Nanofomulation of zinc oxide and chitosan zinc sustain oxidative stress and alter secondary metabolite profile in tobacco

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Abstract

Advancement in nanotechnology has improved ways for large-scale production and characterization of nanoparticles of physiologically important metals. The current study explores the impact of Zinc Oxide Nanoparticles (ZnO-NP) and Chitosan-Zinc oxide nano-bioformulation (CH-ZnO) in tissue culture raised callus of *Nicotiana benthamiana*. Results indicated augmented biomass in CH-ZnO treated callus, while a reduced biomass was observed in ZnO-NP treated callus, at all the concentrations tested. Higher chlorophyll and carotenoid content were recorded in callus treated with 800 ppm CH-ZnO as compared to ZnO-NP treated callus. A higher accumulation of proline was observed in CH-ZnO treated callus when compared to ZnO-NP treatment, which was significantly higher at 50, 200 and 400 ppm CH-ZnO treatment. A maximum reduction in malondialdehyde (MDA) content was recorded at 800 ppm, for both the nano-formulations tested. Likewise, a significant reduction in the H₂O₂ levels was observed in all the treatments, while the callus treated with 400 ppm ZnO-NP and 800 ppm CH-ZnO recorded the highest reduction. Phenylalanine Ammonia-Lyase (PAL), activity increased significantly in callus treated with 400 ppm concentration for both ZnO-NP and CH-ZnO with respect to control. An increased level of tannin and nicotine were recorded in callus supplemented with 50, 200 and 400 ppm CH-ZnO. Notably, a significant decline of 94 and 52% in tannin content and 25 and 50% in nicotine content was recorded in the callus treated with 800 ppm CH-ZnO and ZnO-NP, respectively. The findings of this study suggest that an optimized dosage of these nano-bioformulations could be utilized to regulate the nicotine content and stress tolerance level.

Keywords Chitosan · Nano biotechnology · Nicotine · Oxidative stress · Zinc oxide nano-bioformulation

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Introduction

Nanotechnology is a promising technique holding a dominant position in transforming agriculture and food production in a sustainable manner. Nanoparticles have potential applications in agriculture system viz., controlled delivery of pesticides, fertilizers and vital nutrients (Ghormade et al. 2011). The nano-based fertilizers can be rapidly absorbed by plants due to an active metabolic and water/mineral conduction. Nano-fertilizers improve soil health and nutrient transport and minimizes the harmful effects of chemical fertilizers. However, the composition of nanoparticles (NPs) plays a significant role in their application. Recently polymer-coated nanoparticles are being utilized as agrochemical carriers due to their controlled-release ability. Moreover, metal nanoparticles show size-dependent properties that have applications in agrochemical degradation, soil remediation, and regulation of plant metabolism (Ghormade et al. 2011). Chitosan, a biopolymer, is a deacetylated form of chitin that is widely

used in agriculture, food, medicine and bioengineering industries, and for enzyme immobilization. The amino-functional group of chitosan makes it suitable for immobilization (Verma et al. 2019). In agriculture, chitosan is majorly utilized as a biopolymer and for plant disease control-management (EI hadrami et al. 2010). Enhanced thermal stability was reported for trypsin during immobilization study based on linoleic acid-modified chitosan NPs (Liu et al. 2005). Agricultural application of chitosan is mainly in the form of delivery systems, due to its cationic properties and increased solubility in acidic solution (Kananont et al. 2010). The chitosan based NPs induce numerous biological responses in plants and possess a broad-spectrum anti-pathogenic activity against several phytopathogens (Malerba and Cerana 2016). The amine group of chitosan forms complex with a wide range of oppositely charged polymers. Additionally, chitosan gets easily absorbed to plant surfaces thus prolonging the contact time between plant surface and agrochemical. In biological systems, zinc is an essential micronutrient and is an integral component of many enzyme structures, cofactors and is the only metal to be represented in all six enzyme classes (Brown et al. 1993). Zinc is essentially required for normal growth, development, and sustained health of plants. Zinc deficiency causes rapid inhibition of plant growth and development, and final yield (Cakmak 2000). Even a very little reduction in dosage leads to physiological imbalances and affects enzyme activities and other metabolic processes (Baybordi 2006). Studies confirm that zinc oxide derived NPs have the potential to boost the yield and growth of food crops, via promoting the germination of seeds, as well as in through disease suppression (Sabir et al. 2014; Siddiqi et al. 2018). Zinc also has an important role in the management of reactive oxygen species (ROS) and the protection of plant cells against oxidative stress. Moreover, zinc oxide (ZnO) is currently listed as a “generally recognized as safe (GRAS)” material by the Food and Drug Administration and is also used as a food additive (Sabir et al. 2014).

Nano agriculture also involves the employment of nanoparticle and similar nano-formulations in agriculture, for imparting a positive effect in plants by reducing the overall impact of environmental stresses. One of the consequences of stress is an increase in the cellular concentration of ROS which is later converted to hydrogen peroxide (H_2O_2) which is detrimental above certain levels. ROS specifically is used to describe free radicals that play a key role as an inducer of oxidative damage. However, an excess concentration of ROS results in oxidative damage or apoptotic death of cells (Sharma et al. 2012). Depending on the concentration, ROS may play a dual role in plants, as being detrimental as well as beneficial. As excess ROS badly affect the plant growth and productivity, therefore, any treatment that can help to reduce the ROS load would prove beneficial in improving the overall growth and productivity. Moreover, plants can produce

a large and diverse array of organic compounds termed as secondary metabolites that appear to have no direct function in their growth and development. However, these secondary metabolites are known to play a substantial role in defence mechanisms in plants. Notably, *Nicotiana benthamiana* is the most widely used experimental host making this species a cornerstone of host–pathogen research, particularly in context to defense and stress signaling. It contains various types of secondary metabolites like isoprenoids, alkaloids, flavonoids, and anthocyanin etc., whose concentration is said to vary with fluctuating environmental stresses (Akula and Ravishankar 2011). Nicotine represents the majority of alkaloids in tobacco leaf tissues, where anatabine, anabasine, and nornicotine are the minor components of the alkaloid fraction (Saitoh et al. 1985; Chintapakorn and Hamill 2003). Commercial use of tobacco is in cigarette preparation. Smoking cigarettes affect human health directly as well as indirectly, as nicotine is the fundamental component that leads to addiction. There are several tobacco products, which are marketed and promoted as “nicotine-free”; however, such products have often been found to contain at least measurable amounts of nicotine, which are considered as addictive levels of nicotine. Methods have been developed in the past to lower the nicotine content in tobacco which mostly involves chemical extraction of nicotine from tobacco. Frequently, these methods produce less satisfactory tobacco products, since other ingredients in addition to nicotine are also removed from the tobacco.

