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Original Research

Genotoxic Potentials of Biosynthesized Zinc Oxide Nanoparticles

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Abstract

There are various studies on the toxicological potentials of conventionally synthesized zinc oxide (ZnO) nanoparticles, which are useful tools for many industrial applications. However, knowledge about the biologically synthesized ones is still limited. The current study was designed to biologically synthesize ZnO nanoparticles from zinc acetate, zinc chloride, zinc nitrate hexahydrate and zinc sulfate heptahydrate precursors by *Rhodococcus erythropolis* K85 and assess their toxicological potentials on *Triticum aestivum*. ZnO nanoparticles were successfully synthesized from each precursor and characterized by scanning electron microscopy (SEM) observations and energy-dispersive X-ray spectroscopy (EDAX) analysis. The size of produced nanoparticles ranged from 50 to 150 nm and none of the test groups affected seed germination at 0.1 mg/ml concentration. However, seedling growth was significantly affected by ZnO nanoparticle exposure. Besides, random amplified polymorphic DNA (RAPD) analysis results showed a conformity to the seedling growth results and all of the test groups caused significant changes in the RAPD profiles for OPA-2, OPA-13, OPH-19, OPW-6, OPW-11, OPW-17, OPW-18 and OPY-8 primers. This resulted in a significant decrease in genomic template stability percentage (GTS%) and an increase in polymorphism percentage values. In conclusion, this study confirms that ZnO nanoparticles may show significant toxicological features – even if they are biologically synthesized and it is necessary to determine their hazardous potential before use.

Keywords: biosynthesis, genotoxicity, RAPD, *Rhodococcus erythropolis*, ZnO nanoparticle

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Introduction

Zinc (Zn), known as one of the important essential elements for living organisms including bacteria, plants and animals, has various roles in many critical metabolic pathways such as homeostasis, immune-response, oxidative stress, cellular differentiation and aging [1-3]. Besides, cells require Zn or Zn-derived compounds for maintaining activity of the mammalian androgen receptors, synthesis and stability of new DNA/RNA molecules and related repair enzymes, and regulation of transcription [4]. These valuable properties of Zn in living organisms have placed it among the most popular research topics for many decades [5-6].

On the other hand, apart from the important roles in the vital activities, engineered/artificial Zn-derived compounds have attracted much attention due to their unique features used for a wide range of agricultural, environmental, industrial and medicinal applications [7-10]. Among these compounds, especially ZnO nanoparticles have become prominent because of their versatile properties mostly applicable for cutting-edge technological research and development (R&D; also known in Europe as research and technical, or technological, development, or RTD) approaches [11-13]. These nanoparticles belong to a group of rare compounds that have piezoelectricity, semi-conductivity and optical features in the same time [14]. Furthermore, they show valuable antimicrobial, dermatologic, electronic, optic and photocatalytic activities that enable broad range use of ZnO nanoparticles in cosmetic, medicine, pharmacy, textile, etc. [15-17].

Although common use of ZnO nanoparticles with various specifications has been beneficial and rapidly expanded in recent decades, the lack of sufficient information on their hazardous potential on living organisms and the environment has increased concerns in public and scientific societies [18-21]. In this regard, many reports on the toxicological effects of ZnO nanoparticles produced by using conventional methods including chemical and physical routes has been published and corroborated the importance of the present concerns, which has resulted in some restrictions on the use of ZnO nanoparticles and the search for alternative synthesis methods that produce less harmful compounds [22-26].

Recently, the biosynthesis of ZnO nanoparticles has been more attractive and biosynthetically produced nanoparticles have been mainly considered as safe owing to the fact that many biological production processes occur under mild conditions and produce biocompatible compounds and environmentally friend byproducts [24, 27-29]. Contrary to this common thought, current studies have noticed that biologically synthesized nanoparticles may also show hazardous effects on living organisms and the environment; likewise, conventionally produced ones do [18, 30-32]. This reveals that there is a necessity for toxicological assessments of biologically synthesized nanoparticles before their use.

In this perspective, our present study was designed to contribute to the knowledge on the hazard potential of biosynthetically produced ZnO nanoparticles. Thus, ZnO nanoparticles were biologically synthesized by using *Rhodococcus erythropolis* K85 from Zn-based precursors, and the toxicological potentials of the produced nanoparticles were assessed by seed germination assay and a random amplified polymorphic DNA (RAPD) test system in the present study.

