Biofabricated zinc oxide nanoparticles coated with phycomolecules as novel micronutrient catalysts for stimulating plant growth of cotton

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Abstract
This study describes the bioengineering of phycomolecule-coated zinc oxide nanoparticles (ZnO NPs) as a novel type of plant-growth-enhancing micronutrient catalyst aimed at increasing crop productivity. The impact of natural engineered phycomolecule-loaded ZnO NPs on plant growth characteristics and biochemical changes in Gossypium hirsutum L. plants was investigated after 21 days of exposure to a wide range of concentrations (0, 25, 50, 75, 100, and 200 mg l⁻¹). ZnO NP exposure significantly enhanced growth and biomass by 125.4% and 132.8%, respectively, in the treated plants compared to the untreated control. Interestingly, photosynthetic pigments, namely, chlorophyll a (134.7%), chlorophyll b (132.6%), carotenoids (160.1%), and total soluble protein contents (165.4%) increased significantly, but the level of malondialdehyde (MDA) content (73.8%) decreased in the ZnO-NP-exposed plants compared to the control. The results showed that there were significant increases in superoxide dismutase (SOD, 267.8%) and peroxidase (POX, 174.5%) enzyme activity, whereas decreased catalase (CAT, 83.2%) activity was recorded in the NP-treated plants compared to the control. ZnO NP treatment did not show distinct alterations (the presence or absence of DNA) in a random amplified polymorphic DNA (RAPD) banding pattern. These results suggest that bioengineered ZnO NPs coated with natural phycochemicals display different biochemical effects associated with enhanced growth and biomass in G. hirsutum. Our results imply that ZnO NPs have tremendous potential in their use as an effective plant-growth-promoting micronutrient catalyst in agriculture.

Keywords: antioxidative enzymes, Gossypium hirsutum, zinc oxide nanoparticles, plant-growth-stimulating nanocatalyst, phycomolecules

Classification numbers: 2. 04, 2.10, 4.02

1. Introduction

Engineered nanomaterials are considered for crop improvement programs in the field of nanogriculture [1]. The application of nanotechnology could increase agricultural productivity substantially by developing suitable nanogrowth-promoting compounds and nanofertilizer [2, 3]. Fertilizers have also played a vital role in promoting plant growth and increasing crop productivity in the recent past. The major problem encountered is that the applied fertilizers are often exhausted due to rapid degradation by various chemical reactions such as photolysis, hydrolysis and decomposition [3]. To overcome this problem, novel nanomaterial-based
micronutrient fertilizers could have some important properties, including the targeting of specific soils and the slow release of essential nutrients into the soil. Engineered nanomaterials could have great potential in nanofertilizers and are likely to encounter challenges regarding higher fertilizer consumption and agricultural soil pollution. Recently, the application of nanomaterial-based nanofertilizers has improved the absorption of soil nutrients, including P and water, with increased crop productivity [4, 5].

In recent years, different types of nanomaterials have become more attractive in agronanotechnology because of their promising effects on crop plants [6]. The nanomaterials are easily absorbed—similar to organic molecules and inorganic ions from the nutrient solution—inducing various physiochemical changes such as the growth and development of plants and biomass etc. However, the influence of nanomaterials can vary greatly and depends on the dose, type, shape, structure, duration and solubility of the nanoparticles used [7]. The development of low-cost nanomaterials which are not toxic to plants is highly essential for the effective use of nanotechnology in agriculture. Nanoagriculture is mainly aimed at crop improvement relating to superior plant growth with outstanding yield. The role of different nanomaterials on various plants has been studied and most have reported phytotoxicity effects [7–11]. However, most of the reports describe the impact of different nanomaterials at the seedling stage.

The occurrence of oxidative stress is one of the major biochemical alterations following nanoparticle exposure, and it disturbs the balance between cell function and antioxidative defense mechanisms. Biochemical changes mainly cause the generation of excess reactive oxygen species (ROS), affecting membrane transport mechanisms, oxidative damage to the cell membrane, genotoxicity etc. Plants have potential antioxidant defense mechanisms which eliminate the excess production of ROS, including \( \text{H}_2\text{O}_2, \text{OH}^-, \) and \( \text{O}_2^- \) free radicals. Enhanced levels of antioxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX), can assist plant cells in alleviating the oxidative stress induced by nanomaterials. The effects of nanoparticle-induced antioxidant changes and their role in the alleviation of phytotoxicity in plant cells have been reported previously [8, 9, 11, 12]; however, biochemical studies on the long-term effects of nanomaterial exposure to crop plants are limited at present. Recently, nanomaterials have been tested as nano-micronutrient fertilizers in \textit{Brassica} species [11], \textit{Solanum lycopersicum} [3], \textit{Coriandrum sativum} and \textit{Allium sativum} [2].