Currently, there are several methods available to treat nicotine addiction, including pharmaceutical approaches such as nicotine replacement therapy, other methods include generation of transgenic tobacco lines via RNA silencing, and antisense RNA technology, co-suppression technology, and conventional breeding are also often used. However, all these methods are time-consuming and require recombinant DNA technology. Further studies are required to reduce the nicotine content naturally. It is reported that zinc oxide nano particles have the efficiency to alter the ROS generation and scavenging (Panwar et al. 2012). Furthermore, Zarei et al. (2014) demonstrated that chitosan has a tremendous ability to form metal complexes with zinc metal because of its amine groups and hydroxyl groups; therefore, the major emphasis of our study was to formulate chitosan mediated ZnO nano-bioformulation. Therefore, it was hypothesized that zinc chitosan based nano-bioformulation may help in alleviating oxidative stress via modulation of redox status which may positively correlate with altered secondary metabolite levels. The aim of this study was to assess the dose-dependent effects of ZnO-NP and CH-ZnO nano-bioformulation in ameliorating oxidative stress and altering the levels of commercially important secondary metabolites, with major emphasis on the reducing the nicotine content in *Nicotiana benthamiana*.

Materials and methods

Preparation of zinc oxide nanoparticle (ZnO-NP)

Zinc oxide nanoparticles were synthesized through chemical reduction of zinc nitrate as described by Yadav et al. (2006) with minor modifications. Polyethylene glycol (0.2%) and polyvinylpyrrolidone (1%) was added in 100 mM zinc nitrate solution (Loba Chemie Pvt. Ltd., India) and stirred constantly for 5 min at 60 °C. Furthermore, 30 ml sodium hydroxide solution (Loba Chemie Pvt. Ltd., India) was added dropwise until white precipitate appears, followed by gradual heating of solution at 60 °C for 2 h. The solution was subjected to centrifugation at 2000 rpm for 10 min. The supernatant was discarded and the pellet was washed twice with 2 ml distilled water and 2 ml alcohol. Finally, the pellet was dissolved in 1 ml distilled water.

Preparation of chitosan zinc oxide nano-bioformulation (CH-ZnO)

The methodology for preparation of CH-ZnO nano-bioformulation was adopted from Dhillon et al. (2014) with minor modifications. Zinc oxide powder (0.75 g) was dissolved in 100 ml of acetic acid (1% v/v) solution followed by addition of 1 g of chitosan powder (poly [β -2-amino-2-deoxy-(1-4)-D glucopyranose]; molecular weight 50,000–190,000 Da; deacetylation degree of 75–85%; Sigma-Aldrich®, Bangalore, India) and 1% w/v citric acid powder (Loba Chemie Pvt. Ltd., India). Furthermore, this solution was subjected to sonication for 1 h and 1 M sodium hydroxide solution was used to attain the pH 10. The solution was kept at 80 °C for 3 h and filtered and washed thrice with 2 ml distilled water and then dried in an oven at 50 °C for 1 h.

Characterization of nanoparticles

The synthesized zinc oxide nanoparticles were characterized by ultra violet visible (UV-vis) spectroscopy, Zeta potential and Particle size analysis through Dynamic light scattering (DLS). The diffusion coefficients of nanoparticles were determined, and the average diameters of the particles were estimated using the Stokes–Einstein relationship as described by Berne and Pecora (2000).

Plant material and growth conditions

Seed germination

After sterilization, seeds of *Nicotiana benthamiana* were inoculated in MS (Murashige and Skoog) basal media for

further germination. Seed germination was initiated after 12–15 days of inoculation. After 25 day post-inoculation, leaves started to appear from seedlings which were used as explants for callus proliferation. The seedlings were grown at 25 ± 1 °C temperature under cool white fluorescent lights (Phillips Champion, PHILLIPS, India), 50 $\mu\text{mol}/\text{m}^2/\text{s}$ photosynthetic photon flux density, under 16 h photoperiod and relative humidity of 70%. For callus formation, MS basal medium was supplemented with growth regulators, i.e., α naphthalene acetic acid (NAA) and 2 isopentyl adenine (2iP) (3.0: 0.3 mg/L concentration).

Callus induction

Callus induction was done in MS media supplemented with NAA and 2iP by inoculating leaf discs prepared using leaves from 25-day-old *in-vitro* raised seedlings. These plant growth regulators were optimized for promoting growth of the callus. Leaf curling initiated after 20 days and green fragile callus was obtained after 4–5 weeks of inoculation. Furthermore, the callus was treated with different concentrations (50, 200, 400, and 800 ppm) of both ZnO-NP and CH-ZnO nano-bioformulation and these treated calluses were used for further experiments along with untreated callus (0 ppm) as control.

Treatment of callus with nanoparticles

Initially, stock solutions of 1 mg/ml of both ZnO-NP and CH-ZnO nano-bioformulation were prepared in sterile distilled water which was subsequently diluted to the desired concentration (50, 200, 400 and 800 ppm). UV radiation is one of the methods to sterilize the NPs (Tapia-Guerrero et al. 2020). The non-greasy formulation of ZnO-NP does not degrade with UV radiation exposure (Hanigan et al. 2018). Therefore, the NPs were sterilized by UV radiation for 1 h before adding in the MS media, to maintain aseptic conditions during callus treatment. The required concentrations of nanoparticles were added to the MS media after autoclaving, but just before solidification of the media. The control tube contains no nanoparticles. The media was gently shaken to prevent the settling down of nanoparticles. Fragile callus was divided in six equal parts and inoculated in test tubes containing MS media with different concentrations of nanoparticles. These tubes were incubated for 7 days at 25 ± 1 °C in 16/8 h light and dark cycle. (Supplementary Fig. 1).

Growth profile measurement

Biomass content

To measure biomass, equal amount of treated callus was kept at 60 °C in an air circulation oven until a constant weight

was obtained. Furthermore, this dry weight was considered as biomass.