Experimental

Chemicals and Microorganisms

Agarose (CAS#9012-36-6), CTAB (CAS#57-09-0), Magnesium sulfate heptahydrate (CAS#10034-99-8), potassium phosphate monobasic (CAS#7778-77-0) and potassium phosphate dibasic (CAS#7758-11-4) were purchased from Merck; 2-mercaptoethanol (CAS#60-24-2), 2-propanol (CAS#67-63-0), chloroform:isoamyl alcohol 24:1 (C0549), dNTP mix (D7295), EDTA (CAS#60-00-4), ethidium bromide (CAS#1239-45-8), magnesium chloride (CAS#7786-30-3), PCR buffer without $MgCl_2$ (P2317), phenol:chloroform:isoamyl alcohol 25:24:1 (CAS#136112-00-0), PVP (CAS#9003-39-8), SDS (CAS#151-21-3), sodium chloride (CAS#7647-14-5), Taq DNA polymerase (CAS#9012-90-2), TBE buffer (T4415), TE buffer (93283), Tris-HCl (CAS#1185-53-1), wide-range DNA marker (D7058), zinc acetate (CAS#557-34-6), zinc chloride (CAS#7646-85-7), zinc nitrate hexahydrate (CAS#10196-18-6) and zinc sulfate heptahydrate (CAS#7446-20-0) from Sigma-Aldrich; and nutrient broth (CM0001) from Oxoid.

The bacterial strain *Rhodococcus erythropolis* K85 (NCBI GeneBank accession number: KF976880) was provided from the culture collection in the Molecular Biology and Bacteriology Research Laboratory of the Biology Department at Atatürk University.

Synthesis and Characterization of ZnO Nanoparticles

Synthesis of ZnO nanoparticles was done according to the protocol previously described by Kundu et al. with some minor modifications [23]. Briefly in this procedure, 50 ml of sterilized liquid mineral salt basal medium (MSB-Broth, l^{-1}: 0.75 g K_2HPO_4, 0.2 g KH_2PO_4 and 0.09 g $MgSO_4 \cdot 7H_2O$) with varying concentrations of each precursor (0, 10, 50 and 100 mM) was inoculated with an overnight culture of nutrient broth with *R. erythropolis* K85 (0.5 OD units – mid-log phase; ~10^7 CFU ml^{-1}) for determining bacterial tolerance for zinc acetate, zinc chloride, zinc nitrate hexahydrate, and zinc sulfate heptahydrate. The culture bottles were incubated at 30ºC for 72 h at 120 rpm, sampling steps were aseptically performed at regular time-points and the samples were optically monitored for bacterial growth at 600 nm.

Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDAX) techniques were used for characterizing produced ZnO nanoparticles.

Determining Test Concentrations

Five test solutions at different concentrations (0.01, 0.05, 0.1, 0.5 and 1 mg/ml) were prepared for the nanoparticles produced from each precursor. *Triticum aestivum* L. seeds were treated with these solutions. After the germination and growth periods, the minimum concentration where observable phenotypic changes occur (0.1 mg/ml) was chosen for additional assays.

Seed Germination and Seedling Growth

T. aestivum L. seeds with uniform genophond characteristics were obtained from the Faculty of Agriculture at Atatürk University. For germination studies, the seeds were soaked in test material solutions at 0.1 mg/mL concentration for 48 h in the dark at room temperature with gentle shaking on an orbital shaker at 150 rpm. After the incubation period, the seeds were washed with distilled water and transferred to 10-mm Petri dishes containing a piece of filter paper (90 mm) and 5 mL of distilled water. The seeds were tested for germination in a growth chamber under a range of conditions established by the OECD guidelines: 24ºC, humidity 70±25%, photoperiod 18 h light, and light intensity 300 µE m^{-2} s^{-1} with protection from drying. In this experiment, each Petri dish (n = 10) contained 5 seeds, and germination rates were investigated for 3 days. Distilled water is also used as a negative control [33].

After 7 days of germination, seedling samples were collected for each treatment, morphologically examined and stored at -80ºC until DNA isolation studies [34-35].

