation of ZnO NPs in the culture medium exerted a significant and concentration-dependent impact on all *in vitro* root development parameters of banana plantlets compared to the control (0 ppm ZnO NPs). The most pronounced positive effects were observed at a concentration of 300 ppm ZnO NPs. Plantlets cultured on medium supplemented with 300 ppm ZnO NPs exhibited a significant

enhancement in all measured root attributes (Table 5). Root length was maximized at this concentration, showing a 31.4 % increase over the control (Fig. 4a). Similarly, the number of roots per plantlet was highest in the 300 ppm treatment, being 60% greater than that of the control (Fig. 4b). This robust root development directly translated into a significant increase in biomass, with root fresh weight and root dry weight reaching their peak values at 300 ppm, significantly surpassing the weights recorded for the control, 100 ppm, and 200 ppm treatments (Fig. 4c). Furthermore, plantlets in the 300 ppm treatment maintained significantly higher RWC**,** indicating improved overall water status and reduced transplant stress potential. A critical finding was the effect of ZnO NPs on the speed of root initiation. The number of days to root initiation was significantly reduced in the 300 ppm treatment compared to all other concentrations, including the control (Fig. 4d). Root primordia emerged 5 days earlier than in the control, demonstrating that 300 ppm ZnO NPs not only improved the quality and quantity of roots but also accelerated the rooting process. Conversely, the effects at lower concentrations (100 and 200 ppm) were less pronounced. While these treatments often resulted in modest improvements in some parameters compared to the control, the differences were either statistically insignificant or markedly lower than the enhancements achieved with 300 ppm ZnO NPs. A prominent negative effect was observed at the highest concentration tested. The supplementation of 400 ppm ZnO NPs proved inhibitory and often toxic to root development. This treatment resulted in a significant reduction in root length, root number, and root biomass (both fresh and dry weight) compared to all other treatments, including the control. The RWC was also lowest at this concentration. Furthermore, root initiation was severely delayed in the 400 ppm treatment, with plantlets taking the longest time to develop visible roots. The influence of ZnO NP supplementation on the biochemical profile of *in vitro*-cultured banana plantlets was significant and concentration-dependent. The most favourable biochemical responses were elicited at a concentration of 300 ppm ZnO NPs. Plantlets grown on medium supplemented with 300 ppm ZnO NPs exhibited a marked up regulation in key antioxidant enzyme activities. Specifically, the activities of POX and SOD were significantly higher in this treatment group compared to the control and the 100 ppm and 200 ppm treatments (Table 5). This enhanced enzymatic activity corresponded with a significant increase in the total antioxidant potential of the plantlets, indicating a more robust capacity to mitigate oxidative stress. Concurrently, the soluble protein content was highest in the 300 ppm treatment. In contrast, the total phenolic content was significantly lower in plantlets treated with 300 ppm ZnO NPs compared to all other treatments, including the control. Treatments with lower concentrations of ZnO NPs (100 and 200 ppm) induced moderate changes in the measured parameters. While some increases in POX and SOD activity were noted compared to the control, these enhancements were consistently and significantly less pronounced than those achieved with the 300 ppm supplementation. Conversely, the application of 400 ppm ZnO NPs had a profoundly negative impacts on all assessed biochemical parameters. This high concentration resulted in a sharp decline in the activities of POX and SOD. The total antioxidant potential and soluble protein content were also significantly reduced to levels below those of the control group. In contrast to the trend observed at 300 ppm, the total phenolic content was highest in the 400 ppm treatment.

Table 5 : Impact of different concentration of ZnO NPs on the Biochemical parameters of *in vitro* grown banana cultures in shooting stage

	Rooting				
	Control	**100 PPM**	**200 PPM**	**300 PPM**	**400 PPM**
POX (U/mg Protein)	0.22±0.01	0.25±0.011	0.33±0.02	0.4±0.006	0.18±0.03
SOD (U/mg Protein)	44±2.0	52±2.4	59±3.4	69±3.5	22±1.6
TAP (µmol AA /g FW)	57.2±3.0	60.0±3.0	63.3±2.5	67.8±4.0	18.0±0.8
TPC (mg/g GAE)	4.7±0.22	4.0±0.2	3.8±0.81	3.1±0.74	5.4±0.22
SP (mg/g FW)	14.4±0.51	15.7±0.77	17.4±1.0	19.5±1.2	7.2±0.31