Chlorophyll content

The chlorophyll content was measured according to Arnon (1949) with slight modifications. 0.5 g of callus was homogenized using 5 ml of 80% chilled acetone to extract chlorophyll and centrifuged at 5000 rpm for 5 min and the supernatant was collected. The extraction step was repeated until the residue became colourless. Absorbance was measured at 663 nm for chlorophyll a and 645 nm for chlorophyll b. Total chlorophyll concentration ($\mu\text{g/g}$) in the callus was calculated according to the following equation:

$$\text{Total chlorophyll } (\mu\text{g/g fwt.}) = [(20.2 \times A_{645}) + (8.02 \times A_{663}) / 1000 \times W] V$$

where W is fresh weight of sample and V is the extraction volume.

Carotenoid content

The methodology for the estimation of carotenoid content was adopted from Shaw and Hossain (2013). In brief, 0.5 g of callus was homogenized using 80% acetone for carotenoid extraction and was centrifuged at 5000 rpm for 5 min. The supernatant was collected and the extraction was repeated until the residue became colourless. Absorbance was measured at 470 nm and 80% acetone was used as a blank. Total carotenoid content was calculated using the following formula:

$$\text{Total carotenoid } (\mu\text{g/gm fwt.}) = (1000A_{470} - C_{ab}) / 198$$

where C_{ab} is absorbance of total chlorophyll

Biochemical parameters and enzyme assays

Malondialdehyde content

The procedure of Ghosh et al (2015) was followed for measuring malondialdehyde (MDA) content. Callus (0.5 g) was homogenized with 10 ml of sodium phosphate buffer (50 mM) solution and centrifuged at 10,000 rpm for 30 min at 4 °C. Furthermore, 4 ml of 20% (v/v) trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid (TBA) was added to 1 ml of supernatant. The homogenate was heated at 95 °C for 30 min and centrifuged at 8000 rpm for 30 min. The MDA concentration was calculated using the extinction coefficient $155 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed in terms of μM

g^{-1} fresh tissue. The following formula was used to estimate the MDA content:

$$\text{MDA } (\mu\text{M/g fwt.}) = [(A_{532} - A_{600}) / 155V] \times W \times 1000$$

where V is volume of extract, W is Fresh weight of seedling and $155 \text{ mM}^{-1} \text{ cm}^{-1}$ is co-efficient.

Hydrogen peroxide content

Hydrogen peroxide (H_2O_2) was measured as per the protocol given by Alexieva et al. (2001). In brief, callus (0.5 g) was homogenized in 10 ml of 0.1% (w/v) aqueous TCA and centrifuged at 10,000 rpm for 30 min at 4 °C. Furthermore, 1 ml of 0.1 M potassium phosphate buffer and 1 ml of 1 M potassium iodide (KI) was added in 1 ml of supernatant for reaction preparation. The blank consisted of 0.1% TCA instead of callus extract. The reaction was allowed to develop for 1 h in dark and absorbance was measured at 390 nm. The amount of H_2O_2 was calculated using standard curve prepared with different dilutions of a working standard of 100 μM of H_2O_2 .

Free proline content

The methodology for the estimation of free proline content was adopted from Bates et al. (1973) with minor modifications. Callus was homogenized in 3% sulfosalicylic acid and centrifuged at 8000 rpm for 15 min and the crude enzyme extract was collected as supernatant. The enzyme extract was mixed with equal quantities of glacial acetic acid and acid ninhydrin reagent and incubated for 1 h at 100 °C. Furthermore, this was kept in ice to terminate the reaction. To each tube of the reaction mixture, 4 ml of toluene was added and followed by vigorous stirring on a vortex mixer to extract the red coloured chromophore. The absorbance of chromophore was measured at 520 nm. Blank constitute of 1 ml of 3% sulfosalicylic acid (SSA) in place of the supernatant. Concentration of proline in the sample was computed from standard curve of L-proline.

Tannin content

The procedure for the determination of tannin was followed as described by Price and Butler (1977). In brief, 0.5 g of callus was homogenized using 1 ml of 1% potassium ferricyanide and 1 ml of 1% ferric chloride. Distilled water was added to make up the solution up to 10 ml followed by incubation at room temperature for 5 min. Absorbance was measured at 720 nm against the reagent blank. The tannin content was expressed as milligram of

tannic acid (TAE) (200–1000 µg) equivalence per gram of extract.

Extraction of nicotine from callus

The methodology for nicotine extraction was adopted from Rai et al. (1994) with minor modifications. Tobacco callus (1 g) was placed in 100 ml beaker and methanol (10 ml) was added followed by incubation at room temperature for 30 min. Furthermore, 25 ml of distilled water and 1 ml of sodium hydroxide (2 M) were added. This mixture was kept in a boiling water bath for 1 min. and filtered through a Whatman filter paper. The beaker was rinsed using 5 ml of distilled water and the resulting solution was filtered and mixed with original filtrate and the final volume was made up to 25 ml using distilled water. Then, zinc acetate (1 ml) and potassium hexacyanoferrate (1 ml) solutions were added and centrifuged at 2000 rpm for 5 min. The supernatant was transferred into a beaker and 0.5 g of fuller's earth was added. The mixture was shaken thoroughly and centrifuged at 5000 rpm for 10 min. The clean supernatant was discarded and the residue was treated with 0.01 M sodium hydroxide solution and filtered. To this filtrate, 14 ml of 1 mol/l HCL was added and the solution was made up to 100 ml with water. An aliquot of this solution was used for further quantification.

Quantification of nicotine

Quantification of nicotine content was based on the oxidation of nicotine by potassium permanganate in an alkaline medium to form a green product. The nicotine quantification was performed as described by Al-Tamrah (1999). In brief, 1 ml of 1.25×10^2 M potassium permanganate and 2 ml of 6.25 M sodium hydroxide was gently mixed and the desired amount of callus extract was added. The final volume of 20 ml was adjusted using distilled water and incubated at 100 °C for 7.5 min. in a water bath. After cooling, again the final volume of 20 ml was adjusted using distilled water. The absorbance was measured in a 1 cm cell at 610 nm against a reagent blank. Commercially available nicotine was used to prepare the standard curve.