RAPD Analysis

DNA Extraction

T. aestivum L. seedlings after seed germination assay were chosen for DNA extraction. Genomic DNA was extracted from the root tips of the seedlings after liquid nitrogen treatment and powdering according to a modified method [36]. In this method, approximately 10-15 mg tissue samples were placed in 2 ml Eppendorf tubes; 1000 µl DNA extraction buffer [100 mM Tris-HCl (pH 8.0); 50 mM EDTA (pH 8.0); 500 mM NaCl; 2% SDS (w/v); 2% 2-mercaptoethanol (v/v); and 1% PVP (w/v)] was added and mixed well. The tube was placed in a water bath at 65ºC and was incubated for 40 min and shaken briefly at 5-min intervals. The sample was centrifuged at 12 000 × g for 15 min at 4ºC. The supernatant was transferred into a new 1.5 ml tube and mixed with equal volume of phenol:chloroform:isoamylalcohol (25:24:1), and then centrifuged. The supernatant was transferred and mixed with 1/10 volume 10% CTAB-0.7 M NaCl in a new tube. After centrifugation, the supernatant was added with equal volume of chloroform:isoamylalchol (24:1). The DNA was precipitated by the addition of 0.6 volume of freezer-cold isopropanol left at -20ºC for 10 min. The DNA was pelleted by centrifugation (12 000 × g, 10 min) and the isopropanol was poured off carefully. The sample was allowed to air-dry before being dissolved in 100 ml of TE buffer.

RAPD PCR Procedure

Samples were screened for RAPD variation using standard 10-base primers supplied by Macrogen – Humanizing Genomics (Amsterdam, The Netherlands). Sequences (5'→3') of primers were TGCCGAGCTG (OPA-2), CAGCACCCAC (OPA-13), CTGACCAGCC (OPH-19), AGGCCCGATG (OPW-6), CTGATGCGTG (OPW-11), GTCCTGGGTT (OPW-17), TTCAGGGCAC (OPW-18) and AGGCAGAGCA (OPY-8). PCR amplifications were carried out in a total volume of 20 µl, containing 50 ng genomic DNA, 1×PCR buffer (without $MgCl_2$), 0.25 µM dNTP, 0.25 mM primer, 2.5 mM $MgCl_2$, and 1.5 U Taq DNA polymerase. The thermal cycle was as follows: 5 minutes at 95ºC, an initial denaturation step; 42 cycles at 94ºC for 1 min, in different annealing temperature for each primer for 1 min, 72ºC for 2 min, penultimate step 15 min at 72ºC, and a final extension of 10 min at 4ºC.

Evaluation of the Amplified DNA Products and Data Analysis

RAPD PCR products were separated by electrophoresis using 1% agarose gel in 0.5 × TBE buffer with constant voltage of 80 V for 150 min. Then gels stained with ethidium bromide, visualized under UV light and photographed using the gel visualization system. The sizes of the fragments were estimated based on a DNA ladder of 50-10.000 bp. The bands were primarily detected by using the bio doc image analysis system with the Uvisoft analysis package (Cambridge Electronic Design Ltd., Cambridge, UK). Then RAPD patterns were further evaluated using TotalLab TL120 computer software (TotalLab, Phonetix 1D). The evaluation of polymorphism included the disappearance of a normal band and the appearance of a new band in comparison with the control. The genomic template stability (GTS; %) was calculated as follows: GTS% = [1-(a/n)] × 100 (a is the average number of polymorphic bands detected in each treated sample and n is the count of total bands in the control) and polymorphism% = (1-GTS) × 100.

Results and Discussion

Although the cells of *R. erythropolis* K85 exhibited tolerance even up to 100 mM concentration of

Fig. 1. Blurred pale white color of the culture media and the coalescent white deposition at the bottom of the bottle indicating the synthesis of the ZnO nanoparticles from the precursor.

precursors, 50 mM concentration, where the maximum cell density was observed for each precursor, was chosen as the experimental dose for biological production of ZnO nanoparticles. Visual examinations of the cultures confirmed the synthesis of ZnO nanoparticles, in which the color of the cultivation media was changed from colorless transparent to a blurred pale white color during the reaction, and finally distinct coalescent white deposition was observed at the bottom of the culture bottle, indicating the synthesis of ZnO nanoparticles (Fig. 1). Generally, the size of nanoparticles synthesized from each precursor ranged from 50 to 150 nm. However, much larger or much smaller structures were also observed in the SEM analysis results (Fig. 2). EDAX analysis results also confirmed that "Zn" and "O" are the basic units of these nanoparticles (Fig. 3).