The reports on nanomaterial-induced phytotoxicity that have been published until now have tested the use of chemically synthesized nanomaterials, for which various noxious chemicals are used in the fabrication process. Therefore, it is presumed that the occurrence of nanomaterial-induced phytotoxicity symptoms in plants are mostly due to the hazardous substances coated on the surface of the engineered nanoparticles. In addition, the plant growth and development depend on the physical and biochemical interaction of plant cells with the nanomaterials altering the biological pathways by the regulation of gene expression. The use of nanofertilizers as micronutrients in agriculture is one of the significant strategies for enhancing the crop productivity of a nutritionally enriched global food supply [5, 13]. The major advantage of nanofertilizers is that not only do they serve as the best micronutrient for a plant but that they also rejuvenate soil fertility to organic condition without causing any of the detrimental effects of chemical fertilizer. Natural phyto-compounds from various seaweeds have been reported to enhance seedling growth and photosynthetic pigment content, as well as the yield potential in crops [14]. \textit{G. hirsutum} \( L. \) is one of the world’s major natural commercial textile fiber crops, and a severe yield loss has been recorded (40%) due to poor soil. Samples \textit{et al} [15] reported that plant growth regulator application is highly essential in cotton cultivation in order to manage vegetative growth for the reduction of crop yield loss in the USA. Also, no reports are available which show the role of bioengineered zinc oxide nanoparticles (ZnO NPs) coated with phycomolecules on growth-promoting responses in crop plants. Hence, phycomolecule-coated ZnO NPs were engineered and tested in the present experiment.

The major goals of the present study were to examine the impact of bioengineered ZnO NPs coated with phycomolecules on (1) seedling growth response, the level of photosynthetic pigments and the total soluble proteins, (2) membrane lipid peroxidation, (3) antioxidant enzyme activity, and (4) genomic changes in cotton plants (\textit{G. hirsutum} \( L. \)) exposed to different doses under a hydroponic system.

2. Experimental

2.1. Bioengineering and characterization of zinc oxide nanoparticles

Green macroalga, \textit{Halimeda tuna}, collected from the Rameswaram coastal region of Tamil Nadu, India, was thoroughly washed several times with deionized water and the shade-dried algae were powdered and stored at \(-20^\circ\text{C}\). About 20 g of algae powder was mixed with 200 ml distilled water, and boiled under microwave irradiation for 15 min. The algae extract was filtered and the algal extract was collected by centrifugation and thoroughly washed several times with sterile distilled water. The dried nanoparticles were further characterized by Fourier transform infrared spectroscopy (FTIR, Perkin-Elmer Spectrum RXI model), x-ray diffraction (XRD) analysis, field emission scanning electron microscopy (FESEM, Carl Zeiss Ultra 55 model) along with energy dispersive x-ray (EDX) spectra analysis. To determine the size of the zinc oxide nanoparticles (ZnO NPs), transmission electron microscopy (TEM) was performed and selected area electron diffraction (SAED) pattern
analysis was carried out to determine the crystalline nature of the nanoparticles.

2.2. Plant growth conditions and ZnO NP exposure

Seven-day-old cotton (*G. hirsutum* L.) seedlings were collected from trays without damaging the roots, washed thoroughly with distilled water and transferred to plastic cups containing a Hoagland nutrient medium (500 ml) under greenhouse conditions. Seedlings with a uniform size (8.5 cm) were placed in each cup and the plants were allowed to acclimatize for one week with continuous aeration before nanoparticle exposure. For the nanoparticle treatment, various doses of ZnO NPs (25, 50, 75, 100, and 200 mg l\(^{-1}\)) were added to the nutrient medium connected by an aerator to supply proper oxygen to the growing plant roots, and also to maintain the continuous mixing of the nanoparticles in the medium. A nutrient medium without the addition of ZnO NPs served as a control. The nutrient medium was changed at intervals once every three days for all treatments, including the control experiment. For each experiment, triplicates were used for all the treatments and the experiments were repeated thrice. A completely randomized block design was adopted for the present study.

2.3. Measurement of plant growth response and biomass

The nanoparticle-exposed seedlings, along with the control, were collected and washed thoroughly with distilled water to remove traces of any nanoparticles that had adhered to the surface of the plants. Immediately, the seedlings were divided into two groups and one group was stored in an ultra-deep freezer at \(-80^\circ\text{C}\) for biochemical and molecular analysis. The second group of seedlings was used for the measurement of seedling growth, biomass, the level of photosynthetic pigment contents, and lipid peroxidation (MDA content). The fresh weight (FW) biomass per plant was immediately quantified using an electronic balance (Shimadzu, Tokyo, Japan) and it was dried in a hot air oven at 65 °C for 48 h for the dry weight (DW) biomass (in grams) measurement. The shoot and root length of the seedlings was measured and the growth tolerance index (GTI) percentage was determined using the Wilkins method [16]:

\[
\text{GTI}(\%) = \frac{\text{Growth in medium} - \text{NPs}}{\text{Growth in medium} + \text{NPs}} \times 100. \tag{1}
\]

