Application of Zinc Oxide Nanoparticles and Plant Growth Promoting Bacteria Reduces Genetic Impairment under Salt Stress in Tomato (*Solanum lycopersicum* L. ‘Linda’)

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Abstract: Salinity is an edaphic stress that dramatically restricts worldwide crop production. Nanomaterials and plant growth-promoting bacteria (PGPB) are currently used to alleviate the negative effects of various stresses on plant growth and development. This study investigates the protective effects of different levels of zinc oxide nanoparticles (ZnO-NPs) (0, 20, and 40 mg L$^{-1}$) and PGPBs (no bacteria, *Bacillus subtilis*, *Lactobacillus casei*, *Bacillus pumilus*) on DNA damage and cytosine methylation changes in the tomato (*Solanum lycopersicum* L. ‘Linda’) seedlings under salinity stress (250 mM NaCl). Coupled Restriction Enzyme Digestion-Random Amplification (CRED-RA) and Randomly Amplified Polymorphic DNA (RAPD) approaches were used to analyze changes in cytosine methylation and to determine how genotoxic effects influence genomic stability. Salinity stress increased the polymorphism rate assessed by RAPD, while PGBP and ZnO-NPs reduced the adverse effects of salinity stress. Genomic template stability was increased by the PGPBs and ZnO-NPs application; this increase was significant when *Lactobacillus casei* and 40 mg L$^{-1}$ of ZnO-NPs were used. A decreased level of DNA methylation was observed in all treatments. Taken together, the use of PGPB and ZnO-NPs had a general positive effect under salinity stress reducing genetic impairment in tomato seedlings.

Keywords: DNA methylation; genomic instability; PGPB; salt stress; ZnO-NP

1. Introduction

Salinity is one of the most significant abiotic stresses that limits the availability of soil water and inhibits germination and growth, ultimately leading to decreased crop production worldwide [1]. Various stresses lead to enhanced accumulation of reactive oxygen species (ROS) and induce oxidative stress. Under these conditions, plants activate their antioxidant systems to lower over-accumulation
of ROS and maintain a balanced reduction-oxidation state. Under these conditions, plants activate antioxidant systems to lower accumulation of ROS and maintain a balanced reduction-oxidation (redox) state. At the cellular level, salinity stress induces the generation of ROS in various parts of plant cells and tissues. ROS play a vital role in the activation of intracellular redox signaling systems and antioxidant resistance mechanisms. ROS are known as a secondary internal stress and induce various changes in cellular metabolism such as protein denaturation, lipid peroxidation, and DNA mutation [2]. Local chromatin changes and DNA methylation in response to salinity have been studied, and the significance of epigenetic regulation has been emphasized [3]. DNA methylation is an evolutionarily conserved epigenetic mechanism that controls numerous biological processes, including gene imprinting, tissue-specific gene expression, inactivation of transposable elements (TEs), paramutation, and stress responses. In plants, DNA methylation occurs in the CG, CHG, and CHH contexts (where H represents A, C, or T). The methylation level is dynamically controlled by establishment, maintenance, and removal of cytosine methylation (DNA demethylation). The establishment and maintenance of DNA methylation in plants are well understood and have been comprehensively reviewed. The elucidation of mechanisms for active DNA demethylation in plants provides more opportunity to explore the function of active DNA demethylation in gene regulation and plant development [4,5].

Tomato (Solanum lycopersicum L.) is one of the major food crops and a main source of several phytonutrients that provide important nutritional value to the human diet [1]. This plant is highly susceptible to salinity. Therefore, germplasm improvement through a combination of classical breeding and new biotechnological tools has become a key focus in tomato production [6].

In the past decade, some strategies, including plant genetic engineering [7] and plant growth-promoting bacteria (PGPB) [8], have been used to alleviate plant stress caused by salinity [9]. Recently, nanodevices and nanomaterials have been developed and introduced as potential novel technologies to solve these agricultural issues [10]. In comparison with the application of fertilizers and pesticides, the use of nanomaterials can improve seed germination, nutrient utilization, plant tolerance to abiotic and biotic stresses, and plant growth with reduced environmental impact [11]. Zinc oxide nanoparticles (ZnO-NPs) play an important role in the different mechanisms of plants to recognize and respond to abiotic stresses [12]. Although there are several reports regarding the interaction between salinity and ZnO in higher plants, there is insufficient information available about the possible positive effects of ZnO-NP applications to reduce damage due to salinity stress. In addition, such strategies as plant genetic engineering [7] and use of PGPBs [8] can be applied to alleviate plant stress caused by salinity [9].