The *in vitro* seed germination assay results showed that any test compound did not affect the germination rates of the *T. aestivum* L. seeds at the test concentration (0.1 mg/mL). However, all of the test groups clearly decreased growth rates of the seedlings in comparison to the control group (Fig. 4). On the other hand, RAPD analysis showed conformity to the seedling growth results and that the genomic materials from *T. aestivum* seedlings were affected by the treatment of test materials. According to the data, all of the test groups caused significant changes in the RAPD profiles for OPA-2, OPA-13, OPH-19, OPW-6, OPW-11, OPW-17, OPW-18 and OPY-8 primers. These changes included loss of normal bands and the appearance of new bands in comparison to the control group (Fig. 5 and Table 1). As a consequence of the present results, the genomic template stability (GTS) was also affected by the treatments and significant changes were observed in the GTS and polymorphism values (Table 2).

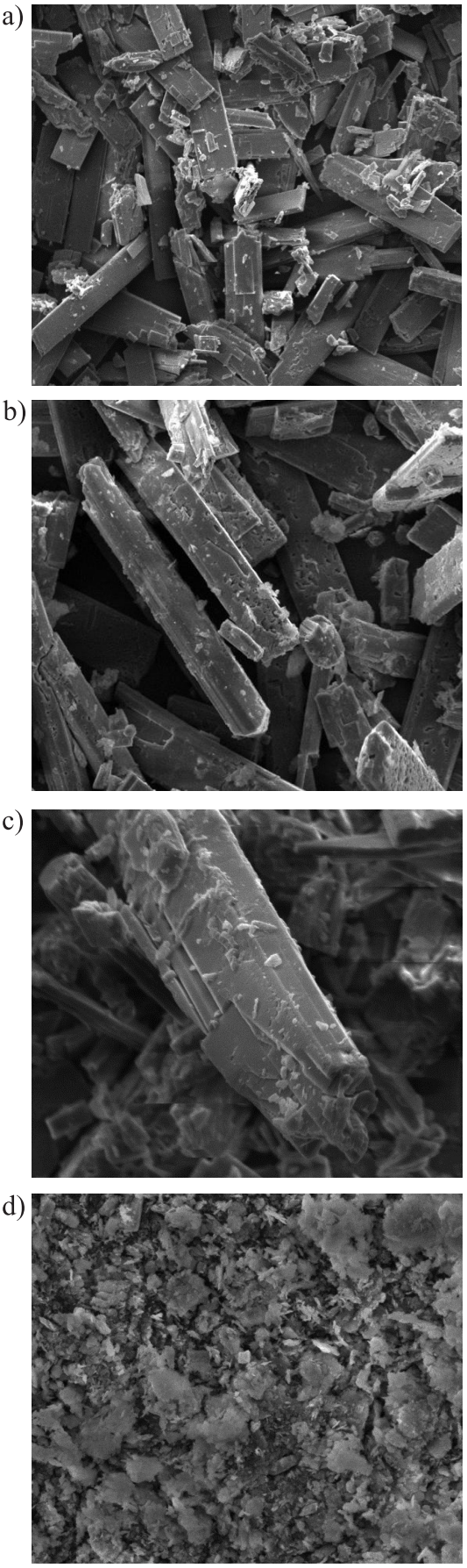

Fig. 2. SEM micrographs of biologically synthesized ZnO nanoparticles from precursors: a) Zn-sulfate·$7H_2O$, b) Zn-chloride, c) Zn-nitrate·$6H_2O$ and d) Zn-acetate.