2.4. Estimation of lipid peroxidation (MDA content)

The level of lipid peroxidation was quantified and expressed as an amount of malondialdehyde (MDA) content using the method of Davenport et al [17]. The leaf samples (0.2 g) were homogenized with 2 ml of 5% (w/v) trichloroacetic acid (TCA) in an ice bath using a pestle and mortar, and the extract was transferred into sterile tubes and centrifuged at 10,000 rpm for 10 min. The resultant supernatant (2 ml) was carefully collected in a fresh tube and mixed with 2 ml of 0.67% (w/v) thiobarbituric acid (TBA). Then, the tubes containing the mixture were placed in a boiling water bath for 30 min and cooled. The supernatant was collected in a fresh tube after centrifugation, and used for the lipid peroxidation measurement. The absorbance was observed at 450, 532, and 600 nm (denoted as \(A_{450}, A_{532}\) and \(A_{600}\), respectively). The level of MDA content (\(C_{MDA}\)) was calculated using the equation proposed by Michael and Krishnaswamy [18] and expressed as \(\mu\text{mol g}^{-1}\) fresh weight.

\[
C_{MDA} = \frac{6.45(A_{532} - A_{600}) - 0.56A_{450}}{W} V_f \tag{2}
\]

where \(V_f = 0.0021, W = 0.2\) g.

2.5. Quantification of total soluble protein content

The photosynthetic pigment levels, namely chlorophyll \(a\) (Chl \(a\)), chlorophyll \(b\) (Chl \(b\)) and carotenoid (Car), were measured using the method described by Arnon [20]. The level of chlorophyll \(a\), chlorophyll \(b\) and carotenoids was estimated using a double-beam UV-visible spectrophotometer (Spectrophotometer UV-1800, Shimadzu Tokyo, Japan) at wavelengths of 663, 645 and 470 nm, respectively. The chlorophyll \(a, b\) and carotenoid level was calculated using the formulas proposed by Lichtenthaler *et al* [21], and expressed as mg g\(^{-1}\) fresh weight.

\[
\text{Chl} a = 12.25 A_{663} - 2.79 A_{645}, \tag{3}
\]

\[
\text{Chl} b = 21.50 A_{645} - 5.10 A_{663}, \tag{4}
\]

\[
\text{Car} = \frac{1000 A_{470} - 1.82 \text{ Chl} a - 85.02 \text{ Chl} b}{198} \tag{5}
\]

2.7. Detection of antioxidant enzyme activity

For the preparation of enzyme extracts, the leaf tissue (0.1 g) from each experiment was separately ground using a pre-chilled pestle and mortar with 1 ml of ice-cold potassium phosphate buffer (50 mM, pH 7.4 containing 0.5 mM ethylenediaminetetraacetic acid (EDTA)). The homogenate was transferred to sterile microfuge tubes and spun at 10,000 rpm for 15 min at 4 °C. The supernatant was collected in fresh tubes and stored in ice-cold conditions for the determination of SOD, CAT and POX enzyme activity and isozyme banding patterns by native polyacrylamide gel electrophoresis (PAGE) analysis, as described below.

2.7.1. Superoxide dismutase enzyme assay. The superoxide dismutase (SOD, EC 1.15.1.1) enzyme activity was estimated...
by measuring the decrease in absorbance of formazone formed by the superoxide radical and nitro-blue tetrazolium (NBT) dye by the enzyme [22]. The reaction mixture (3 ml) consisted of a 100 mM potassium phosphate buffer (pH 7.8), 200 mM methionine, 2.25 mM NBT, 3 mM EDTA, 1.5 M sodium carbonate, 60 μM riboflavin and the enzyme extract. After mixing the content, the reaction mixture was exposed to white fluorescent tube light for 15 min in the chamber. A complete reaction mixture without the enzyme extract served as the control, while an unexposed complete reaction mixture served as the blank. The enzyme reaction was stopped by storing the tubes in dark conditions, and the absorbance was measured at 560 nm against the blank using a UV–vis spectrophotometer. One unit of SOD enzyme activity was defined as the quantity of SOD needed to produce a 50% reduction of NBT under reaction conditions. The enzyme activity was expressed as units/mg/protein.

2.7.2. Catalase enzyme assay. The catalase (CAT, EC 1.11.1.6) enzyme activity was determined by recording the decomposition of hydrogen peroxide according to the protocol of Aebi [23]. The enzyme extract was aliquoted into the reaction mixture (3 ml) containing a 100 mM potassium phosphate buffer (pH 7.0) and 75 mM hydrogen peroxide. The enzyme activity was measured by recording the decrease in absorbance at 240 nm using a UV–vis spectrophotometer. The CAT activity was calculated using the extinction coefficient of 39.4 mM⁻¹ cm⁻¹. One unit of CAT activity was defined as the amount of enzyme required to decay 1 μmol of hydrogen peroxide (min mg⁻¹) protein under assay conditions.

