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EFFECT OF ZNO, SIO_2 AND COMPOSITE NANOPARTICLES ON *ARABIDOPSIS THALIANA* AND INVOLVEMENT OF ETHYLENE AND CYTOKININ SIGNALING PATHWAYS

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Abstract

This study encompasses the synthesis and characterization of ZnO, SiO_2 and ZnO/SiO_2 composite NPs by X-ray diffraction (XRD) and Fourier Transform infrared spectroscopy (FTIR) followed by analyzing the effects of these NPs on growth of *Arabidopsis thaliana* at physiological and molecular levels by transcript analysis of selected genes of ethylene / cytokinin pathways. Our data showed toxic effects of ZnO at all concentrations except 10mg/L, while SiO_2 promoted the seedlings growth at almost every concentration. The transcript analysis of ethylene and cytokinin regulated genes provided evidences that ZnO was toxic at all levels except 10mg/L as it induced the expression of ethylene response genes; ethylene being a stress hormone and reduced the expression of cytokinin response genes, cytokinin being positive regulator of growth through involvement in chlorophyll synthesis. SiO_2 application was nontoxic at all concentrations, determined through less expression of ethylene biosynthesis genes and higher expression of cytokinin genes that showed more than two-fold induction in SiO_2 NP treated seedlings. The chlorophyll contents were decreased to almost 50 percent in response to different concentrations of ZnO while remained unaffected or increased by applying SiO_2 NPs. Combined effect of ZnO and SiO_2 i.e. Composite NPs reduced the toxicity caused by ZnO alone which was responsible in reducing the chlorophyll content, overall growth of plant and ethylene production determined through the expression of ethylene regulated genes.

Key words: Absorption, Metal oxide, Nano agriculture, Silicon, Toxicity, Zinc.

Abbreviations: NPs: Nanoparticles, XRD: X-ray Diffraction, FTIR: Fourier Transform Infrared Spectroscopy

Introduction

Nanoagriculture utilizes nanotechnology to increase plant yield for fuel, food and other purposes and opens up an extensive range of possible usages in various areas of science and industry, such as medicine, electronics, biology, pharmacology, and plant breeding (Begum *et al.*, 2014). However, the ever increasing use of nanoparticles can lead to nanotoxicolgy posing a serious threat to human health when released in to atmosphere (Shvedova *et al.*, 2010). Traditionally fertilizers are used for plant growth and development and they still have an important role but most of these fertilizers are inaccessible to plants because of degradation by photolysis, hydrolysis, decomposition and various other factors (Siddiqui *et al*., 2015). Application of nanotechnology and nanoparticles can be helpful to abate nutrient loses and increase crop yield (Siddiqui *et al*., 2015). Nanoparticles are extremely reactive and their size ranges from 1-100nm with one or more dimensions (Hasan, 2015), with prominent physical and chemical properties such as high surface to volume ratio (Stampoulis *et al*., 2019). Nanotechnology on one hand facilitates agriculture and diminishes environmental pollution produced in the manufacturing of chemical fertilizers and pesticides by using the nanoparticles and nano capsules (Sharon *et al.*, 2010) but on the other hand lead to nanotoxicology, as the main route of nanoparticles to enter the soil is via fertilizers and pesticides (Sun *et al.*, 2014). Several metal oxide nanoparticles have been implicated in agriculture to abate nutrient loses.

Zinc as a plant micronutrient serves important role in plant growth and physiological functions and zinc deficiency can lead to stunted growth and less crop yield (Hafeez *et al.*, 2014). Zn is also crucial for human immune system and its deficiency is associated with various problems including memory loss, skin problems and weakened body muscles (Hafeez *et al*., 2008). Zn interacts with multiple nutrients in soil most importantly with nitrogen and phosphorus (Loneragan & Webb, 1993). High phosphorus concentrations are associated with Zn deficiency while zinc depressed the amount of Cu absorption that resulted in less grain yield in wheat (Loneragan & Webb, 1993). The excessive use of these nanoparticles has also proven to be toxic as low concentration of ZnO nanoparticles exhibited beneficial effects on plant growth and development while higher concentrations impaired seed germination (Siddiqui *et al.*, 2015). Similarly, Silicon, though not considered as essential element is advantageous for plant growth as it enhances the photosynthetic rate and helps in fighting various biotic and abiotic stresses such as disease, cold, heat heavy metal and salinity stress (Ma & Yamaji, 2004). SiO_2 nanoparticles improved seed germination in maize by increasing nutrients availability (Suriyaprabha *et al*., 2012). Exogenous application of SiO_2 NPs increased growth of Changbai larch (*Larixolgensis*) seedlings mainly by improving root growth and enhanced chlorophyll synthesis (Bao-shan *et al*., 2004). Zn in combination with Silicon in form of Zn-silicate has been reported as part of heavy metal tolerance (Neumann & Nieden, 2001). It was recommended that it may be useful for the mitigation of Zn toxicity in *Cardaminopsis* (Neumann & Nieden, 2001).

Plant hormones are dynamic organic materials that regulate plant physiological responses and assists the reactions to challenges that a plant could encounter in an environment (Santner *et al.*, 2009). The level and activity of plant hormones reflects a significant index of toxicity in plants (Gui *et al.*, 2015). The toxic effects of γFe2O3 Nanoparticles was evident by increased levels of IAA and ABA levels in the roots of transgenic and non-transgenic rice (Gui *et al.*, 2015) while CeO2 NPs treated leaves of Bt-transgenic and conventional cotton had no significant difference on IAA:indole-3-acetic acid, ABA:abscisic acid and GA:Gibberellic acid as compared to control group (Nhan *et al.*, 2016). ZnO NPs induced toxicity and inhibited *Arabidopsis* growth via involvement of ethylene signaling by accumulation of ROS and increased lipid peroxidation while the ethylene insensitive mutants showed tolerance against oxidative damage due to ZnO NPs (Khan *et al.*, 2019).

Our explicit aim was to find the effects of different concentration of nanoparticles ranging from 0-500mg/L and finding the best working concentration in terms of growth in plants. Furthermore, we aimed to find if ZnO/SiO2 composite NPs can provide a solution to reduce zinc toxicity and can be used more efficiently together by determining the physiological effects i.e. root growth and chlorophyll synthesis along with the expression analysis to assess the involvement of phytohormones mainly ethylene and cytokinin which influence physiological processes in plants and being chemical messengers coordinate cellular activities of plants as an aspect of finding toxicity or growth promotion of plants induced due to application of variable concentration of nanoparticles.