Effect of ZnONPs on the rooted shoots of banana grown under *in vitro* condition

The supplementation of ZnO NPs in the culture medium exerted a significant and concentration-dependent impact on the sapling development of banana compared to the control (0 ppm ZnO NPs). Gradual increment in the saplings fresh weight and dry weight was recorded upto 300 ppm of ZnONPs. A highest fresh weight and dry weight were recorded in the saplings developed in the medium supplemented with 300 PPM of ZnO NPs (Fig. 5a, b). However, a lowest fresh and dry weight were recorded in the

saplings grown under 400 ppm ZnO NPs treatment. A highest fresh weight and dry weight was recorded as 6.4g and 0.53g respectively in the 300 ppm ZNO NPs treatment, which was 25.5% and 12.8% higher than that of control treatment respectively (Fig. 5a, b). However, under 400 ppm of ZnO NPs the fresh and dry weight showed non-significant change as compared to the control treatment (Fig. 5a, b). However non-significant differences were observed among control and 100 ppm, 200 ppm and 300 ppm ZnO NPs treatments and it was recorded as more than 95% (fig. 5c). However, under 400 ppm ZnO NPs treatment the RWC content (80%) was recorded as 16.6% lower than control treatment

Discussion

In the present study, we investigated the hormetic effect of ZnO NPs on the direct organogenesis of Cavendish sub-group of banana (cv. Grand Nain). We investigated the impact of four different concentrations of ZnO NPs in four different developmental stages (initiation, multiplication, shooting and rooting) across different morphological parameters (days to response, shooting and rooting attributes, leaf number and total biomass accumulation), physiological parameters RWC, chlorophyll, and root/shoot fresh and dry mass), and redox biochemistry (POX and SOD activities, total soluble protein, antioxidant capacity and phenolics content). The hormetic response recorded in this study underscores the fundamental principle that the biological effect of a substance is entirely dependent on its concentration. The lower concentrations of ZnO NPs induced eustress on the different morphological, physiological and biochemical parameters in all the four *in vitro* developmental stage. The reduction in the number of days to initiation, with enhanced biomass and RWC observed in sucker explants, points accelerated metabolic and cellular processes which emphasized the transition from a quiescent explant to an actively growing culture. The observed phenomenon can be attributed to the multifaceted role of zinc, delivered in a nanoparticulate form, in stimulating key physiological and molecular pathways to break apical dominance and swift entry into the initiation phase. The primary mechanism behind the accelerated response could be attributed to the essential function of zinc (Zn^{2+}) such as a catalytic and structural cofactor for different enzymes, involved in the biosynthesis of auxins, particularly tryptophan synthase (Cakmak, 2000). ZnO NPs, potentially dissolve at the explant interface, and provide a highly bioavailable source of Zn^{2+} ions (Ma *et al.*, 2013). Swamy *et al.* (2016) reported an enhanced pool of endogenous IAA at the cut ends of the explant which could immediately stimulate cell division and organogenesis, led to the visibly faster bud break and shoot emergence. This bypasses the lag phase associated with explants mobilizing their limited internal zinc reserves or adapting to suboptimal zinc levels in the basal culture medium. Simultaneously Zn is a vital component to enhance energy metabolism and nutrient assimilation as it catalyzes the reversible hydration of carbon dioxide by helping carbonic anhydrase, an enzyme that play a critical role in photosynthesis and respiration (Hafeez *et al.*, 2013). The supplementation of Zno NPs in the culture medium might be associated with more efficient respiration, ATP generation for biosynthesis and active transport in incubated explants in all the four developmental satage. Furthermore, zinc is also a co-factor for nitrate reductase, which involved in nitrogen metabolism and protein synthesis (Alloway *et al.*, 2008). Activation of this enzyme might help the explants in efficient utilization of nitrogen present in the culture medium, which could result in utilization of the building blocks for proteins and nucleic acids necessary for the new growth. These rapid establishments of an efficient metabolic state could be a cause of shorter lag period and a visible response in fewer days. Rastogi *et al.* (2019) describe that due to the high surface area-to-volume ratio of ZnO NPs could facilitate stronger interactions with the cell wall and plasma membrane of the explant tissues and this interaction may act as an elicitor, which influence growth responses. The increment in shoot multiplication could be due to fundamental stimulation of meristematic activity and a shift in the physiological state of the *in vitro* cultures of banana. This effect is not merely a gross nutritional supplement but is likely a multi-faceted response orchestrated by the unique properties of nanoparticles interacting with plant cellular machinery. The axillary bud break and shoot proliferation under *in vitro* condition is the balance between cytokinins and auxins. Zinc is an essential cofactor for the enzyme tryptophan synthase, which is critical for the production of Indole-3-acetic acid (IAA). In the present study an optimal supply of Zn^{2+} ions, released from the dissolution of ZnO NPs, can enhance the biosynthesis of endogenous IAA up to a beneficial level. A specific, low level of endogenous auxin is known to promote bud break by overcoming apical dominance (Muller and Leyser' 2011). However, a high auxin-to-cytokinin ratio typically suppresses shoot formation. This study postulates that the ZnO NP-induced auxin signal is sufficient to initiate bud break and might create a physiological