2.7.3. Peroxidase enzyme assay. The peroxidase (POX, EC 1.11.1.7) enzyme activity was estimated as described by Castillo et al [24]. The enzyme extract was transferred to the reaction mixture (3 ml) containing a 100 mM potassium phosphate buffer (pH 6.1), 12 mM hydrogen peroxide and 96 mM guaiacol. The oxidation of guaiacol was measured by the increase in absorbance at 470 nm using a UV–vis spectrophotometer. The POX activity was calculated using the extinction coefficient of 25.5 mM⁻¹ cm⁻¹ and expressed in units/mg/protein. One unit of POX activity was defined as the estimated consumption of 1 μmol of hydrogen peroxide per min under assay conditions.

2.8. Identification of isoenzyme expression pattern by native PAGE analysis

The isoenzyme banding patterns of SOD and POX were detected on polyacrylamide gels (stacking gel 6% and 7.5%) under non-denaturing conditions. For each lane, a 20 μg protein sample was applied and electrophoresis was performed using a constant power supply at 50 V. The activity of the SOD isozymes in the gel was determined as per the method described by Beauchamp and Fridovich [25]. POX isoenzyme separation and staining were carried out according to the method of Anderson et al [26]. The isoenzyme banding patterns were documented by using an Alpha Innotech gel imager system (USA).

2.9. Detection of genomic changes by RAPD-PCR analysis

The total genomic DNA was isolated from the treated nanoparticles and control leaf tissues by using the modified CTAB method [27]. The leaf samples (0.1 g) were homogenized with 1 ml of 2× CTAB buffer (2% w/v) hexadecyl triethyl-ammonium bromide, 1.4 M NaCl, 20 mM EDTA (pH 8.0), 0.1 M tris-HCl (pH 8.0), 1% (w/v) polyvinyl polypyrrolidone (PVPP), 1% (w/v) 2-mercaptoethanol using a pestle and mortar and the homogenate was incubated in a water bath at 65 °C for 30 min. The leaf extract was spun at 8000 rpm for 10 min and the aqueous phase was re-extracted with an equal volume of phenol:chloroform:isoamyl alcohol, 25:24:1, and centrifuged as above. The RNA was removed by RNAsure (10 mg ml⁻¹) treatment and re-extracted with equal volumes of chloroform:isoamyl alcohol, 24:1, as described above. For DNA precipitation, a 0.6 volume of ice-cold isopropyl alcohol (100%) was added to the supernatant and stored at −20 °C for 20 min. After being spun at 10 000 rpm for 10 min, the DNA pellet was washed with 70% (v/v) ethanol and air dried. The DNA was dissolved in a TE buffer and stored at −20 °C until polymerase chain reaction (PCR) amplification. The random primers were procured from Operon Technologies Inc., (Alameda, CA, USA). The DNA samples from the control (unexposed), as well as the treated nanoparticles, were used for PCR amplification in a 20 μl reaction volume, which consisted of 10 mM tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 1 mM dNTP, 0.5U Taq DNA polymerase enzyme, 25 ng of template DNA and 250 μM of random primer. The PCR reactions were carried out in a thermal cycler machine (Cyber Cycler-P series of a PCR Peltier model P + 96, USA). The DNA amplification conditions consisted of an initial denaturation at 94 °C for 4 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 37 °C for 1.5 min, extension at 72 °C for 2 min with a final extension at 72 °C for 7 min, and the DNA amplicons were stored at 4 °C before electrophoretic analysis. The amplified DNA products were mixed with a loading buffer and analyzed on a 1.5% (w/v) agarose gel. Electrophoresis was carried out with a 50 V power supply until the bromophenol blue front had migrated to the bottom of the gel. The molecular size marker used was the lambda DNA double digested by EcoRI/HindIII. The DNA bands were visualized under UV light and the images were captured using an Alpha Innotech Gel Documentation system, USA.

2.10. Statistical analysis

All the experiments were conducted in triplicate and the data was taken for the measurement of plant growth, biomass, level of MDA content, photosynthetic pigment contents (chlorophyll a, b, and carotenoids), total soluble protein content and antioxidant enzyme activity. The experimental data was computed using one-way analysis of variance (ANOVA) and the mean differences were compared for pair
3. Results and discussions