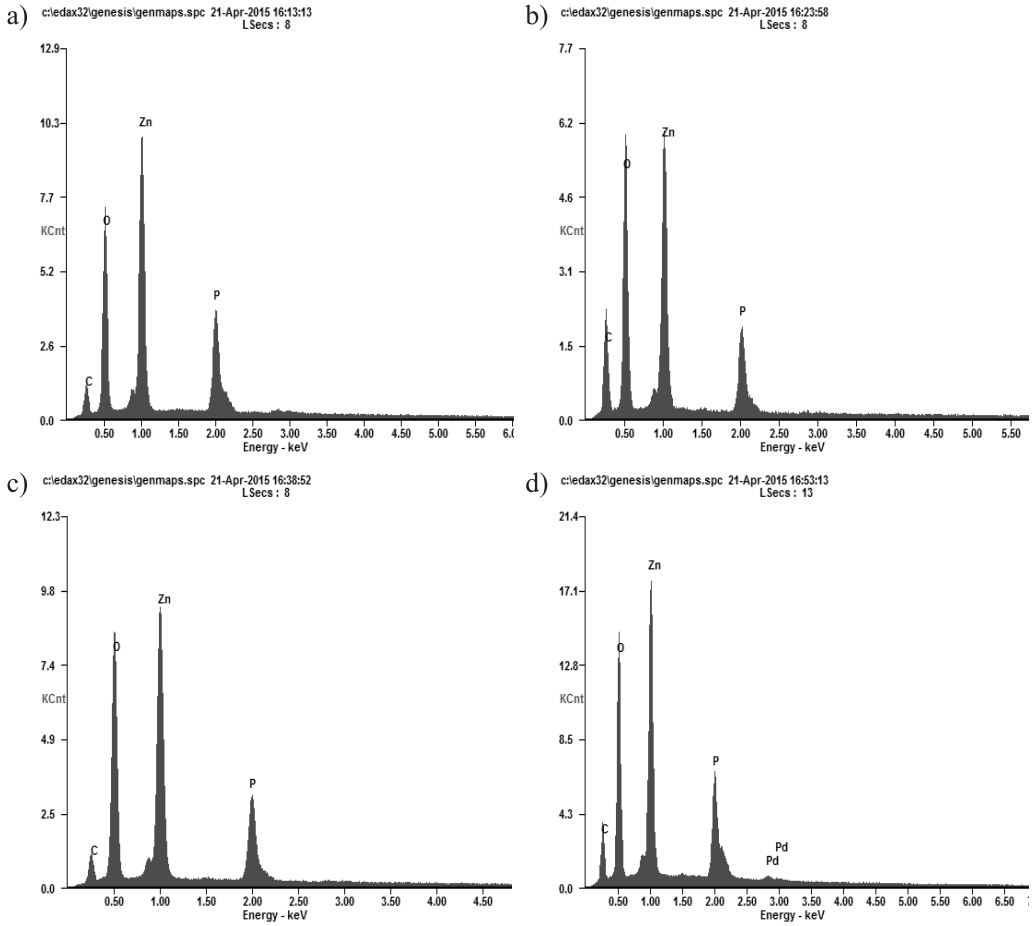

Fig. 3. EDAX results of biologically synthesized ZnO nanoparticles from precursors: a) Zn-sulfate·$7H_2O$, b) Zn-chloride, c) Zn-nitrate·$6H_2O$ and d) Zn-acetate.

Recently ZnO nanostructures with a great variety of characteristics and unique properties have been used in various industrial applications and these got involved in valuable products associated with people, other organisms and the environment directly or indirectly [8, 10]. Especially, these nanoparticles are thought of as promising nanomaterials for bioimaging and drug/gene delivery studies in pharmaceutical and medicinal research. Moreover, they have potential use as biosensors for glucose, phenol, H_2O_2, cholesterol

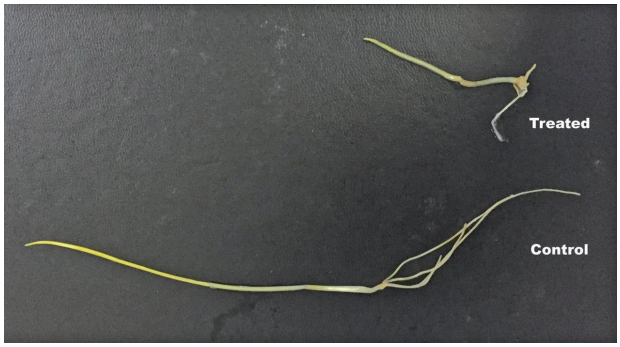

Fig. 4. Inhibitory effects of ZnO nanoparticles on the growth of *T. aestivum* seedlings.