Antioxidant enzyme assay

Phenylalanine ammonia lyase (PAL) (EC 1.10.3.3)

PAL activity was measured according to Brueske (1980). Callus (0.5 g) was homogenized using a mortar pestle in 5 ml of cold 25 mM borate-HCL buffer (pH-8.8) containing 5 mM mercaptoethanol (0.4 ml/L). This homogenate was centrifuged at 10,000 rpm for 20 min at 4 °C and supernatant was used as enzyme source. For enzyme assay, 0.2 ml

of enzyme solution was added in 0.5 ml of borate buffer and 1.3 ml of water followed by the addition of 1 ml of L-phenylalanine. Furthermore, the reaction mixture was incubated for 60 min at 32 °C. After incubation, the reaction was stopped by addition of 0.5 ml of 1 M trichloroacetic acid. For blank, the reaction mixture was prepared without L-phenylalanine and was added after the addition of trichloroacetic acid. The absorbance was measured at 290 nm. The decrease in absorbance was recorded and the specific activity of PAL was calculated and expressed in $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein using the extinction coefficient as $9.6 \text{ mM}^{-1} \text{cm}^{-1}$ at 290 nm.

Ascorbate oxidase (AO) (EC 1.10.3.3)

Activity of ascorbate oxidase was measured by adopting the procedure described by Nakamura et al. (1968), with minor changes. Callus (0.5 g) was homogenized in mortar pestle with 0.1 M phosphate buffer having pH of 6.5. This homogenate was centrifuged at 9000 rpm for 30 min at 4 °C and the supernatant was transferred in a chilled centrifuge tube. This crude extract was used as an enzyme for performing the enzyme assay. Furthermore, 0.5 ml of 1 mM ascorbic acid solution, (stock solution prepared in 1.0 mM HCL solution containing 1.0 mM EDTA and dilute this stock solution to tenfold volume with 0.2 M KH_2PO_4 solution containing 1.0 mM EDTA), was added to 0.5 ml of 10 mM Na_2HPO_4 solution, and 0.1 ml of the enzyme solution was added lastly. The resulting solution was mixed gently and incubated at 37 °C for 5 min. After incubation, the reaction was stopped by adding 3.0 ml of 0.2 N HCL solutions. The absorbance was measured at 245 nm against water (OD test). At the same time, the blank was prepared by mixing the reaction mixture with 3.0 ml of HCL solution after 5 min. incubation at 37 °C, followed by adding the enzyme solution (OD blank).

Statistical analysis

The experiment was designed as completely randomized design (CRD) using Genstat 14Ed (Rothamsted Experimental Station, Harpenden, UK). Statistical analysis was performed using two-way analysis of variance (ANOVA) with treatment and concentration as factors. Tukey's HSD Post hoc test was performed to compare individual means.

Results and discussion

Several reports have utilized zinc and chitosan nanoparticles within a broad range of 2–2000 ppm in various crops like rice (Chen et al. 2015); wheat (Tripathi et al. 2017); tomato (Wang et al. 2018); and maize (Choudhary et al. 2019).

Notably, most of the reports utilizing ZnO-NP suggested a higher level of toxicity at an increased dosage beyond 800–1000 ppm. (Chen et al. 2015; Wang et al. 2018; García-López et al. 2019). Therefore, because of an enhanced toxicity beyond 800 ppm, we planned to utilize the following dosage (50, 200, 500, 800 ppm) concentration of both ZnO-NP and CH-ZnO nano-bioformulation.

Characterization of nanoparticles

Both zinc oxide nanoparticles (ZnO-NP) and chitosan zinc oxide nano-bioformulation (CH-ZnO) were synthesized by precipitation method. The NPs formation was ascertained by recording the absorption spectra of the synthesized

particles within a range of 300–500 nm and the absorption maxima ($\lambda_{\max}$) was recorded at 372 and 360 nm, respectively (Fig. 1a). The dynamic light scattering (DLS) technique was used to determine the size distribution profile of synthesized NPs. DLS is widely used as an effective technique for determining the average secondary particle size of brownian nanoparticles in colloidal suspensions in nano and submicron range (Berne and Pecora 2000; Kato et al. 2009). The average particle size obtained, of ZnO-NP and CH-ZnO nano-bioformulation, was 327 and 534 nm, respectively (Fig. 1b, c). Zeta potential measurement is a key indicator of the stability of colloidal dispersions. NPs are very small in size for which they are energetically very unstable. Therefore, the particles undergo agglomeration to stabilize themselves

Fig. 1 Characterization of Zinc Oxide Nanoparticles (ZnO-NPs) and Chitosan-Zinc oxide (CH-ZnO) nano-bioformulation. **a** UV-spectrum of ZnO-NPs and CH-ZnO nano-bioformulation; **b** Hydrodynamic diameter (particle size) of ZnO-NPs using size distribution

determined by dynamic light scattering (DLS); **c** Hydrodynamic diameter (particle size) of CH-ZnO nano-bioformulation using size distribution determined by dynamic light scattering (DLS). Each result represents mean ($n = 3$) with $\pm$ standard deviation

as there are some potential charges on the surface of the nanoparticles which are responsible for providing stability. These charge potential were analysed by determining their Zeta potential. Charge of -40.9 for ZnO-NP and -1.31 mV (Milli volt) for CH-ZnO nano-bioformulation was obtained which further strengthen the fact that the synthesized nanoparticles are stable.