3.1. Bioengineering and characterization of ZnO NPs

In the present investigation, it is hypothesized that the growth-enhancing effects of phycomolecule-loaded ZnO-NP-mediated plants can be accomplished by alleviating phytoxicity using one of these mechanisms: (a) ZnO NP amendment increases the plant growth rate associated with enhanced biomass, (b) decreased ROS production in the cells due to the presence of low MDA content, (c) an elevated level of antioxidant enzyme activity for effective scavenging of ROS in cells, and (d) differential expression pattern of isoenzymes for regulating the multiple biochemical mechanisms involved in rapid plant growth and developmental process. Natural phycomolecule-coated ZnO nanoparticles (ZnO NPs) were engineered using *H. tuna* algae extracts and characterized by FTIR, XRD, FESEM, TEM and EDX analysis. The major characteristic peaks of the ZnO NPs observed by FTIR analysis were: 3417.6 cm\(^{-1}\) (N–H amine stretching), 2923.9 cm\(^{-1}\) (C–H alkyl methylene stretch), 1787 cm\(^{-1}\) (C=O aldehyde stretch), 1487.9 cm\(^{-1}\) (C=C aromatic stretch), 1082.7 cm\(^{-1}\) (C–N amine stretch), 1045.7 cm\(^{-1}\) (C–O alcohol bond), 854.8 cm\(^{-1}\) (C–H aromatic benzene bond), and 712.8 cm\(^{-1}\) (C–H aromatic bond). The XRD analysis confirmed the crystalline nature of the engineered nanoparticles and FESEM revealed that the zinc oxide nanoparticles were spherical and rod-shaped with a size ranging from 2 to 54 nm (figure 1). The EDX analysis exhibited strong peaks at 1.4 and 8.6 keV, confirming the presence of zinc elements in the NPs. The TEM bright field image of the synthesized ZnO nanoparticles is depicted in figure 1(e) and equipped with a selected area electron diffraction pattern (SAED) in figure 1(f). The results illustrate that the surface layer of the synthesized nanoparticles exhibit a single crystalline wurtzite structure with a diameter of 20 nm. The corresponding SAED pattern confirms that the synthesized products are crystalline in nature, as shown in figure 1(e). The obtained SAED pattern is consistent with the images from the TEM observation. The present results are in agreement with the earlier findings by Raliya and Tarafdar [28] and Tarafdar *et al* [29], who also reported ZnO NPs of different shapes and sizes.

3.2. Effect of ZnO NPs on plant growth and biomass

In order to assess the plant-growth-stimulating role of phycomolecule-loaded ZnO nanoparticles in cotton, the physiochemical characteristics, such as plant growth, biomass, level of MDA content, photosynthetic pigment content, total soluble protein content, and SOD, CAT, POX antioxidant enzymes activity, were investigated in the present experiment. Furthermore, the nanoparticle-induced molecular alterations were also examined by using an isozyme expression pattern and DNA finger printing analysis after 21 days of exposure. The impact of different levels of ZnO nanoparticles on the plant growth, biomass and growth tolerance index (GTI) of cotton is depicted in table 1. The results show that shoot and root lengths, total plant biomass and GTI were enhanced in a dose-dependent manner, and that the growth rate increase was between 114.7%–125.4% and 112.8%–141.5% for shoots and roots, respectively, over the untreated control (table 1). The growth percentage was found to be significantly higher in the root tissue following ZnO NP exposure with respect to the control. It has been reported previously that nanoparticles promote the growth of cluster bean [28], pearl millet [29], soybean [13], tomato [30], and coriander seedlings [2]. All these studies showed phytotoxicity symptoms at higher doses besides the seedling growth increase recorded at a lower concentration. It is worth mentioning that the increased growth rate correlated positively with ZnO NP exposure over the control without showing any toxicity symptoms in the present study.

An increased rate of biomass was recorded from 117.2%–134.2% and 120.9%–136.2% for fresh weight per plant and dry weight per plant, respectively, in the ZnO-nanoparticle-treated plants compared to the control (table 1). The plant biomass was found to increase significantly with an increasing concentration of ZnO NPs up to 100 mg l\(^{-1}\), while it decreased slightly at a higher dose (200 mg l\(^{-1}\)). Although the biomass decreased slightly at a higher dose of ZnO NP treatment, it was significantly higher than the untreated control. In addition, the growth tolerance index of the seedlings was promoted by 125.4% and 141.5% for the shoot and root, respectively, in the ZnO-NP-treated plants (200 mg l\(^{-1}\)) over the unexposed control (table 1). Earlier studies also showed that nanoparticle exposure at lower doses enhanced the plant biomass in pearl millet [29], coriander and garlic [2], and green pea [7]. The present study clearly indicates that the growth rate is stimulated significantly, with a high biomass in cotton plants exposed to biologically engineered ZnO NPs. Moreover, the possible reason for the plant-growth-promoting role of the ZnO NPs carrying the phycomolecule ligands is that they are coated with natural growth stimulating substances, including proteins.