environment where the exogenous cytokinin present in the multiplication medium becomes more effective in stimulating the outgrowth of those activated buds, leading to a greater number of shoots per explant. Simultaneously, Zinc is a vital component of numerous enzymes central to primary metabolism. It is a key cofactor for carbonic anhydrase, which facilitates CO_2 hydration and is crucial for photosynthesis and respiration (Hafeez *et al*., 2013). The bioavailability of zinc from NPs likely enhances the activity of these enzymes, leading to improved carbon assimilation and sugar metabolism, providing the necessary carbon skeletons for new growth, enhanced respiratory efficiency and increased rates of protein and nucleic acid synthesis to support rapid cell division. This boosted metabolic state provides the energetic foundation required to support the enhancement of shoot length, root length, number of leaves, and roots during shoot and root induction stage. Moreover, *in vitro* environment imposes significant abiotic stress on plant tissues. While high concentrations of NPs cause damaging oxidative stress (Javed *et al*., 2018); an optimal concentration can induce a beneficial, low-level oxidative challenge (Shehzad *et al*., 2021). A specific, low dose of ZnO NPs can trigger a mild, transient burst of Reactive Oxygen Species (ROS). This ROS burst does not cause damage but acts as a signaling molecule that pre-activates the plant's antioxidant defense system, including enzymes like SOD and POX (Maity *et al*., 2018; Agathokleous *et al*., 2019).

A significant increase in SOD and POX activities at concentrations below 300 ppm indicates a state of mild, non-damaging oxidative stress that effectively primes the plant's antioxidant defense system. ZnO NPs, even at low doses, are known to partially dissolve, releasing Zn^{2+} ions, and can also generate reactive oxygen species (ROS) on their surface or upon interaction with cellular components (Calabrese *et al*., 2017; Alscher *et al*., 2002). The concomitant rise in POX activity represents the secondary, complementary response which is responsible for scavenging the resulting H_2O_2, converting it into water and oxygen, thus preventing the formation of more harmful hydroxyl radicals via the Fenton reaction (Passardi *et al*., 2005). This coordinated enhancement of both SOD and POX activities suggests a successful and adaptive metabolic adjustment by the plantlet. As increased SOD and POX activities are biomarkers of this successful acclimation. At 300 ppm, the same nanoparticles induce distress, however a decline in enzymatic activity at 400 ppm ZnO NPs, falling below the levels of the control, signifies a breakdown of this adaptive mechanism and a transition into a state of chronic toxicity. This inhibition is not indicative of a lack of oxidative stress on the contrary, the stress level is likely severe but rather reflects a catastrophic failure of the detoxification system (Mittler, 2002; Broadley *et al*., 2007; Wang *et al.,* 2016). The TAP and TPC of the cultures in all growth stage were influenced by the ZnO NPs. The data indicates that a higher dose of ZnO NPs positively influences the production of phenolic compounds in cultures however antioxidant potential. Similar trends in the quantities of TPC in response to ZnO-NPs at varying concentrations in different cultures were observed in other important medicinal plant species such as *Thymus daenensis*, *Stevia rebaudiana Bertoni, Linum usitatissimum, and Silybum marianum* (Javed *et al*., 2018; Mosavat *et al*., 2019; Kazmi *et al*., 2019; Shehzad *et al*., 2021).

Simultaneously optimal concentration of ZnO NPs enhances the overall antioxidant potential while reducing the total phenolic content in *in vitro* plant cultures. As described earlier that optimum concentration of ZnO NPs enhances the activity of two key enzymes POX and SOD. Mittler (2002) reported that SOD is the first line of defense, convert superoxide radicals into hydrogen peroxide (H_2O_2). POD and CAT then rapidly detoxify H_2O_2 into water and oxygen. These enzymatic pathways are highly efficient and rapidly inducible. The measured antioxidant potential in assays captures the cumulative reducing capacity of the cell extract, which is highly boosted by the surge in activity of these highly active enzymes. The plant, in this primed state, effectively manages oxidative pressure with this enzymatic first response and it might be because enhanced antioxidant potential. Moreover, significant reduction in phenolic content could be explained by a shift in the plant's metabolic strategy under optimal, primed conditions (Naik and Al-Khayri, 2016). Under severe stress, the enzymatic system may be overwhelmed, which enable plants to synthesize a wide range of secondary metabolites, including phenolics, which often leads to a spike in phenolic content. However, at the optimal hormetic dose, the highly efficient enzymatic system is sufficient to manage the oxidative challenge and it controls the production of phenolic compounds.