Materials and Methods

Synthesis of nanoparticles: NPs were synthesized by following two different procedures i.e. co-precipitation and sol-gel method. Zinc oxide NPs were prepared by using co precipitation method with zinc sulfate and sodium hydroxide as starting materials. 1M aqueous solution of Zinc sulfate was added dropwise into 2M sodium hydroxide under vigorous stirring for 12 hours. Filtration was carried out to obtain precipitates and washed completely with deionized water. The obtained precipitates were dried at 100ºC and ground to fine powder using agate mortar. The obtained powder was calcinated at 100º for 2 hours (Sebők *et al*., 2008). For synthesis of NPs analytical grade chemicals were used and all reagents solutions were prepared in deionized water. For the preparation of SiO_2NPs by sol gel method hydrolysis and condensation of Tetra ethylorthosilicate (TEOS) takes place as a precursor of Silica. Tetra Ethylorthosilicate was mixed in absolute ethanol with the subsequent addition of tartaric acid, water and ammonia solution. The resulting filtrate was dried in oven at 100ºC to obtain SiO_2 NPs (Hench & West 1990). ZnO/SiO_2 Composite NPs were prepared by refluxing of ZnO and SiO_2NPs dissolved in (Tetrahydrofuran) THF for 6 hours at 80ºC with constant stirring then filtered and dried at 100ºC.

Characterization of nanoparticles: To characterize NPs, FTIR and XRD was used for confirmation of their chemical structure, purity, crystallinity, phase distribution, size and shape. FTIR and XRD was performed using Thermo Scientific, Nicolet 6700 USA and X'Pert PRO, PANalytical, the Netherlands (Holland) respectively for the confirmation of chemical structure of all ZnO, SiO_2, ZnO/SiO_2 and composite NPs.

Plant material and growth conditions

Seed sterilization: ***Arabidopsis*** *thaliana* ecotype Columbia wild type was used for all the experiments. Seeds were sterilized by one-minute wash with 70% ethanol followed by wash with 20% bleach solution containing 1% Triton X- 100 for 20 minutes. Subsequent washes with sterile deionized distilled water were done to ensure complete removal of bleach.

Growth conditions: The effects of ZnO, SiO_2, and ZnO/SiO_2 composite were tested on Arabidopsis seedlings by growing them in the presence of increasing concentrations of the NPs (0,10,50,100 and 500mg/L). For each concentration of the NPs used, the NPs were dissolved in half strength Murashige and Skoog media with Gamborg's vitamins (plant growth medium, Sigma Aldrich, Catalogue # M0404), and the pH was set to 5.7 with 1M KOH. For complete dispersion of NPs in media, sonication was done for 30 min in a water bath. 0.8% (w/v) phytoagar (SKU# 40100072) was added and autoclaved afterwards. Square plates (25*15mm) were prepared from the media for each concentration ranging from 0-500mg/L of ZnO, SiO2 and composite NPs and seeds were plated. After two of day's stratification at 4°C, plates were placed in percival growth chamber and grown for 10 days at 22°C with 16 h day/ 8 h night cycle and light intensity of 200 $\mu M/cm^2/s^1$. The experiment was done in triplicates, for each concentration three plates were grown under same conditions for all the nanoparticles.

Root growth measurement: Seedings were scanned after growing them for 10 days in light. Root length was measured with ImageJ software version: 20 seedlings for each concentration of nanoparticles were analyzed for root growth.

Chlorophyll measurement: To determine the effect of ZnO, SiO_2, and ZnO/SiO_2 composite NPs on photosynthetic activity, Chlorophyll a b levels were determined in triplicates by the protocol described by Lichtenthaler (1987) . Chlorophyll was extracted from finely ground leaves with 95% (v/v) ethanol incubated at 4°C overnight in dark on shaker incubator. The supernatant was taken and OD at 664 and 648 was measured by using the BIORAD SmartSpec Plus spectrophotometer.

Quantitative real time PCR: To analyze gene expression total RNA was isolated from 10 days old Arabidopsis seedlings grown +/- NPs with three biological replicates for each treatment by using E.Z.N.A

Omega kit for RNA extraction (cat# R6827). To prepare cDNA from extracted RNA was first given DNase treatment with TURBO DNA-free kit (Amibion by Thermofisher Scientific) according to manufacturer's instruction. Then First strand cDNA synthesis was done using BIORAD iScript III cDNA synthesis kit. *β* tubulin was used as control gene to normalize the expression of ethylene/cytokinin primary response genes.

RT-qPCR was carried out in a 96-well reaction plate by preparing reaction mixture containing 5uliTaq Universal SybergreenSupermix (BioRad), 3ul deionized distilled H_2O, 1ul Forward + Reverse primer and 1ul of diluted cDNA. For relative expression *ß*-tubulin was run as an internal control for each target gene by determining the fold difference between the internal control and the target genes. All the transcripts were amplified in triplicates using gene specific (Table 1). Statistical analysis was done using One-way ANOVA with post-hoc Tukey HSD Test and Bonferroni and Holm Test calculator between all the treatments and control. (https://astatsa.com/OneWay_Anova_with_TukeyHSD/).

Statistical analysis

For qRT-PCR and chlorophyll content, one-way analysis of variance (ANOVA) was used to measure differences between treatments followed by Bonferroni and Holm post-hoc test where significant differences were found at (p<0.05).

Results

Synthesis and characterization of nanoparticles: Zinc oxide (ZnO) NPs were synthesized by using a simple precipitation method (Sebők *et al*., 2008). Sol-gel method was applied for preparation of Silica NPs (Hench & West 1990). Similarly, ZnO/SiO_2 Composite NPs were prepared by refluxing of ZnO and SiO_2NPs dissolved in (Tetrahydrofuran) THF. NPs were first optically characterized by FTIR spectroscopic technique then morphological characterization done by XRD analysis.

FTIR (Fourier Transform Infrared Spectroscopy) Spectra of newly synthesized NPs has confirmed the expected chemical structure: Fourier transform infrared spectroscopy (FTIR) spectrum was done for the confirmation of chemical structures of all ZnO, SiO_2 and ZnO/SiO_2 composite NPs. FTIR gives the information about functional groups present in the synthesized NPs for understanding their transformation from simple inorganic chemicals to elemental NPs. The FTIR spectrum for ZnO displayed a broad peak at the region of 3241 cm^{-1} which showed the O−H bond stretching vibration. The characteristic absorption peaks at 1509 cm^{-1} and 725 cm^{-1} specified the Zn−S stretching vibration (Fig. 1A). The absorption spectrum for SiO_2 presented peaks at 1055 to 938 cm^{-1} and 2360 cm^{-1} that corresponded to the Si−O stretching vibration. The characteristic absorption peaks at 795 cm^{-1} and 3244 cm^{-1} showed Si−O and O−H stretching vibration respectively. The presence of characteristic peak at 1661 cm^{-1} was as a result of stretching and bending vibration of H−O−H (H_2O) (Fig. 1B). FTIR spectra of composite NPs showed characteristic absorption peaks at 3285 cm^{-1} specified the O−H stretching vibration present in ZnO/SiO_2 composite NPs (Fig. 1C). The presence of characteristic absorbance peak at 2357 cm^{-1} was due to of Zn−S. The sharp absorbance peaks at the region of 1059 cm^{-1} and 799 cm^{-1} showed Si−OH and Si−O stretching vibration respectively.