3.3. Effect of ZnO NPs on MDA content, photosynthetic pigments and protein level

To examine the effects of ZnO NP exposure on membrane damage (lipid peroxidation) and photosynthesis, the level of MDA content, pigment content and total soluble protein content in the leaf tissue were measured, with the results illustrated in figure 2(a). The MDA content level correlated negatively with ZnO NP exposure, and a significant reduction of 73.8% was noticed in the leaves grown with 200 mg l\(^{-1}\) ZnO NP treatment compared to the control. Nanomaterial exposure might induce oxidative stress in plants by the generation of excess reactive oxygen species that can severely alter the level of lipid peroxidation, photosynthetic pigments, and protein contents [31]. The present results clearly indicate that ZnO NP treatment exhibits a declined level of MDA.
Bioengineered ZnO nanoparticles have the ability to protect cell membrane integrity due to the presence of natural bioactive compounds in the algal extracts from the NP-induced oxidative stress, as evidenced by a decreased level of MDA content, which correlates positively with the absence of excess ROS in NP-exposed plants. In contrast, an increased level of MDA content (lipid peroxidation) resulted in the loss of cell membrane integrity in CeO-nanoparticle-treated corn plants [32] and in Ag-nanoparticle-exposed rice [33].

In the present study, ZnO NP treatment increased the level of photosynthetic pigment content, including chlorophyll a, b and carotenoids in the leaves of the cotton plants (figure 2(b)). The increased percentage of photosynthetic pigment content was directly proportional to the ZnO NP doses, and it was enhanced from 108.2%–134.7%, 122.2%–132.6%, and 123.8%–160.1% for chlorophyll a, b and carotenoids, respectively, over the control. Interestingly, the maximum level of photosynthetic pigment content observed was 134.7%, 132.6%, and 160.1% for chlorophyll a, b, and
carotenoids, respectively, for the plants exposed to 200 mg l\(^{-1}\) ZnO NPs compared to the control (figure 2(b)). Similarly, applications of ZnO NPs at a lower concentration increased the photosynthetic pigments in cluster bean [28], pearl millet [29], soybean [13], and green pea [7]. However, bioengineered ZnO NPs increased the rate of photosynthetic pigments significantly in cotton, even at higher doses, and this might be due to the presence of phycomolecule ligands on the ZnO NPs.

The level of total soluble protein in the leaves of cotton plants exposed to various concentrations of ZnO NPs is presented in figure 2(c). Interestingly, ZnO NP exposure enhanced the level of total soluble protein content, ranging from 126.0%–165.4% in the treated plants over the control. The increased total soluble protein content correlated positively to the nanoparticle dose and it declined slightly following treatment with 200 mg l\(^{-1}\) ZnO NP. Although the protein content decreased slightly at a higher dose, it was

| ZnO NP dose (mg l\(^{-1}\)) | Shoot length (cm) | Root length (cm) | Growth tolerance index (%) | Plant biomass (g/plant) |
|-----------------------------|-------------------|------------------|----------------------------|------------------------|
|                             | Shoot tissue      | Root tissue      |                           | Fresh weight | Dry weight |
| Control                     | 7.83 ± 0.18\(^{d}\)| 5.36 ± 0.16\(^{a}\)| 100.0                     | 0.61 ± 0.031\(^{d}\) | 0.58 ± 0.035\(^{a}\) |
| 25                          | 8.95 ± 0.19\(^{c}\)| 5.98 ± 0.35\(^{d}\)| 114.7                     | 0.71 ± 0.045\(^{c}\) | 0.69 ± 0.051\(^{d}\) |
| 50                          | 9.47 ± 0.18\(^{b}\)| 6.58 ± 0.27\(^{c}\)| 120.7                     | 0.73 ± 0.073\(^{c}\) | 0.71 ± 0.043\(^{c}\) |
| 75                          | 9.42 ± 0.16\(^{b}\)| 7.76 ± 0.26\(^{a}\)| 121.4                     | 0.76 ± 0.081\(^{b}\) | 0.74 ± 0.068\(^{bc}\) |
| 100                         | 9.65 ± 0.19\(^{a}\)| 7.81 ± 0.28\(^{a}\)| 123.7                     | 0.81 ± 0.061\(^{a}\) | 0.78 ± 0.037\(^{a}\) |
| 200                         | 9.78 ± 0.14\(^{a}\)| 7.50 ± 0.23\(^{b}\)| 125.4                     | 0.78 ± 0.095\(^{b}\) | 0.76 ± 0.084\(^{b}\) |

\(^{a}\) Each value represents the mean of three replicates ±SE (n = 3). The different letters in each row are statistically significant at a level of \(p < 0.05\).
found to be significantly higher than the control. Similarly, the ZnO NPs at lower doses enhanced the total soluble protein content in cluster bean [28], pearl millet [29], and green pea [7]. The present findings suggest that the up-regulated expression of proteins might protect the cells from any oxidative stress caused by NPs in cotton plants. Notably, the enhanced photosynthetic pigment content promoted an increase in the growth rate and biomass, eventually altering the level of protein, starch and yield in the crops [7, 10, 13, 29].