Conclusion

This study conclusively demonstrates that ZnO NPs exert a pronounced hormetic effect on the *in vitro* micropropagation of *Musa sp.* cv. Grand Nain. The observed biphasic response across morphological, physiological, and biochemical parameters underscores

the critical importance of dosage in nanoparticle applications. The superior performance at 300 ppm is attributed to its role as a potent physiological primer. This concentration effectively induced a state of eustress, which stimulated the plant's growth and development without causing toxicity. The resultant upregulation of antioxidant enzymes enhanced the plantlets' ability to manage oxidative stress, thereby creating a more favorable cellular environment for growth. The findings of this research have substantial practical implications for commercial banana micropropagation. The application of 300 ppm ZnO NPs presents a viable, efficient strategy to shorten production cycles, increase multiplication rates, and improve the overall quality and hardiness of *in vitro* plants. This nano-elicitation technique could significantly reduce costs and improve output for tissue culture industries.

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Informed consent

The written informed consent obtained from all participants involved in this study. The consent process adhered to the journal's requirements, ensuring participants' voluntary involvement and their comprehensive understanding of the research objectives and potential outcomes. This study was conducted in compliance with ethical standards and guidelines set forth by the journal.

Ethical approval

All experiments involving banana plants were conducted following institutional guidelines for plant research. The application and handling of ZnO nanoparticles (ZnO NPs) were carried out according to institutional safety and environmental regulations. No human participants or animals were involved in this study

Data availability statement

The data supporting the conclusions of this study can be found within this manuscript and additional data can be made available upon reasonable request.

Disclosure of potential conflicts of interest

The authors declare there are no competing interests.

Author Contributions

- **Conceptualization:** Pradeep Kumar Shukla, Pragati Shukla,
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- **Data Analysis:** Pragya Srivastava,
- **Writing - Original Draft:** Pragya Srivastava,
- **Writing - Review & Editing:** Pradeep kumar Shukla, Pragati Shukla**,**
- **Supervision:** Pragati Shukla
- **Resources:** Pragati Shukla