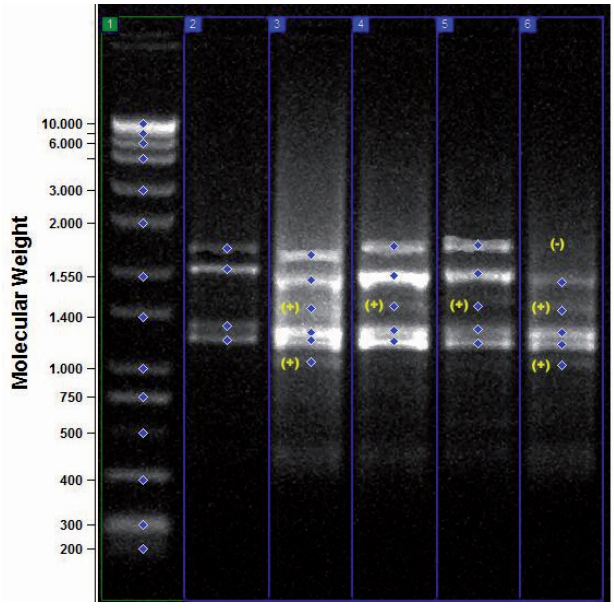

Fig. 5. Example evaluation of RAPD profiles of ZnO nanoparticle exposed and non-exposed *T. aestivum* seedlings (Primer: OPW-11, +: appearance of a new band, –: disappearance of a normal band, Lines 1: Marker; 2: Control; 3, 4, 5 and 6: ZnO nanoparticles synthesized from precursors Zn-sulfate·$7H_2O$, Zn-chloride, Zn-nitrate·$6H_2O$ and Zn-acetate, respectively).

Table 1. Molecular sizes (bp) of appeared/disappeared (+/-) bands in RAPD profiles of ZnO nanoparticle-treated *T. aestivum* seedlings.

Primers	Control	+/-	ZnO nanoparticles synthesized from precursors:			
			Zn-sulfate · $7H_2O$	Zn-chloride	Zn-nitrate · $6H_2O$	Zn-acetate
OPA-2	11	+	-	-	1470	-
		-	1564 1206	1206	2695 1206 1116	2695 1206
OPA-13	7	+	618	1663 600	-	-
		-	-	2543	2543	2543
OPH-19	12	+	2978 768	-	-	-
		-	2222 1444	2222 1572 1550 1516 1444 1233 980	3214 2651 2222 1729 1572 1516 1347 980	3214 2222 1516 1347
OPW-6	8	+	-	-	-	1883
		-	923	2292 1166 923	3163 2292 1166 1107 923 783	3163 2292 923
OPW-11	4	+	1298 1062	1463	1463	1439 1031
		-	-	-	-	1601
OPW-17	3	+	6543 1546	4207	-	-
		-	2575	3715 2575	3715 2575	6263 1555
OPW-18	4	+	1771 1581 1444	1843 1457	1817 1463	1817 1463
		-	1605	-	-	-
OPY-8	7	+	406	412	-	417
		-	-	1720 944	1720 944	1720 944

and urea [37]. On the other hand, apart from these useful properties of ZnO nanoparticles, the large-scale production and use of these nanomaterials have raised concerns regarding the difficulties in the control of their exposure and inadequate information on the possible risks to human health and the environment [18, 20, 38]. Indeed, the hazardous potential of ZnO nanoparticles, synthesized by traditional methods such as thermal decomposition, laser ablation, microwave irradiation, sonochemical, reverse micelles process, chemical reduction, ultrasonic irradiation and radiolysis on various cell cultures, animal and plant species have been shown in recent studies [18, 31, 39-40]. This resulted in redirecting related studies to biosynthesis routes of ZnO nanoparticles as an alternative solution that generally allows for the production of nanomaterials with high biocompatibility. However, counteracting the common thought on the safety of biosynthesized nanoparticles, present knowledge about hazardous potentials of them is still limited and their large-scale production may exert inevitable impacts on living organisms in the near future [31, 41].

In the present study conducted within this framework, ZnO nanoparticles were biosynthesized

Table 2. Individual and average (%) GTS and polymorphism values of ZnO nanoparticle-treated seedlings.

Primers	ZnO nanoparticles synthesized from precursors:			
	Zn-sulfate · $7H_2O$	Zn-chloride	Zn-nitrate · $6H_2O$	Zn-acetate
OPA-2	81.81	90.90	63.63	81.81
OPA-13	85.71	57.14	85.71	85.71
OPH-19	66.66	41.66	33.33	66.66
OPW-6	75	62.5	25	50
OPW-11	50	75	75	25
OPW-17	0	0	33.33	33.33
OPW-18	0	50	50	50
OPY-8	85.71	57.14	71.42	57.14
GTS %	55.61	54.29	54.67	56.20
Polymorphism %	44.39	45.71	45.33	43.80

from each of zinc acetate, zinc chloride, zinc nitrate hexahydrate and zinc sulfate heptahydrate precursors by *R. erythropolis* K85, and their toxicological potentials were investigated.