3.4. Influence of ZnO NPs on antioxidative defense enzyme activity

The antioxidative defense system responsible for scavenging excess ROS can be activated to alleviate nanomaterial-induced toxicity in plants. SOD is the first defense enzyme that acts against ROS to convert them from superoxide anions (O$_2^-$) to less toxic hydrogen peroxide (H$_2$O$_2$) and oxygen (O$_2$) [31]. The effect of various doses of ZnO NP exposure on the superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) enzyme activity in the leaves of cotton plants is depicted in figure 3. The SOD activity in leaves was increased to 174.6%, 252.5%, 267.8%, 223.7%, 242.4% against 25, 50, 75, 100, 200 mg l$^{-1}$ ZnO NP treatment, respectively, when compared to the unexposed control (figure 3(a)). The results show that SOD activity was found to increase with increasing concentrations of ZnO NPs up to 75 mg l$^{-1}$, while it decreased at higher doses. It is worth mentioning that the SOD activity declined slightly at higher doses of ZnO NPs, but that overall, activity was enhanced by nanoparticle exposure relative to the untreated control. The level of antioxidant enzyme activity differs significantly depending on the plant species, nanoparticle type, duration and dose [31]. The present findings are in agreement with Kouhi et al [11], and Kim et al [12], who observed increased SOD activity at lower doses but slightly decreased activity at higher ones. Decreased SOD activity might be due to the lower production of superoxide (O$_2^-$) from oxidative stress caused by nanomaterials [12]. Hu et al [9] also demonstrated increased SOD enzyme activity after ZnO NP exposure, showing an amplification of the ROS scavenging process in Spirodela polyrhiza.

The CAT and POX are notable antioxidant defense enzymes involved in the detoxification of H$_2$O$_2$ by converting free radicals to water (H$_2$O) and oxygen (O$_2$) [31]. The impact of ZnO NP treatment on CAT enzyme activity in the leaf
Figure 4. The effects of various concentrations of ZnO nanoparticles on isoenzyme expression: (A) the SOD isozyme banding pattern, and (B) the POX isozyme banding pattern established from the leaf tissue of cotton (G. hirsutum L.) seedlings. Lanes: C—Control, and 1–5 ZnO nanoparticle doses (25, 50, 75, 100, and 200 mg l−1). The arrow indicates the number of expressed isoenzyme bands.

tissue of cotton is shown in figure 3(b). As the ZnO NP dose was increased in the medium, CAT activity decreased significantly. Interestingly, CAT activity increased slightly (106.9%) at 25 mg l−1, while it decreased significantly to 92.0%, 93.4%, 86.1%, and 83.2% in the leaf tissue against the 50, 75, 100, and 200 mg l−1 ZnO NP treatments, respectively, compared to the untreated control (figure 3(b)). Similarly, Kim et al [12] reported decreased CAT activity in ZnO-NP-treated Cucumis sativus plants. In contrast, ZnO NPs increased CAT activity in Prosopis juliflora-velutina [8] and in Spirodela polyrhiza [9]. The results suggest that ZnO NPs decreased CAT activity with an increase in the dose, indicating the existence of low oxidative stress (lower MDA content) in cotton plants.

The effect of various doses of ZnO NPs on peroxidase (POX) activity in the leaves of cotton seedlings is illustrated in figure 3(c). The addition of ZnO NPs enhanced POX activity by 131.8%, 145.5%, 163.6%, 174.5%, and 159.1% for the 25, 50, 75, 100, and 200 mg l−1 treatments, respectively, over the untreated control (figure 3(c)). It is worth recording that the POX activity was found to increase significantly in leaf tissue exposed to ZnO NPs over the untreated control, while it decreased at higher doses. The present results are in agreement with earlier findings by Hu et al [9], and Kim et al [12], who reported decreased POX activity in leaves treated with higher doses of nanoparticles. At higher doses of ZnO NP treatment, SOD, CAT, and POX enzyme levels exhibited a concomitant decrease in cotton leaves with a low level of MDA content, which suggests that cotton plants elevate the antioxidant enzyme level in order to remove excess H2O2. Interestingly, increased levels of SOD and POX activity in ZnO-NP-treated plants show the ability to nullify ROS-mediated oxidative-stress-induced damage in G. hirsutum.