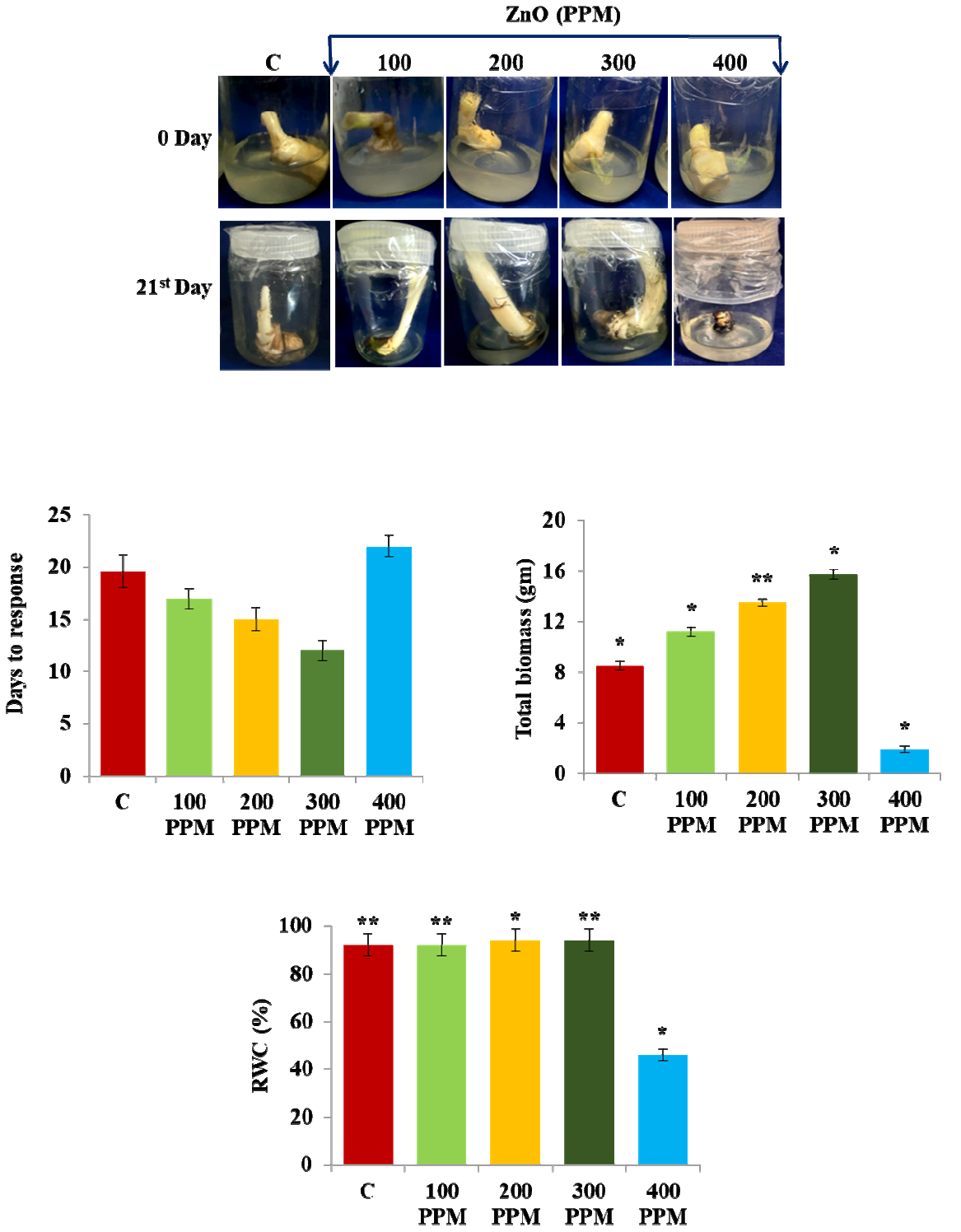

Fig. 1 : Effect of different concentration of ZnO NPs on the morphological developmental pattern of banana grown under initiation stage.

a. Suckers explant on the zero day of inoculation.
b. Morphological changes after 21st days of inoculation.
c. Graphical representation of days to response under different concentration of ZnO NPs.
d. Graphical representation of accumulation of total biomass under different concentration of ZnO NPs.
e. Graphical representation of relative water content in the initiation culture after 21 days of inculation in the medium supplemented with different concentrations of ZnO NPs.
Data represent the mean of three replicates (n=3), with error bars representing standard deviation of the mean ($p \leq 0.05$ *; $p \leq 0.005$ **).