XRD Spectrum of newly synthesized NPs has confirmed the nature of particles: Among the group of non-destructive characterization techniques, X-ray scattering technique or XRD is usually used to study crystalline nature, physical properties and chemical composition of thin films as well as other materials. This technique relies on recording the intensity of scattered X-ray beam falling on sample as a function of energy or wavelength, polarization and incident and scattered angle.

XRD patterns of all the three types of particles were synthesized at distinct temperatures (Fig. 2). Series of the obtained diffraction peaks at 20°, 27°, 31°, 34°, 36°, 47°, 56°, 62° and 68° correspond to the (100), (002), (101), (102), (110), (103), (200), (112) and (201) planes were observed in XRD spectrum of ZnO NPs (Fig. 2A) and these peaks agreed to the ZnO structure (International Center for Diffraction Data, JCPDS 5-0664). There were no impurity peaks seen that provideed evidences of highly pure synthesis of ZnO NPs. Further, the peaks were widened (Radzimska & Jesionowski, 2014) suggesting that the small size of the particles (the average crystallite size was 41±1 nm).

Table 1. List of selected primers used for ethylene and cytokinin pathway genes.

Gene	Forward primer	Reverse primer
B-tubulin	TGGTGGAGCCTTACAACGCTACTT	TTCACAGCAAGCTTACGGAGGTCA
ACS	TTACGGAGAAGTACATTAGG	AACCTCCTTCGTCGGTCCAT
ACO-2	GAGTGTGCTGCACCGTGTGG	CATTGCTGCGAACCGTGGCT
ETR2	AGAGAAACTCGGGTGCGATGT	TCACTGTCGTCGCCACCATC
ERF1	TCTAATCGAGCAGTCCACGCAACA	AACGTCCCGAGCCAAACCCTAATA
ARR7	AGAGTGGAACTAGGGCTTTGC	CTCCTTCTTTGAGACATTCTTGT
ARR15	CTGCTTGTAAAGTGACGACTGT	AGTTCATATCCTGTTAGTCCCG

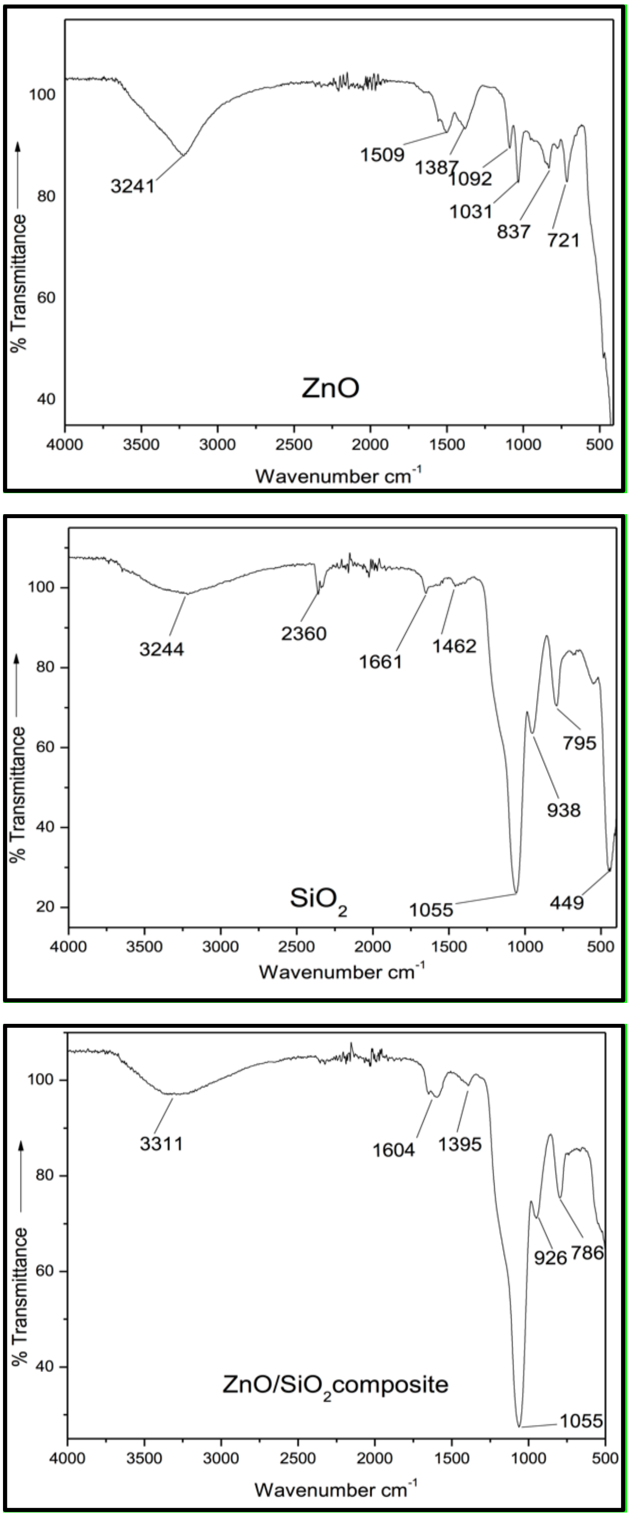

Fig. 1. FTIR Spectrum of NPs, (A) ZnO NPs showing various Zn−O and Zn−S stretching vibration modes and the contribution of the ambient zinc sulfate ($ZnSO_4$), sodium hydroxide (NaOH) and water. (B) SiO_2 showing various Si−O vibration modes and the contribution of the ambient carbon dioxide and water. (C) ZnO/ SiO_2 Composite NPs displaying different characteristic peaks of Si−O and Zn−S stretching vibration modes and the contribution of the carbon dioxide and water.