Growth profile measurement

Biomass content

A significant treatment, concentration and (treatment $\times$ concentration) ($P < 0.001$) effect was observed for biomass (Supplementary Table 1). Higher biomass was recorded in callus treated with CH-ZnO nano-bioformulation as

compared to ZnO-NP. The CH-ZnO treated callus demonstrated a higher biomass at 50 ppm (5.3%); 200 ppm (8.89%); 400 ppm (23.77%) and 800 ppm (9.69%) concentration as compared with control. However, ZnO-NP treated callus demonstrated a reduced biomass at 50 ppm (29.74); 200 (19.26%); 400 (10.07%) and 800 ppm (40.01%) concentration then control (Fig. 2a). In our study, the application of CH-ZnO nano-bioformulation positively influenced growth of tobacco callus and produced more favourable effects than ZnO-NP. Both positive and negative effects of ZnO-NP on plant growth and metabolism at various developmental periods have been documented in several studies. Notably, a major chunk of reports suggests a negative role of ZnO-NP in crops (Boonyanitipong et al. 2011; Khare et al. 2014; Thunugunta et al. 2018; Wang et al. 2018). The present study underlines a positive effect of chitosan based

Fig. 2 Response of *Nicotiana benthamiana* callus grown in media supplemented without and with different concentrations (50, 200, 400, and 800 ppm) of Zinc Oxide Nanoparticles (ZnO-NP) and Chitosan-Zinc oxide nano-bioformulation (CH-ZnO). **a** Biomass, **b** Chlorophyll, **c** Carotenoid and **d** Proline content in the treated callus along

with control (without NPs). Each vertical column represents mean of three replicates. Bars indicate means $\pm$ standard error. Tukey's HSD Post hoc test was performed to compare means. Columns with different letters are significantly different ($P < 0.05$)

nano-bioformulation (CH-ZnO) used in the study, as they enhanced the plant biomass at all dosage tested; however, the maximum increase was recorded at 400 ppm CH-ZnO (Fig. 2a). Our results are consistent with Guan et al. (2009), in which they have highlighted the role of chitosan NPs in enhancing biomass via reducing the transpiration rate by maintaining cell osmotic pressure and in facilitating water and nutrient uptake. Furthermore, they emphasized on chitosan NPs mediated reduction in the accumulation of harmful free radicals via modulating the antioxidants and enzyme activities, could be one of the reasons of augmented biomass observed in this study.

Chlorophyll content

A significant treatment, concentration and (treatment $\times$ concentration) ($P < 0.001$) effect was observed for chlorophyll content (Supplementary Table 1). Chlorophyll is a vital pigment for photosynthesis, which allows plants to absorb energy from light. The function of the reaction center of chlorophyll is to absorb light energy and transfer it to other parts of the photosystem. The chlorophyll donates the high energy electron to a series of molecular intermediates called an electron transport chain (Carter et al. 1996). The results of this study indicated a reduction in chlorophyll content of ZnO-NP and CH-ZnO treated callus as compare to control. Though, the greater reduction was recorded in the callus supplemented with ZnO-NP with respect to CH-ZnO treated callus. The reduction of (90, 78, 54 and 80%) in chlorophyll content was recorded in the callus treated with 50, 200, 400 and 800 ppm ZnO-NP, respectively, as compared to control callus (Fig. 2b). While in CH-ZnO treated callus a relatively less reduction of (25, 24 and 9%) in the chlorophyll content was recorded in 50, 200 and 400 ppm treatment, respectively. In a similar study conducted in maize plants, authors have evaluated the impact of ZnO-NP and observed a significant reduction in chlorophyll content in plants treated with an increased dose of ZnO-NP (Choudhary et al. 2019). In our study ZnO-NP mediated decline in chlorophyll content could be a result of the internalization of these NPs, and their accumulation in the tissue and the subsequent dissolution of the zinc ions (Ma et al. 2013), which indicate that ZnO-NP can induce toxicity during a prolonged period through the activity of the ions that are released, consequently inducing a greater stimulation in the ROS generation (Choudhary et al. 2019).

Similar results were obtained by Wang et al. (2016), where a decrease in chlorophyll content was observed in *Arabidopsis* plant treated with 200 ppm ZnO-NP with respect to control plants. The observed decrease in the chlorophyll are in line with the fact that zinc, if supplemented, at a higher dosage it is known to induce cellular toxicity (Hou et al. 2018). Even though several studies report toxicity

via enhanced zinc accumulation but zinc is attributed as an essential micronutrient for plant growth and metabolism (Narendhran et al. 2016), and it is a prerequisite for chlorophyll production (Cakmak 2008). In our study, 800 ppm treatment of CH-ZnO treated callus resulted in a significant increase of 192% in chlorophyll content (Fig. 2b) which may be attributed to the slow and sustained release of zinc from chitosan matrix of CH-ZnO nano-bioformulations, which is reported a major advantage of encapsulating zinc in chitosan nanomaterial as described by Cornelis et al. (2014) and Choudhary et al. (2019). This may be further corroborated with the findings of Chibu and Shibayama (2001), where they have linked chitosan NPs mediated increase in chlorophyll synthesis and growth, with the release of amino compounds present in chitosan, which further assist the regulation of endogenous levels of cytokinins. In addition, the increased chlorophyll content can also be associated with the nitrogen (N) content of chitosan that has a vital role in tetrapyrrole ring of chlorophyll (Behboudi et al. 2019). Similar results of enhanced pigment were observed in the leaf of coffee seedlings, where coffee seedlings supplemented with chitosan NPs resulted in a significant enhancement in the levels of chlorophyll a, b and carotenoid pigments (Van et al. 2013). Furthermore, Dzung and Thang (2004) and Dzung (2005) also reported that chitosan oligomer was able to significantly increase the chlorophyll content in soybean and peanut plants. Moreover, zinc is reported to have an imperative role in influencing the activity of carbonic anhydrase enzyme that contains a Zn atom which catalyses the hydration of CO_2 and facilitates the diffusion of carbon dioxide to the carboxylation sites in plants (Badger and Price 1994; Pullagurala et al. 2018). Reports reveal that application ZnO-NP at 100, 200, and 400 mg kg^{-1} increased the relative chlorophyll content by 41, 37, and 58%, respectively (Pullagurala et al. 2018). Interestingly in our study, a similar trend of increased pigment content was also recorded in another pigment namely carotenoid, where a substantial increase in carotenoid content was observed in CH-ZnO nano-bioformulation treated callus as compared to ZnO-NP treated callus.