ZnO nanoparticles having different physical characteristics in shape and size were obtained in the biosynthesis steps for each precursor. These results cohered with the data in the literature that explains the biosynthesis of ZnO nanoparticles from zinc sulfate by *R. pyridinivorans* NT2 [23]. Besides, the occurrence of aggregates and microstructures together with ZnO nanoparticles may be explained by the assemblage of several nanoparticles to form a stable, non-uniform secondary size particle. This phenomenon arises from large specific surface area and high surface energy level of nanoparticles and results in the formation of more stable aggregates and secondary microstructures [39].

On the other hand, all the synthesized ZnO nanoparticles showed similar toxicological profiles despite their physical differences. In this regard, many toxicity studies performed with nanoparticles have especially focused on plants due to their crucial roles in ecosystems as primary producers of organic compounds from atmospheric or aqueous carbon dioxide. Almost all life on earth is directly or indirectly reliant on this phenomenon [18, 42]. Among these, the results of DNA-based toxicology studies are especially accepted as more adaptable to other species, including human beings due to the universal nature of DNA in all living organisms.

Recent studies have shown that ZnO nanoparticle exposure affects plant species and causes inhibitions in seed germination, gene expression levels, physiological activities, and growth-survival rates of seedlings. For example, Landa et al. (2012) reported that ZnO nanoparticles showed negative effects both on the growth and plant morphology and caused dramatic changes in *Arabidopsis* gene expression [18]. In that study, they also showed that 0.1 mg/ml is applicable for testing hazard potential of ZnO nanoparticles on plants. Similarly, Gunawan et al. (2013) tested the toxicity of ZnO nanoparticles at up to 0.1 mg/ml concentration and showed inhibition of cell proliferation and intracellular ROS generation in algae after ZnO nanoparticle exposure. These negative effects were even observed at lower concentrations such as 0.00001 mg/ml (0.1 mg/L) [19]. In the most recent study of Hossain et al. (2016), the test concentration for ZnO nanoparticles was chosen as 0.5 mg/ml. Plant growth, rigidity of roots, and root cell viability proteomic patterns in soybeans were markedly affected by ZnO nanoparticle treatment [42]. In addition, Vankova et al. (2017) evaluated the impact of ZnO nanoparticles on *Arabidopsis thaliana* plants at up to 0.1 mg/ml concentration and they indicated significant changes in the level of phytohormones such as cytokinins, auxin, abscisic acid, salicylic acid and jasmonic acid [43].

In a similar fashion, *T. aestivum* seedlings were significantly affected by all ZnO nanoparticle treatments in the present study and a significant inhibition was observed in the growth rates for each test group. This inhibition was explained with the induction of apoptosis, polymerization of F-actin, ROS generation, reduced glutathione (GSH) depletions, and morphological changes in nucleus by ZnO nanoparticle exposures. Especially the inductive potential of ZnO nanoparticles on intracellular ROS generation has become prominent because of their disruptive effects on cellular components such as lipids, proteins and nucleic acids, resulting in metabolic changes and eventually cell death [18, 31, 32, 39]. ROS generation and cellular oxidative stress are also known as the main reasons for unscheduled DNA alterations called as mutations in living organisms. In this manner, comparative RAPD profile analysis with known primers, a PCR-based technique that checks genomic material damages and mutational events, serves as a useful tool for determining the genotoxic features of test materials on eukaryotic systems [34-35].

The RAPD assay results of the present study referenced how one possible reason for the inhibition of the growth of treated seedlings may be genotoxic activities of the test materials, which cause changes in DNA sequences of the seedlings and result in differentiations in the RAPD profiles. In addition, the data was also supported by the decrease in the GTS value for each test material.

Conclusions

Results of the present study mainly emphasize that there is an urgent necessity to determine toxicological properties of ZnO nanoparticles and their exact mechanism of action on living organisms and the environment before commercialization, although biosynthesized nanomaterials are generally thought of as safe. On the other hand, this is the first report that ZnO nanoparticles were microbiologically biosynthesized from zinc acetate, zinc chloride and zinc nitrate hexahydrate precursors by using the *R. erythropolis* K85 strain, and this production process can be brought to green synthesis applications after toxicological problems are overcome with the optimization studies to be done.

Conflict of Interest

The authors declare no conflict of interest.

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