The XRD analysis of the SiO_2 NPs showed characteristics diffuse peak at about 2θ=20º (Fig. 2B). Our data suggested that the high percentage of these silica particles were amorphous and few of them were crystalline as observed by Tabatabaei *et al*., (2006). Our composite NPs contained equal amounts of ZnO and SiO_2 NPs, so we were expecting crystalline nature due to ZnO NPs and amorphous nature due to SiO_2NPs. While, the spectrum specific absorption peaks of crystalline ZnO NPs were prominent and showing that presence of SiO_2 nanoparticles but were suppressed by presence of ZnO peaks. The spectrum of composite peaks at 31º, 34º, 36º, 47º, 56º, 62º, 68º and 69º, and all the peaks were pretty in accordance with ZnO NPs structure. This demonstrates that the high percentage of ZnO/SiO_2 composite NPs were crystalline, and few were amorphous in nature (Fig. 2C) as evident from lower basal level of SiO_2in comparisons of the XRD spectra of ZnO, SiO_2 and composite NPs (Fig. 2D). Spectrum of composite displayed diffused peaks of SiO_2NPs that was overlapped by characteristic absorption peaks of ZnO NPs. Absence of any peak of impurities in all the synthesized NPs has confirmed their pure preparations.

Effect of SiO_2, ZnO and composite NPs on *A. thaliana* growth: ZnO possessed toxic effects while SiO_2 promoted seedlings growth, composite showed intermediate effect: To determine the potential effects of the newly synthesized NPs, *Arabidopsis* seedlings were grown on medium containing different concentrations of all the NPs individually, ranging from 0-500 mg/L. Increasing concentration of NPs showed variable effects depending upon the type of NPs. We observed that almost 10mg/L concentration of all the NPSs under study promoted the growth of *Arabidopsis* seedlings, but higher concentrations showed marked difference in growth responses as shown in Fig. 3. ZnO NPs were toxic at higher concentrations while SiO_2 NPs promoted *Arabidopsis* growth in almost all of the concentrations even at 500mg/L while composite NPs which were mixture of equal amounts of ZnO and SiO_2 NPs showed combined effect of both the types of NPs on Arabidopsis growth. Fig. 4A showed the toxic effect of ZnO on seedlings, the root length was significantly reduced with increasing concentration of the NPs, SiO_2 promoted root growth while composite nanoparticles showed less toxic nature as compared to ZnO alone as the root length was significantly greater compared to respective ZnO concentrations. Fig. 4B presents the dose response curves of root length of seedlings grown and exposed for 10 days to each nanoparticle treatment.

ZnO NPs showed evidences of toxicity, SiO_2 NPs promoted the seedlings growth, while composite exhibited the combined effect: We measured chlorophyll contents of leaf tissue from ten days old green seedlings grown on media containing different doses of NPs. ZnO suppressed the levels of chlorophyll a and b with increasing concentration of NPs suggesting toxicity due to application of ZnO. ZnO NPs has led to reduction in chlorophyll a and b, while SiO_2 promoted chlorophyll synthesis even at higher concentrations. The combined effect of ZnO and SiO_2 composite NPs was overpowered by SiO_2 at lower concentrations i.e. the chlorophyll content was higher at 10mg/L, while significant reduction was observed at higher concentrations suggesting the pronounced effect of ZnO toxicity over SiO_2 leading to reduced chlorophyll contents and retarded growth (Fig. 5A&B).

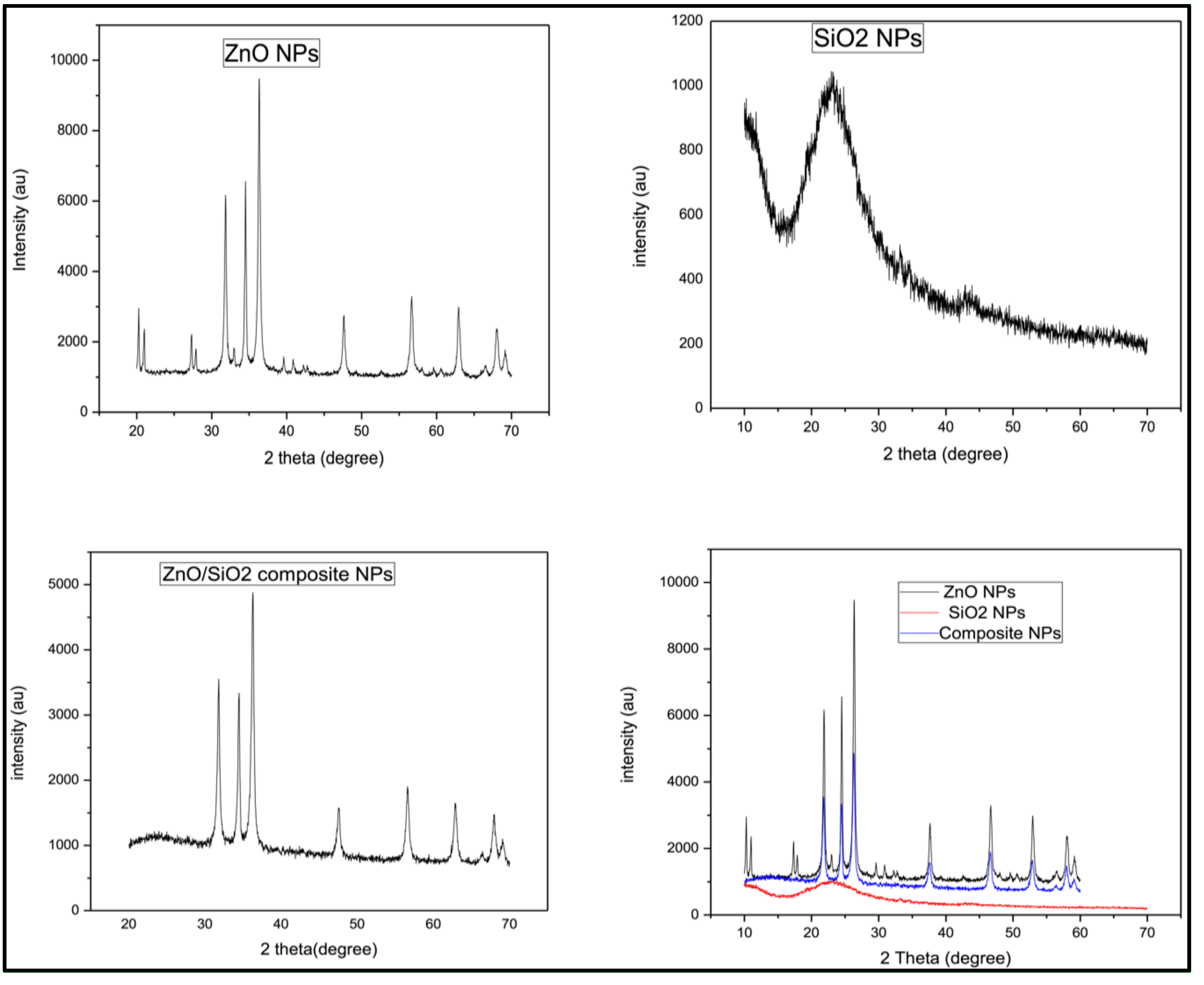

Fig. 2. XRD spectrum of ZnO, SiO_2 and ZnO/ SiO_2 Composite NPs at different temperatures during synthesis. (A) ZnO: Characteristic peaks at specific values of 2theta showing purity and crystalline nature of ZnO NPs. (B) SiO_2: Diffused peaks in SiO_2 spectrum showing the amorphous nature of SiO_2NPs. (C) ZnO/ SiO_2 Composite NPs: Characteristic sharp and diffused peaks in this spectrum showing crystalline and amorphous nature of ZnO/SiO_2 composite. (D) Comparison of XRD spectrum of all the NPs.