Carotenoid content

A significant treatment, concentration and (treatment $\times$ concentration) ($P < 0.001$) effect was observed for carotenoid content (Supplementary Table 1). In our study, a gradual increase in carotenoid content was observed in tobacco callus supplemented both with ZnO-NP and CH-ZnO nano-bioformulation which gradually increased in a range of (75, 87, 150 and 400%) in the callus treated with 50, 200, 400, and 800 ppm CH-ZnO, respectively, as compared to control callus. However, a moderate increase in carotenoid content was also observed in ZnO-NP treated callus,

where the carotenoid content was found to increase (62, 50, 12, and 75%) in the callus treated with 50, 200, 400 and 800 ppm ZnO-NP, respectively, as compared to control callus (Fig. 2c). Carotenoids protect photosynthetic organelles against potentially harmful photo-oxidative processes and are essential structural components of the photosynthetic antenna and reaction center complexes (Bartley 1995). In plants, these compounds are also attributed as precursors of abscisic acid (ABA), a phytohormone that modulates developmental and stress processes (Felemban et al. 2019). The results of this study indicate CH-ZnO treated callus possess a significantly high level of carotenoid as compared to ZnO-NP treated callus which may impart better photo-protection by preventing harmful photodynamic reactions and may also serve as light-harvesting pigment for increased photosynthesis, as evident by our results. Our results corroborated with the findings of Mohsenzadeh and Moosavian (2017), where authors reported increased carotenoid contents in *Rosmarinus officinalis* plant exposed to 100 mg/kg ZnO-NP. This could be attributed to plant response to the inhibition of oxidative damage, which could be caused by reactive oxygen species on photosynthesis (Pullagurala et al. 2018). Similar results were obtained by Wang et al. (2016) in which increased carotenoid content was reported in *Arabidopsis* treated with 200 ppm of ZnO-NP as compared to control plants. As CH-ZnO nano-bioformulation resulted in a significant increase in carotenoid content; therefore, it could be concluded that carotenoid content can be largely affected by CH-ZnO nano-bioformulation as compared to ZnO-NP treatment.

Biochemical and enzymatic assays

Malondialdehyde

A significant treatment, concentration and (treatment $\times$ concentration) ($P < 0.001$) effect was observed for malondialdehyde (Supplementary Table 1). MDA is one of the final products of oxidative modification of lipids and is responsible for cell membrane damage. Lipid peroxidation results in altered membrane fluidity, ion transport, loss of enzyme activity and protein cross-linking thus ultimately resulting in cell death (Sharma et al. 2012). The level of MDA declined significantly in all concentrations of both ZnO-NP and CH-ZnO nano-bioformulation as compared to control. Equal reduction of (66.6%) in MDA content was recorded in 50 and 200 ppm CH-ZnO treated callus followed by (33 and 66.5%) reduction in 400 and 800 ppm of CH-ZnO treated callus, respectively. In ZnO-NP treated callus a maximum decline of 66.5% in MDA level was observed in 800 ppm treated callus, followed by an equal reduction of 33.1% at 50 and 200 ppm treated callus, whereas MDA level remained unchanged in 400 ppm ZnO-NP treated callus (Fig. 3a).

Similarly, a declined level of MDA was also highlighted by Burman et al. (2013), where they have reported a net reduction in lipid peroxidation levels, as evident from lower MDA content, in chickpea seedlings treated with 1.5 and 10 ppm of ZnO-NP. A similar reduction in lipid peroxidation was also reported in green peas, where a 125 and 250 mg/kg (ppm) of ZnO-NP helped in maintaining minimal levels of MDA and H_2O_2 concentration (Mukherjee et al. 2014).

Hydrogen peroxide

A significant treatment, concentration and (treatment $\times$ concentration) ($P < 0.001$) effect was observed for hydrogen peroxide (Supplementary Table 1). H_2O_2 a form of ROS is a free radical which is known to increase in plants under stressful conditions. The biological effect of H_2O_2 not only depends on its concentration but also on the site of production, the developmental stage of the plant and previous exposures to different kinds of stresses. Generally, at low concentrations, it acts as a signalling molecule, while at higher concentrations it provokes the onset of cell death (Gechev and Hille 2005). In the current study, levels of H_2O_2 content were found to be lowest for ZnO-NP treated callus at 400 ppm, whereas at 800 ppm for CH-ZnO NP treatment, with respect to control callus (Fig. 3b). Notably, a nine-fold higher H_2O_2 level was recorded in 400 ppm CH-ZnO treated callus as compared to ZnO-NP treated callus, thus signifying an improved ROS generation and scavenging capability of CH-ZnO nano-bioformulation as compared to ZnO-NP. Remarkably, a reduced H_2O_2 level, as compared to control, was observed in all CH-ZnO and ZnO-NP treatments. The results of our study suggest a better ameliorative effect of supplemented nano-formulations which results in better redox potential via controlling the formation of harmful free radicals (ROS) in plants. Both MDA and H_2O_2 , are a noteworthy index of the chemically reactive molecule responsible for modulating and generating highly unstable ROS molecules; therefore, an increased MDA level and a reduced H_2O_2 level at 400 ppm can be attributed to the increased accumulation of other metabolites obtained in this study, namely nicotine and tannin and increased activity of PAL enzyme at this particular dosage of 400 ppm.

Proline

A significant treatment, concentration and (treatment $\times$ concentration) ($P < 0.001$) effect was observed for proline (Supplementary Table 1). Proline is attributed to be a well-accepted stress marker as multiple studies associated stress ameliorative properties to proline, suggesting its involvement in ROS scavenging acting as a singlet oxygen quencher (Joshi et al. 2011). Proline is a good index of the existing stress experienced by the plant, whereas a declined proline

Fig. 3 Biochemical and enzymatic characterization of *Nicotiana benthamiana* callus under different concentrations (50, 200, 400, and 800 ppm) of Zinc Oxide Nanoparticles (ZnO-NP) and Chitosan-Zinc oxide nano-bioformulation (CH-ZnO). **a** Malondialdehyde (MDA) content, **b** Hydrogen peroxide (H_2O_2) content, **c** Phenylalanine ammonia lyase (PAL) specific activity and **d** Ascorbate oxidase

specific activity in the treated callus along with control (without NPs). Each vertical column represents mean of three replicates. Bars indicate means $\pm$ standard error. Tukey's HSD Post hoc test was performed to compare means. Columns with different letters are significantly different ($P < 0.05$)

level signifies a relief from stress (Monreal et al. 2007). A higher amount of proline content (125, 112, and 12.5%) was recorded in callus supplemented with 50, 200 and 400 ppm CH-ZnO nano-bioformulation, contrarily a 12.5% reduction in proline content was observed at 800 ppm of CH-ZnO nano-bioformulation as compare to control (Fig. 2d). Contrastingly a relatively low increase in proline content was observed in ZnO-NP treated callus, where a mere increase of 25% was observed in callus supplemented with 50 ppm of ZnO-NP which was followed by a gradual decline of (75, 50, and 25%) in callus supplemented with 200, 400 and 800 ppm ZnO-NP, as compare to control callus, suggesting a seemingly less efficient ROS mitigation (Fig. 2d). During stress perception, proline accumulation is reported to act as an antioxidant for neutralizing the toxic effects of ROS and it is also known to maintain the structure of proteins

and membranes in cells (Hayat et al. 2012). Adhikari et al. (2015) also reported the reduction in proline levels in ZnO-NP treated maize plants. An increased amount of proline accumulation was recorded in this study using 50, 200 and 400 ppm of CH-ZnO when compared to a similar concentration of ZnO-NP which further suggests better stress acclimation of CH-ZnO nano-bioformulation in tobacco callus.