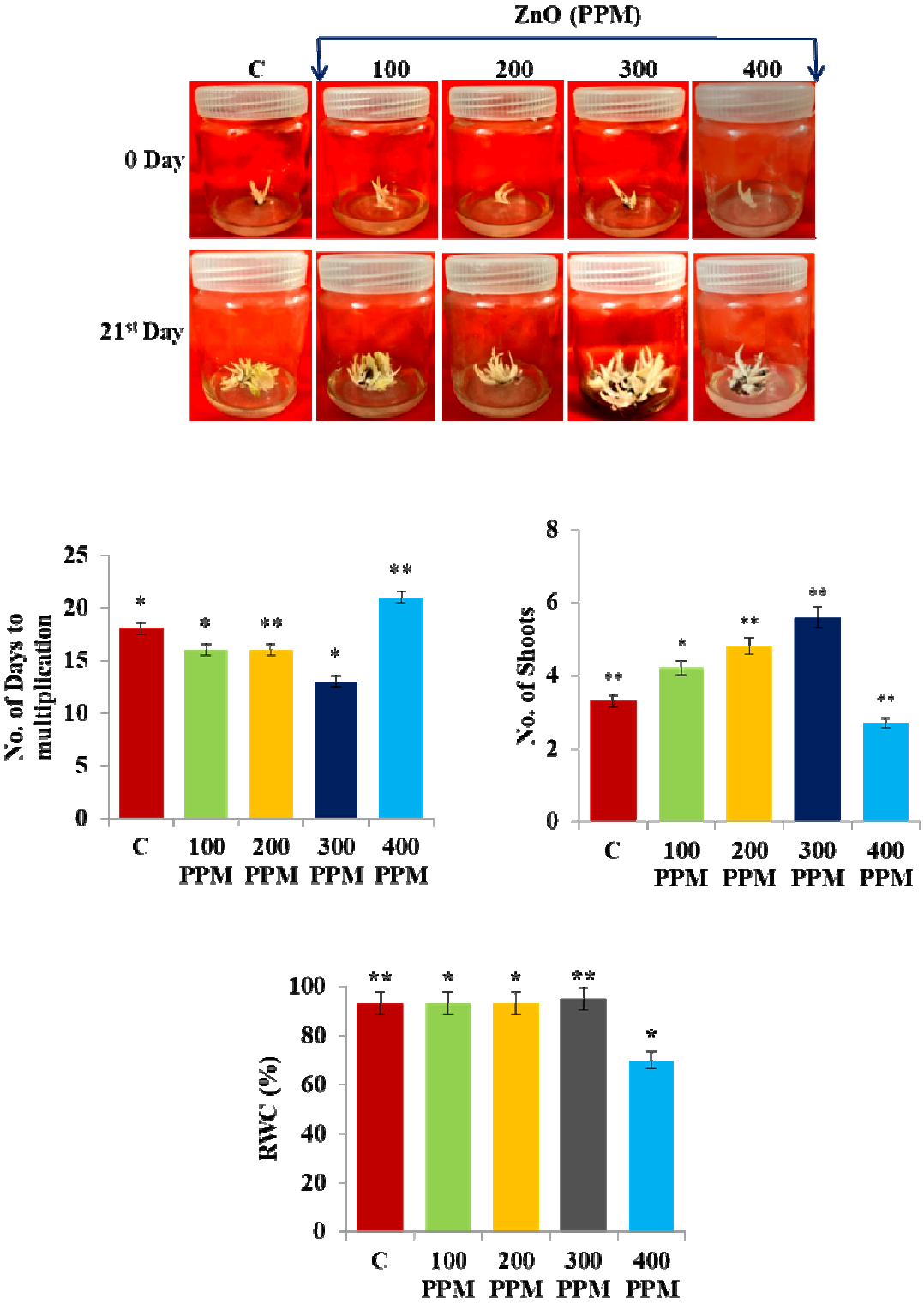

Fig. 2 : Morphological developmental pattern of *in vitro* grown banana cultures in multiplication stage under the influence of different concentration of ZnO NPs.

a. Initiated culture on the zero days of inoculation.
b. Morphological change after 21st days of inoculation.
c. Graphical representation of number of days to initiation of multiplication.
d. Graphical representation of number of shoots proliferated from single explant after 21st days of culture.
e. Graphical representation of relative water content in the multiplication culture after 21 days of multiplication.
Data represent the mean of three replicates (n=3), with error bars representing standard deviation of the mean ($p \leq 0.05$ *; $p \leq 0.005$ **).