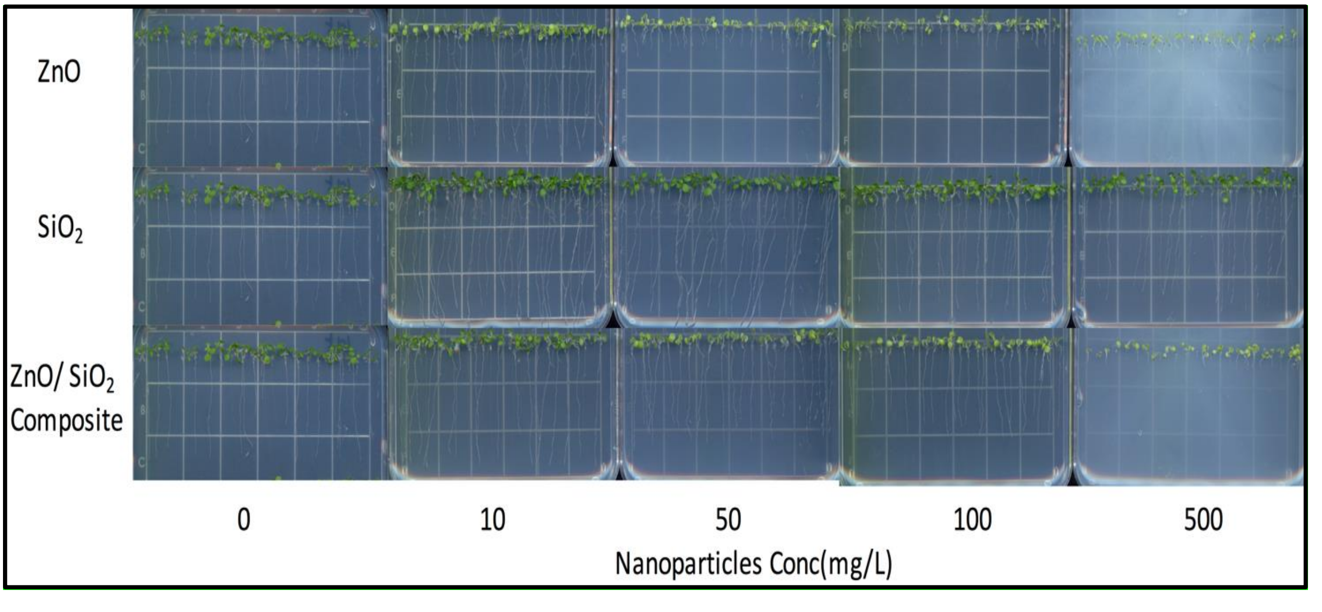

Fig. 3. Overall effect of NPs on *Arabidopsis thaliana* growth. Seedlings grown in the presence of ZnO, SiO_2 and ZnO/ SiO_2 Composite NPs for 10 days in square plates with 1/2MSNS media.

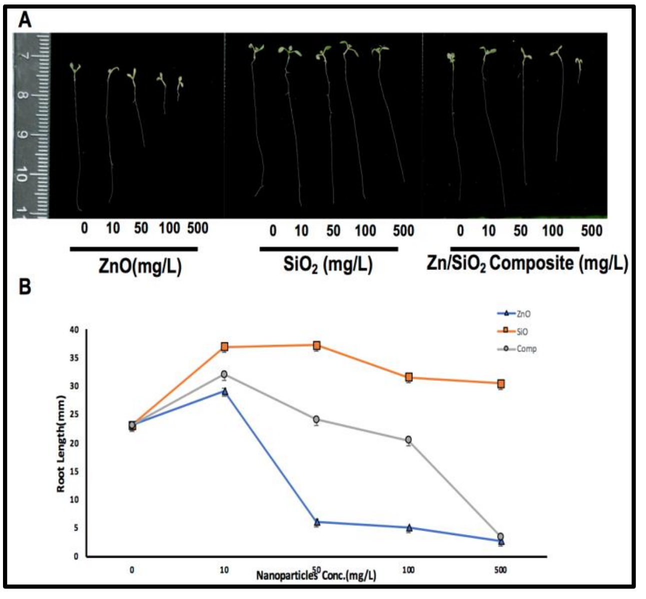

Fig. 4. Effect of NPs on *Arabidopsis thaliana*. A. Seedlings grown in the presence of ZnO, SiO_2 and ZnO/ SiO_2 Composite NPs for 10 days B: Dose-response curves of ZnO, SiO_2 and ZnO/ SiO_2 Composite NPs on root growth of *Arabidopsis thaliana*. The values were given as meanˇSE (standard ERROR) with 20seedlings each, ZnO and composite NPs reduced the root length while SiO_2 has positive effects.

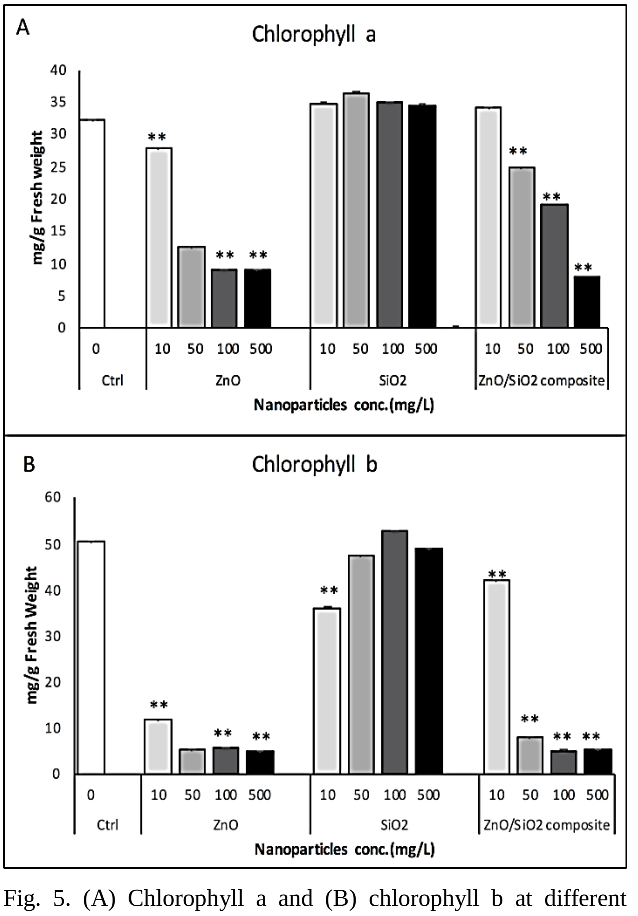

Fig. 5. (A) Chlorophyll a and (B) chlorophyll b at different doses of ZnO, SiO_2 and ZnO/ SiO_2 Composite NPs, showing significant reduction at higher doses of ZnO, increase in chlorophyll b with SiO_2 while composite NPs shows the combined effect .Asterisks (*) represents significant difference in chlorophyll content from control (*; p≤ 0.05, **; p≤ 0.01 from One-way ANOVA with Bonferroni and Holm Test).