3.5. Impact of ZnO NPs on isoenzyme expression pattern

The effect of different doses of ZnO NPs on the SOD and POX isozyme expression pattern in the leaves of cotton plants was studied by native PAGE analysis (figure 4). Two SOD isoforms were observed in the isozyme banding pattern (figure 4(a)). Interestingly, the SOD isoform 1 is displayed in plants treated with ZnO NPs, while the SOD isoform 2 disappears in leaf tissues grown at higher doses (100 and 200 mg l−1) with respect to the control. In the case of the POX isozyme, two isoforms were recorded and a POX isoform 2 band was observed in ZnO-NP-exposed plants, while it was absent in the untreated control (figure 4(b)). Notably, the level of POX isoform 1 expression showed a 2–3-fold increase in leaf tissue grown at higher doses of ZnO NP treatment over the untreated control. The results suggest that the addition of ZnO NPs up-regulates the expression level of SOD and POX isoenzymes at lower doses and decreases slightly at higher doses of NP-exposed cotton plants. The present results are in agreement with earlier findings by Kaveh et al [34], who reported the up-regulated expression level of the SOD, POX and glutathione S-transferase (GST) genes involved in the scavenging of excess ROS in Arabidopsis. Ma et al [31] also noticed InO and CeO in the nanoparticle-induced expression of the glutathione synthase (GS) gene in Arabidopsis. It is interesting to note that the expression level of SOD and POX isoenzymes is on a par with the results of the quantitative analysis of the respective enzymes in the present study.

3.6. Impact of ZnO NPs on genomic DNA fingerprinting

RAPD-DNA fingerprinting analysis was performed to detect the genomic changes that occurred in the cotton plants upon exposure to different doses of ZnO NPs. As seen in figure 5, the RAPD banding patterns pertaining to the impact of ZnO NPs did not exhibit distinct variations except the intensity of DNA amplicons for each primer between the control plants either exposed or unexposed to the NPs. Among the 80 random decamer primers used, 10 primers showed clear DNA fingerprinting patterns, but scorable DNA bands with intensity changes were observed with 4 primers (OPA-08, OPB-12, OPC-05 and OPD-08). Interestingly, four DNA bands (1.0, 1.1, 1.2, and 1.4 kb in size) appeared in the RAPD pattern generated by the OPA-08 primer, but bands at 1.0 and 1.4 kb in size were found to be more intense in the control, while band intensity decreased slightly in the samples treated with higher doses of ZnO NPs when compared to the unexposed control. However, a 1.2 kb DNA band appeared more strongly in the leaf samples treated with a 100 mg l−1 NP dose (figure 5(a)). In the RAPD pattern developed by the OPB-12 primer, three DNA amplicons at sizes of 0.7, 1.0, and 1.1 kb were polymerized in the control plants that were either treated or untreated by ZnO NPs, while the 1.1 kb DNA band was found to be stronger in the leaf samples treated with 200 mg l−1 (figure 5(b)). With the OPC-05 primer, a total of 3 DNA bands (1.0, 1.1, and 1.3 kb in size) were observed in the RAPD banding patterns of the plants that were either treated or untreated by the NPs. It is worth mentioning that the 1.3 kb DNA band was produced more intensely in the leaf sample grown with ZnO NP treatment, when compared to the unexposed control (figure 5(c)). The RAPD pattern generated with the OPD-11 primer revealed 4 DNA bands with 0.9, 1.0, 1.1, and 1.3 kb sizes from the plants that were either treated or untreated with the ZnO NPs. Moreover, the intensity of the
1.1 kb DNA amplicon was found to be low in the leaf samples exposed to ZnO NPs compared to the control (figure 5(d)). RAPD analysis was successfully used as a potential molecular tool to detect a broad range of DNA changes/alterations caused by nanoparticle-mediated genotoxicity [35]. Our results are concurrent with the most recent report by Mattiello et al [36], who reported that TiO NP exposure did not cause any genomic changes in the RAPD banding pattern of Hordeum vulgare. In contrast, TiO NP genotoxicity induced DNA changes in the RAPD profile of Cucurbita pepo plants [35]. In the present study, the possible reasons for the absence of genomic changes in NP-treated cotton plants could be explained by: (a) the engineering of the ZnO nanocomplex capped with natural-growth-promoting compounds/proteins ligands, and (b) the use of phycomolecule-coated ZnO nanoparticles, which can release Zn ions into dividing cells in a slow and sustainable manner without causing genotoxicity to plants.

4. Conclusion

This study reports on the influence of bioengineered zinc oxide nanoparticles coated with natural phycomolecules, and their role in plant growth stimulation in cotton. The results indicate that the addition of ZnO NPs produced morphological, physiological and biochemical changes by activating multiple antioxidant defense pathways, which alleviate the toxicity caused by nanomaterials in G. hirsutum. ZnO NP exposure to the plants significantly promoted the growth rate, biomass, photosynthetic pigment levels and protein content, while MDA production declined compared to the control. Interestingly, the ZnO NPs increased the activity of antioxidant defense enzymes, and up-regulated the expression level of SOD and POX isoenzymes in cotton plants. Notably, ZnO NP treatment did not influence the RAPD banding pattern, indicating the absence of genotoxicity. Our findings suggest that increased levels of SOD and POX enzymes can act together to reduce ROS production. The present study further indicates that engineered ZnO nanocomplexes coated with phycomolecules can be effectively applied as a promising growth-promoting catalyst in agricultural crops.

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