Tannins

A significant treatment, concentration and (treatment $\times$ concentration) ($P < 0.001$) effect was observed for tannins (Supplementary Table 1). Tannins are naturally occurring chemicals that provide mechanical defence in plants and also assist in combating environmental stresses. In their condensed, secondary-metabolite form, tannins are highly

toxic and insoluble compounds, and they bind to proteins and enzymes. The source for these important compounds is a newly discovered small organelle called the tannosome that arises from chloroplasts and is responsible for tannin formation (Brillouet et al. 2013). After the tannins are created, the tannosome is encapsulated in a membrane, allowing the structure to bud off from the chloroplast and to be shuttled to a plant vacuole for safe storage. Tannin content was observed to increase 13 and 5% in 50 and 200 ppm CH-ZnO nano-bioformulation treatment, whereas it decreased from 34 to 94% at 400 and 800 ppm, respectively, as compared to control (Fig. 4b). However, a maximal increase in tannin content was observed when callus was supplemented with 200 ppm of ZnO-NP, where 36% higher tannin content was reported with respect to control callus. A maximum reduction of 94.7% in tannin content was observed at 800 ppm CH-ZnO treatment, whereas a relatively lower reduction of 52.6% was recorded in 800 ppm ZnO-NP treated callus (Fig. 4b). This was similar to results obtained for PAL enzyme which may likely be associated with a sustained release of zinc from the chitosan matrix (Choudhary et al. 2019). In our study, 800 ppm CH-ZnO treated callus, exhibited a reduced H_2O_2 levels and an augmented activity of ascorbate oxidase, an important H_2O_2 metabolising enzyme, signifying better redox homeostasis at a higher dose of 800 ppm. Contrarily, in ZnO-NP supplemented callus, 36.8% higher tannin content was recorded at 200 ppm of ZnO-NP. This significant increase in tannin content was correlated with a higher MDA

content, as evident in our study, which is a result of oxidative stress due to increased ROS generation. These results are in accordance with Zafar et al (2016), where authors highlighted ZnO-NP induced oxidative stress in shoots of *Brassica nigra* and observed an increase in several phenolic compounds as a result of over accumulation of ROS due to oxidative stress.

Nicotine

A significant treatment, concentration and (treatment $\times$ concentration) ($P < 0.001$) effect was observed for nicotine (Supplementary Table 1). Nicotine is an alkaloid, predominantly found in tobacco having commercial utility. Its production is said to increase under stressful conditions to avoid herbivore attack (Saitoh et al. 1985; Chintapakorn and Hamill 2003). A high metabolic cost for nicotine production is reported to be a major reason for a minimal basal level of nicotine under controlled conditions (Liu et al. 2019). However, studies suggest that as a response towards mechanical wounding due to decapitation, or through insect or animal herbivore, a rapid elevation is triggered in the nicotine level. Thus wounding provokes a damage signal which further induces the biosynthesis and transportation of jasmonic acid (JA) and its derivatives, namely methyl jasmonic acid (MeJA), resulting in increased biosynthesis of nicotine and other alkaloids (Baldwin et al. 1994; Baldwin 1996). In the current study,

Fig. 4 Nicotine and Tannin estimation in *Nicotiana benthamiana* callus in response to different concentrations (50, 200, 400, and 800 ppm) of Zinc Oxide Nanoparticles (ZnO-NP) and Chitosan-Zinc oxide nano-bioformulation (CH-ZnO). **a** Nicotine content and **b** Tannin content in the treated callus along with control (without

NPs). Each vertical column represents mean of three replicates. Bars indicate means $\pm$ standard error. Tukey's HSD Post hoc test was performed to compare means. Columns with different letters are significantly different ($P < 0.05$)

a significant increase in nicotine content was observed in both ZnO-NP and CH-ZnO nano-bioformulation treated callus at 50, 200 and 400 ppm treatment, respectively. Results of our study indicated a significantly higher nicotine content at 400 ppm treatment both for ZnO-NP and CH-ZnO treated callus which was (125 and 150%) higher as compared to control callus, devoid of any NPs treatment (Fig. 4a). A higher increase in nicotine content can be attributed to a prolonged availability of zinc from CH-ZnO nano-bioformulations, as an outcome of sustained release of zinc from their chitosan matrix (Cornelis et al. 2014; Choudhary et al. 2019). As the callus were initially chopped and sub-cultured for mass multiplication, which may have induced mechanical wounding and further inclusion of ZnO-NP and CH-ZnO in the media, may have resulted in increased uptake of both the nano-formulations. As wounding induces a damage signal for JA and MeJA biosynthesis, which may further trigger the nicotine biosynthesis (Baldwin et al. 1994). Convincing evidence reveals JA to be a transcriptional inducer for up-regulation of several other genes involved in nicotine biosynthesis, mainly from two distinct families of transcription factors viz. AP2 domain-containing ethylene response factor (ERF) and MYC2-like basic helix–loop–helix (bHLH) gene family (De Sutter et al. 2005; Rushton et al. 2008; Shoji et al. 2010; Todd et al. 2010). In-line with this, Liu et al. (2019) highlighted that exogenous MeJA treatment was able to elevate nicotine production in all transgenic tobacco lines. This could be attributed as a possible reason for enhanced nicotine content up to 400 ppm in both ZnO-NP and CH-ZnO treated callus.