Expression of ethylene and cytokinin regulated genes provided evidences of the involvement of ethylene and cytokinin pathways in NP induced plant responses: In order to evaluate the role of ethylene and cytokinin signaling in response to ZnO, SiO_2 and composite NPs, we analyzed the transcript levels of several ethylene related genes likeAcetyl-CoA Synthetase (ACS) and ACC Oxidase (ACO) involved in ethylene biosynthesis, Ethylene Response 2 (ETR2) and Ethylene Response Factor 1 (ERF1) involved in ethylene perception and signaling, and Response Regulators (ARR7 and ARR15) involved in cytokinin signaling pathway in whole seedlings grown in the presence of different concentrations of ZnO, SiO_2 and ZnO/SiO_2 composite NPs. The expression of all ethylene related genes was significantly increased ($p<0.01$) compared to control in response to all ZnO concentrations except 10mg/L. There was 10 fold inductions in ETR2 at 50mg/L and 100mg/L while 6 folds at 500mg/L (Fig. 6). Similarly, ERF1 showed 50 folds and 80 folds induction at 50mg/L and 100/500mg/L respectively compared to control (Fig. 6). The expression of ACS gene was 10 folds higher at 50mg/L and further increased to almost 25 folds at 100 and 500mg/L while there was 2 to 3 folds induction in ACO2 at higher concentrations of ZnO (Fig. 6). SiO_2 have no significant effect on the expression of ETR2, ERF1 and ACS while ACO2 showed 2-fold induction at higher concentrations (Fig. 5). The expression of ETR2 and ERF1 was significantly increased in response to higher concentrations (100,500mg/L) of composite NPs ($p<0.01$) while ACS and ACO2 were significantly induced at 100mg/L and then turnover started at 500mg/L (Fig. 6).

Arabidopsis response regulators ARR7 and ARR15 involved in cytokinin signaling were downregulated due to application of ZnO NPs. The expression of ARR15 was significantly reduced with increasing concentrations of ZnO ($p<0.01$) (Fig. 7), while there was significant 2 fold induction observed in both ARR7 and ARR15 at 100 and 500mg/L in response to SiO_2 NPs (Fig. 7). No significant difference in ARR7 expression was observed, while transcript level of ARR15 gene was significantly reduced compared to control at all the given concentrations of composite NPs (Fig. 6). Ethylene most commonly known as stress hormone negatively regulates cell growth and development and higher levels of ethylene have been reported under several metal stresses (DalCorso *et al.*, 2010; Khan *et al.*, 2015), here the increased expression of ethylene regulated genes suggested the involvement of ethylene signaling in growth reduction which was consistent with the findings by Khan *et al*., (2019) under ZnO NPs where ZnO was shown in repressing plant growth by inhibiting cell cycle genes, Cytokinins on the other hand positively regulates plant growth thus increase in cytokinin response genes is positively correlated with the growth.

Discussion

NPs have variable effects on plant growth and development depending upon the plant species and properties of nanoparticles i.e. nature, composition, reactivity and the dose of NPs administered (Khodakovskaya *et al.*, 2012). There are several methods to synthesize metal oxides and metal nanoparticles (Aitken *et al.*, 2006). High energy ball milling melt mixing and physical vapor deposition etc are physical methods of ZnO nanoparticles synthesis. Plants, fungi, algae, bacteria, and viruses have also been reported for ZnO synthesis by biological method (Haq *et al.*, 2017). Silica nanoparticles have been synthesized by several methods like reverse microemulsion, flame synthesis, high temperature flame decomposition and the most widely used sol-gel procedure (Rahman & Padavettan, 2012). Here we successfully synthesized ZnO and SiO2 NPs by a simple precipitation method using zinc sulphate and sol gel method respectively. Nanoparticles are characterized by Fourier Transform Infrared Spectroscopy and X-ray Diffraction analysis, that gave the structure and purity of elements present in the respective NPs (Figs. 1&2).

For determining the possible positive or toxic effects of newly synthesized NPs, *Arabidopsis*, seedlings were grown for 10 days on media supplemented with different concentrations (0,10,50,100 and 500mg/L) of ZnO, SiO_2 and composite NPs. ZnO NPs exhibited reduced growth due to toxic effects of ZnO on overall plant growth as NP toxicity is usually characterized by reduced plant/cell size and necrotic leaves. Dose response including the exposure 50, 100 and 500 mg/L of ZnO NPs for ten days resulted in significant decrease in root length, accompanied with yellowing of leaves with increased concentrations of ZnO NPs. Interestingly, no toxic effect of SiO_2 on plant growth was observed for all the concentrations used, although root growth was either enhanced or had no effect of SiO_2 as compared to control seedlings suggesting the beneficial nature of these NPs. Similar kind of effect causing almost 50% and 67% reduction in root length of ZnO NPs treated maize and rice respectively were observed (Yang *et al.*, 2015). SiO_2 NP has also shown positive effects when used at lower concentrations (50 and 100mg/L) on growth of wheat seedlings though higher concentrations showed decrease in growth (Karimi & Mohsenzadeh, 2016). Silicon itself is very important for the plants and several beneficial effects of Si are known for example increased photosynthetic ability, decreased mineral toxicity, increased insect and disease resistance, improved drought and cold tolerance and improvement of nutrient imbalance (Ma *et al.*, 2001). SiO2 NPs have proven to be efficient in controlling drought and salinity stress for wheat in salt effected areas in Pakistan (Mushtaq *et al.*, 2019). Exposure of seedlings to ZnO/SiO_2 composite NPs resulted in decreased plant growth and root length with increasing concentrations, but the level of toxicity was less than caused by ZnO NPs alone showing that SiO_2 complemented the Arabidopsis seedling growth by its positive effects.