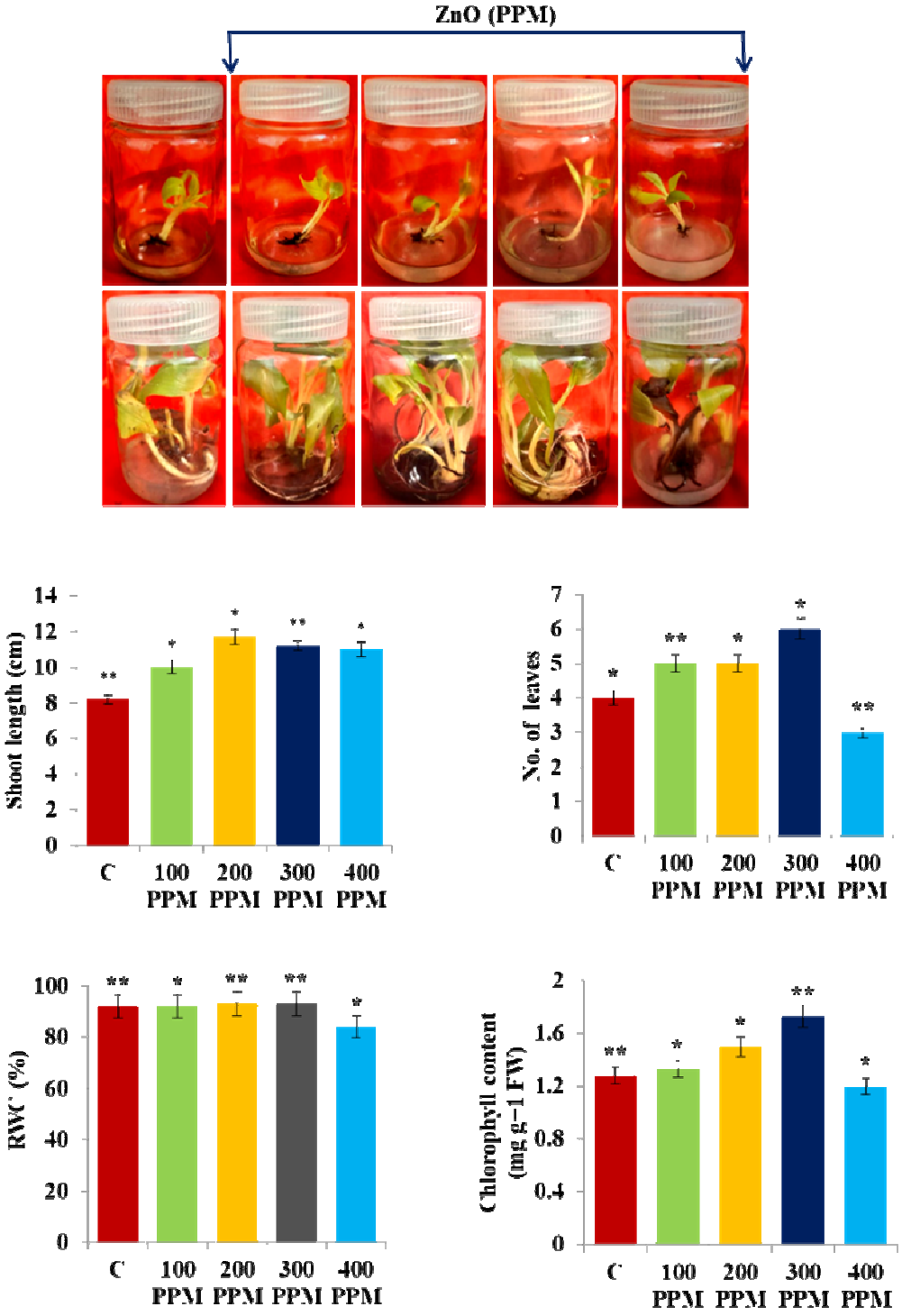

Fig. 3 : Morphological and physiological developmental pattern of *in vitro* grown banana cultures in shooting stage.

a. Single shoot on the zero days of inoculation in the medium.
b. Morphological developmental pattern of shoots after 21st days of inoculation.
c. Graphical representation of shoot length after 21st days of inoculation.
d. Graphical representation of number of new laves in a shoot after 21st days of inoculation.
e. Graphical representation of relative water content in the shooted culture after 21st days of inoculation.
f. Graphical representation of chlorophyll content of the leaves after 21st days of inoculation.

Data represent the mean of three replicates (n=3), with error bars representing standard deviation of the mean ($p \leq 0.05$ *; $p \leq 0.005$ **).