However, a significant reduction of (25 and 50%) in nicotine content was observed in the case of 800 ppm treatment, in callus supplemented with ZnO-NP and CH-ZnO nano-bioformulation, respectively (Fig. 4a). This may due to over accumulation of zinc at a higher dosage, leading to perpetual zinc toxicity which may be negatively influencing the growth and biosynthesis. As evident in our study, reduced biomass, tannin and nicotine content were observed, at 800 ppm treatment, in both ZnO-NP and CH-ZnO nano-bioformulation thus signifying increased toxicity at higher levels. Our results are in accordance with Vankova et al. (2017), where a higher ZnO-NP treatment was reported to decrease the level of JA, a positive regulator of nicotine biosynthesis, where increased suppression in JA levels was recorded in the *Arabidopsis* plants treated with highest ZnO-NP dosage (Vankova et al. 2017). The results of our study signify the potential role of ZnO-NP and CH-ZnO nano-bioformulation in significantly altering the nicotine content in tobacco. In addition, chitin and chitosan can also be used to further influence the total nicotine and tar content in tobacco; however, very few reports exist that highlight its

molecular mechanism of action and to which degree both chitin and chitosan are to be utilized (Austin et al. 1976).

Antioxidant enzyme assay

Phenylalanine ammonia lyase (PAL) (EC 1.10.3.3)

A significant treatment, concentration and (treatment $\times$ concentration) ($P < 0.001$) effect was observed for PAL specific activity (Supplementary Table 1). PAL is the first and committed enzyme of the phenylpropanoid pathway that channels L-Phe (Phenylalanine) from the primary metabolic pool to the synthesis of t-CA (trans-cinnamic acid), which is further transformed in plants into many phenylpropanoid compounds. These phenylpropanoid compounds and their derivatives fulfill several essential functions including mechanical support (lignin) (Whetten and Sederoff 1992), and protectants against biotic and abiotic stress (antioxidants) (Dixon and Paiva 1995). In this report, as compared with the control, the specific activity of PAL was enhanced at 400 ppm concentration by 99 and 213% in ZnO-NP and CH-ZnO treated callus, respectively (Fig. 3c). While maximum reduction (71 and 35%) was observed in ZnO-NP treated callus and (89 and 61%) in CH-ZnO nano-bioformulation treated callus at 50 and 800 ppm, respectively (Fig. 3c). This could be due to relieved stress level at 800 ppm, as evident by other results of our study namely nicotine, H_2O_2 , and MDA content. In this study, nicotine content was positively correlated with enhanced PAL activity at a specific dose. A maximum increase of (99% and 213%) in PAL activity was observed at 400 ppm of ZnO-NP and CH-ZnO nano-bioformulation that perfectly correlates with a higher amount of phenylpropanoid compounds and nicotine content. These findings further signify the role of nanoparticles in stress reduction and enhanced secondary metabolite accumulation. The results of PAL are in agreement with the findings of Pellegrini et al. (1994), where authors reported stimulated levels of PAL and related enzymes of the phenylpropanoid pathway in tobacco (*Nicotiana tabacum* L.) which displayed hypersensitive response against tobacco mosaic virus. Similarly, Hashemi et al. (2016) also observed an increased PAL in soybean treated with 400 ppm of ZnO-NP.

Ascorbate oxidase (AO)

A significant treatment, concentration and (treatment $\times$ concentration) ($P < 0.001$) effect was observed for AO specific activity (Supplementary Table 1). AO is a vital antioxidant enzyme that is known to catalyse the oxidation of ascorbic acid to dehydroascorbic acid via the disproportionation of the monodehydroascorbate radical (De Tullio et al. 2004). Antioxidant enzyme activity further signifies balanced redox homeostasis in plants which implies a significant stress

relief. The specific activity of AO was found to be dose-dependent in CH-ZnO treated callus. AO activity gradually enhanced from (25 to 156%) when the concentration of CH-ZnO nano-bioformulation was increased from 50 to 800 ppm (Fig. 3d). However, in ZnO-NP treated callus, an increase of (80, 26, and 6%) was recorded in the enzyme activity in 50, 200, and 800 ppm treatment, whereas a 16% reduction was observed in 400 ppm ZnO-NP treated callus (Fig. 3d).

The activity of all assessed biochemical parameters, in our study, were found in accordance with AO enzyme activity. AO activity gradually increases in all concentrations of CH-ZnO nano-bioformulation treatment (Fig. 3d). A maximum increase (156%) was reported at 800 ppm of CH-ZnO treated callus. Our study reported gradual enhancement in AO activity with increasing dosage of nanoparticle which proves that AO is a vital enzyme to reduce stress even at higher concentrations. Akram et al. (2017) reported that ascorbic acid can improve tolerance against abiotic stresses by enhancing plant growth, rate of photosynthesis, transpiration, and oxidative defense potential and photosynthetic pigments. Obtained results in our study convincingly justify vital role of AO in improving tolerance against stress even at a higher NP dosage. This further corroborates that increased levels of stress enzymes and biochemical markers are known to alter under stressful regimes and, therefore, substantiate that role of supplemented nano-formulation, in a way, to mitigate the stress levels which could be a potential solution for altering the secondary metabolite levels as well as for achieving enhanced biomass in other crops.

Conclusion

This study experimentally demonstrated the bio-physiological effect of different concentrations of zinc oxide nanoparticles (ZnO-NP) and chitosan-zinc oxide nano-bioformulation (CH-ZnO) on in-vitro grown callus generated from *Nicotiana benthamiana*. The findings of this study suggest that chitosan-based nano-bioformulation showed a greater impact on treated callus by eliciting a better response and also displayed a lesser toxicity when compared to ZnO-NP. Moreover, a significant alteration in level of nicotine and tannin was recorded in the callus treated with NPs. Presently chemical-based bleaching is routinely used to reduce nicotine content which lessens the flavour and texture of products, therefore, utilizing this nano-bioformulation at an optimum dosage, may prove to be viable alternatives for regulating the nicotine content in tobacco. Additionally, as chitosan is widely used for encapsulation and as an elicitor, so future studies may be designed to entrap these nanoparticles using chitosan polymer for enhancing the shelf life of this nano-bioformulations. Further studies are required to understand the action mechanism of chitosan associated

nanoparticles which could be also utilized in other commercially important crops.

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Author contributions DB and KVP participated in planning and conducting the experiments, data interpretation and in drafting the manuscript. MN, MDB helped in finalizing the figures and technical writing. AKD contributed towards data interpretation and helped manuscript corrections. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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