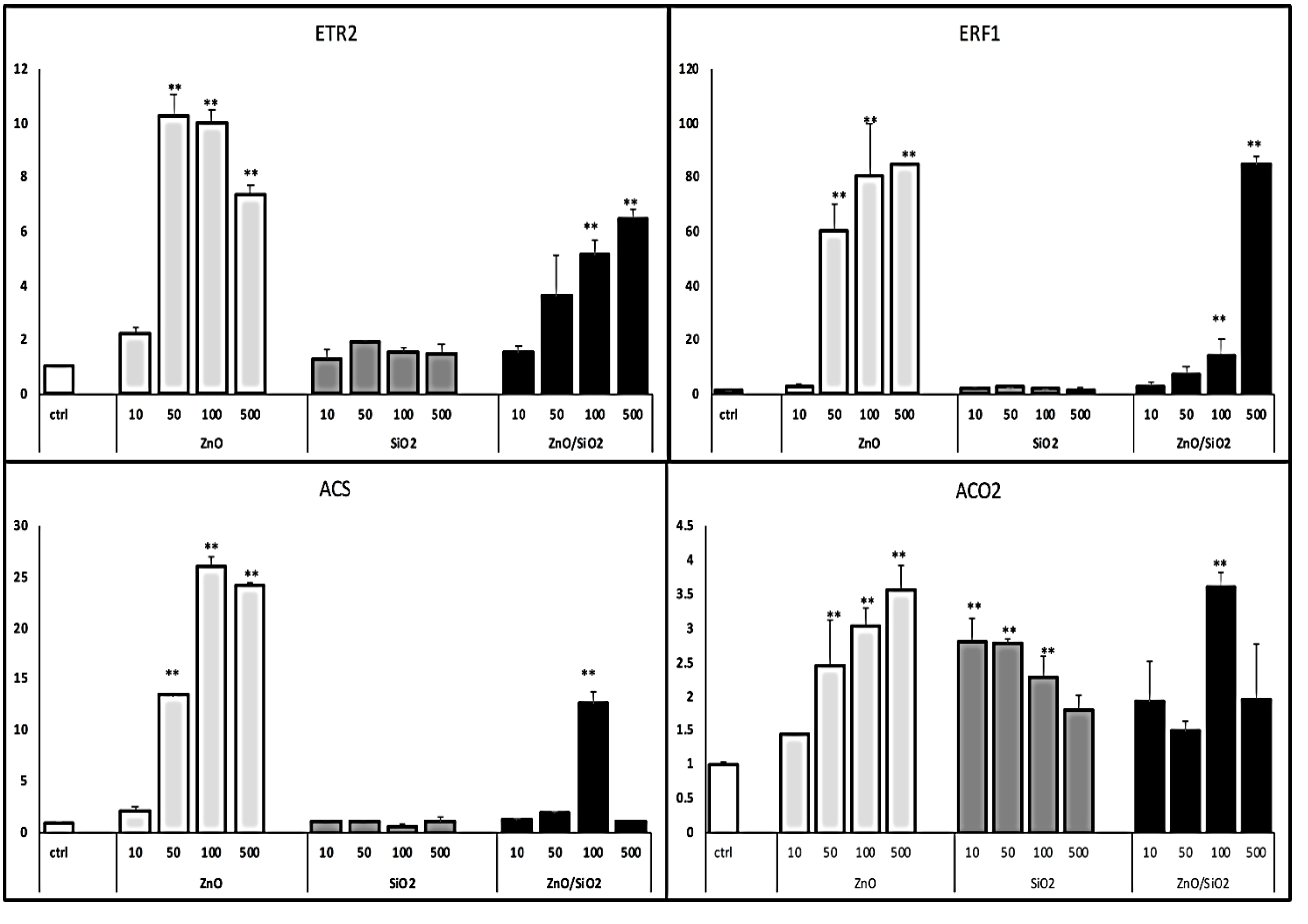

Fig. 6. Transcript levels of ethylene biosynthesis/pathway genes in response to different doses (0,10,50,100,500 mg/L) of ZnO, SiO_2 and ZnO/ SiO_2 Composite NPs. Asterisks (*) represents significant difference in expression from control (*; $P \leq 0.05$, **; $P \leq 0.01$ from One-way ANOVA with Bonferroni and Holm Test).

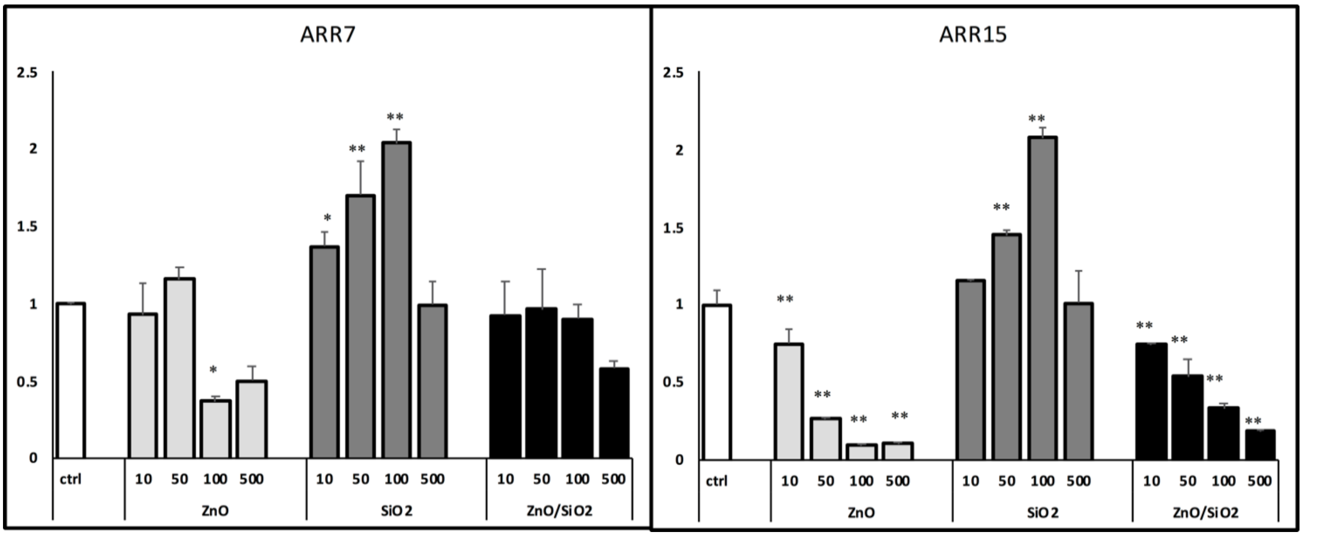

Fig. 7. Transcript patterns of cytokinin regulated and Catalase enzyme gene in response to different doses (0,10,50,100,500 mg/L) of ZnO, SiO_2 and ZnO/ SiO_2 Composite NPs Asterisks (*) represents significant difference in expression in comparisons to control plants (*; $p \leq 0.05$, **; $p \leq 0.01$ from One-way ANOVA with Bonferroni and Holm Test).