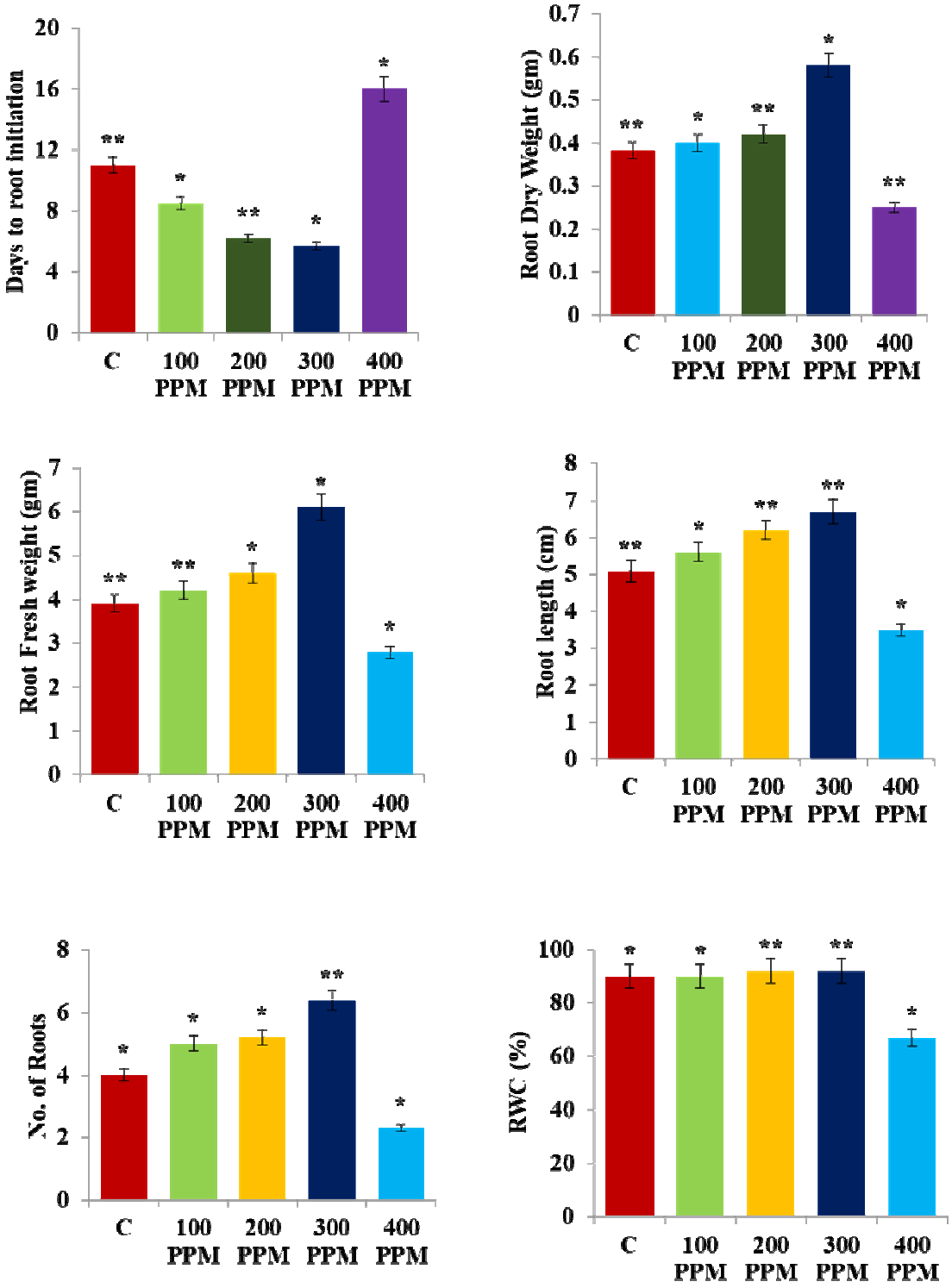

Fig. 4 : Morphological and physiological developmental pattern of *in vitro* grown banana cultures in rooting stage.
a. Graphical representation of days to root initiation in shooted culture of banana.
b. Graphical representation of root length after 21st days of inoculation.
c. Graphical representation of root fresh weight after 21st days of culture.
d. Graphical representation of root dry weight after 21st days of culture.
e. Graphical representation of number of roots after 21st days of culture.
f. Graphical representation of relative water content in the rooted culture after 21st days of inoculation.
Data represent the mean of three replicates (n=3), with error bars representing standard deviation of the mean ($p \leq 0.05$ *; $p \leq 0.005$ **).

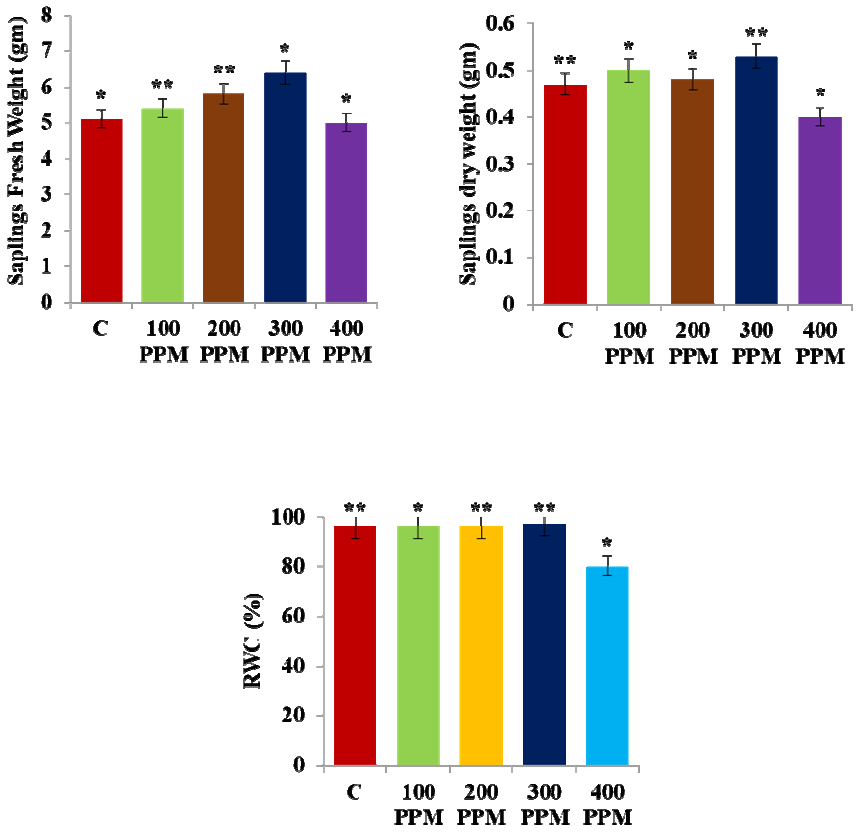

Fig. 5 : Saplings characteristics of *in vitro* grown banana

a. Graphical representation of fresh weight of rooted shoots of banana.
b. Graphical representation of dry weight of rooted shoots of banana.
c. Graphical representation of relative water content in the rooted shots of banana.
Data represent the mean of three replicates (n=3), with error bars representing standard deviation of the mean ($p \leq 0.05$ *; $p \leq 0.005$ **).

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