Plant photosynthetic activity depends upon chlorophyll contents which can also provide an indication of stressed conditions. Reduction in chlorophyll a and b due to ZnO NPs is consistent with chlorophyll degradation in this particular study due to ZnO, may be leading to induced stressed conditions and ethylene production under stressed conditions. Several studies reported chlorophyll degradation and inhibitory effects of NPs on plant growth (Khan *et al.*, 2019) like, application of CuO, TiO2 and CeO_2 NPs significantly reduced the chlorophyll contents in Arabidopsis (Szymańska *et al.*, 2016). The increased chlorophyll content by application of SiO_2 NPs in our experimental conditions was in accordance with the previous studies where SiO_2 promoted plant growth by increasing photosynthesis rate, activity of the photosystem II, stomatal conductance electron transport rate and photochemical quenching (Xie *et al*., 2011; Siddiqui *et al*., 2015).

Phytohormones are crucial for plants interactions under any specific conditions or environment. Similarly, ethylene a simple gaseous hormone is also referred as stress hormone, plays vital role in growth and development of plants (Abeles *et al*., 1992)while cytokinin is known by its ability to promote plant cell divisions , and also regulate the chloroplast development, shoot initiation and delayed senescence (Haberer & Kieber, 2002). As we observed similar kind of hormonal regulated response in our newly synthesized NPs treated seedlings, our data revealed an increased expression of all the ethylene regulated genes in response to ZnO NPs providing an evidence of the toxic nature of these particles which interns induce stress leading to ethylene production. While SiO_2NPs showed no significant difference in the expression of ethylene regulated genes which is coherent with the beneficial nature of SiO_2 NPs (Yang *et al*., 2015). The composite of ZnO and SiO_2 NPs demonstrated relatively less transcript expression of ethylene regulated genes than ZnO alone even at lower concentrations, whereas at the highest concentration i.e. 500mg/L ETR2, ERF1, ACS and ACO2 showed almost the same effect as ZnO alone that was in accordance with the overpowering effect of ZnO on Arabidopsis growth. The ethylene receptor ETR2 is mainly involved in ethylene perception and ERF1 is involved in defense responses related to ethylene signaling. The induction of these genes in response to ZnO is related to increased production of ethylene indicated by the high expression of ethylene biosynthetic genes, ACS and ACO2 followed by growth inhibition due to stress as described by Khan *et al*., (2019). Similarly, ACS is another vital enzyme in the ethylene biosynthesis pathway and its expression increases by the ethylene-mediated processes like wounding and fruit ripening by regulating ethylene production under biotic and abiotic stress (Yang & Hoffman. 1984; Wang *et al*., 2009). ACO, the enzyme that catalyzes the final step in ethylene biosynthesis. Expression of ACO genes is also known to be inducible by ethylene, as well as auxin and gibberellic acid (Calvo *et al*., 1997). The elevated expression of these genes suggests ethylene induced stress in response to ZnO NPs. Upregulation of ethylene regulated genes has already been observed in response to Ag2S- NPs (Wang *et al.*, 2017).

The genes involved in cytokinin regulation such as ARR7 and ARR15 exhibited less expression with increasing concentrations of ZnO NPs while the SiO_2 showed enhanced expression of these genes, following the trend in all other genes the composite NPs had more pronounced effect of ZnO hence decreased transcript of ARR7 and ARR15 with increased concentration of NPs. Elevated level of cytokinin primary response genes indicates up regulation of cytokinin signaling pathway and playing vital role in plants growth by promoting cell division and differentiation. Seedlings treated with SiO_2 had higher chlorophyll content compared to control and other treatments, thus supporting up regulation of cytokinin signaling pathway. Cytokinins are involved in transcriptional induction of GATA NITRATE-INDUCIBLE CARBON-METABOLISM-INVOLVED (GNC) and CYTOKININ-RESPONSIVE GATA1

(CGA1). GNC and CGA1, in turn are involved in development of chloroplast from proplastids and also, they are further involved in growth and division of chloroplasts (Zubo *et al*., 2018). The toxic effects of ZnO and the involvement of ethylene signaling have already been described. The growth inhibition is mainly contributed by ethylene which also induces oxidative damage under ZnO NPs stress, supported by the elevated expression of ethylene related genes in response to ZnO NPs. (Khan *et al.*, 2019). Ethylene modulates the stress responses through ROS scavenging mechanism under salinity stress (Zhang *et al.*, 2016).

Our data provides comprehensive comparison of ZnO NPs with SiO_2 NPs_2 and composite NPs suggesting beneficial effects of SiO_2 at all concentrations as shown by the growth parameters and transcript levels of the ethylene and cytokinin related genes. ZnO/SiO_2 (composite) NPs broadened the range of concentration where ZnO can be used in combination with SiO_2 revealed through the growth and expression analysis so ZnO NPs can be utilized in combination with SiO_2 exploiting their beneficial nature hence reducing the toxicity of ZnO NPs and promoting plant growth by uptaking both nanoparticles. ZnO NPs, most widely used and more toxic in nature at higher concentrations can be used beneficially in combination with SiO_2 NPs that are known for their beneficial effects. Like ZnO/SiO_2 composites, other combinations can also be tested for their possibility to be used together to reduce environmental toxicity, where one or both NPs alone have a predominat toxic nature and a threat to the environment. Also, as the NPs effects are species specific, different crops can be screened out having fewer toxic effects of these NPs and can be planted those in the industrial area or where there is danger of toxicity of these NPs to reduce environmental toxicity.

Conclusion

SiO2 NPs have beneficial effects on plants while ZnO exhibit negative effects on plant growth. The effect of both NPs on the growth of *Arabidopsis thaliana* was examined and it was revealed that ZnO/SiO_2 composite NPs can be used at a wider range in comparison to ZnO alone as Nano fertilizer for increased plant yield. Composite nanoparticles provide a solution used in combination with SiO_2 to reduce toxicity level though at the lower concentrations but offering more possibilities to be used for enhancing plant growth and development. Overall this study provided evidence of involvement of ethylene pathway in ZnO toxicity as the expression level of ethylene biosynthesis genes was significantly enhanced while upregulation of cytokinin pathway genes due to application of SiO_2 also proven by its beneficial effects on growth. Furthermore, research on the molecular mechanisms associated with interaction between ZnO, $SiO_{2,}$ and composite NPs and involvement of phytohormones needs to be conducted.

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