There are several methods, e.g., comet, chromosome aberration, or micronucleus assays, available to detect genotoxic effects [13]. However, these methods have some limitations regarding their sensitivity and detection ability. More sensitive and selective DNA analysis methods have been developed with the advent of molecular marker technology. Molecular markers highlight differences (polymorphisms) between individuals. These differences include mutations such as insertions, deletions, translocations, duplications, and point mutations. They do not, however, comprehend the activity of specific genes. A variety of molecular markers exist to detect polymorphisms. For example, Randomly Amplified Polymorphic DNA (RAPD) can be employed effectively to determine different toxicities in plants [14,15]. The coupled restriction enzyme digestion-random amplification (CRED-RA) method has been successfully used to assess cytosine methylation in the plant genome or toxicity caused by environmental stressors such as salinity [15,16], heavy metals [17,18], and pesticides [14,19,20]. Thus far, no reports exist that describe a protective effect of ZnO-NPs and PGPBs against salinity stress. The current work therefore aimed to determine whether ZnO-NPs and PGPBs have any protective effect against genomic instability and DNA methylation under salinity stress.
2. Materials and Methods

2.1. Plant Material and Growth Conditions

Tomato (*Solanum lycopersicum* L. ‘Linda’) seeds were obtained from the Department of Horticulture, Faculty of Agriculture, Ataturk University (Erzurum, Turkey). Uniform seeds were grown in plastic boxes containing a 1:3 mixture of quartz sand and peat moss. After 14 days of germination, three seedlings were transferred to pots (17 cm × 15.5 cm, 2500 mL) at a 1:3 ratio of quartz sand and peat moss. During growth salinity stress was induced by the application of 20 mL of 250 mM NaCl to each pot three times a week by irrigation. Control plants were grown without the addition of NaCl solution or any PGPB with various concentrations of ZnO-NPs. Before performing the main investigation, a preliminary study was conducted to test whether microorganisms could continue to be viable under nanoparticle and salt mixture. The maximum doses of 20 different PGPRs (1 × 10^9 CFU mL\(^{-1}\)) and 100 mg L\(^{-1}\) ZnO-NPs in different combinations were determined under 250 mM extreme salt stress by the disk diffusion method. Finally, three PGPB were selected from this study (Figure 1).

![Figure 1](image)

**Figure 1.** Testing mixture buffer impregnated to disks for 20 different PGPBs demonstrates (a) microorganisms that maintain their viability against nanoparticle and salt mixture in the selection; (b) microorganisms that do not maintain their viability against nanoparticle and salt mixture; and (c) scanning electron microscopy of image (a), bacteria living on ZnO nanoparticles.

2.2. Phenotypic Assay

This study was conducted as a factorial experiment with completely randomized design and four replications. The first factor consisted of four different PGPB, including no bacteria, *Bacillus subtilis* (Ehrenberg 1835) Cohn 1872 (1 × 10^9 cfu mL\(^{-1}\)), *Lactobacillus casei* (Orla-Jensen 1916) Hansen & Lessel 1971 (1 × 10^9 cfu cfu mL\(^{-1}\)), and *Bacillus pumilus* (1 × 10^9 cfu mL\(^{-1}\)). The second factor included three different concentrations: 0 (control, distilled water), 20, and 40 mg L\(^{-1}\) ZnO-NPs (>100 nm) (Sigma Aldrich, Germany, #1314-13-2). Furthermore, for control treatment, plants received no NaCl solution, PGPB regulators, or ZnO-NP nanoparticles. For salinity; the plants were treated with 250 mM NaCl until the end of the study when the plants needed water. Application of ZnO-NPs with PGPBs was performed into the rhizosphere area of plants in pots by injection (20 mL) twice a week. All plants were grown in controlled growth conditions in a greenhouse with a day/night cycle of 16/8 h natural light, 25/18 °C, and 60/70% relative humidity. To collect phenotypic data, flag leaf width (FLW) (cm), plant height (PH) (cm), stem diameter (SD) (mm), leaf fresh weight (LFW) (g/plant), leaf dry weight (LDW) (g/plant), root fresh weight (RFW) (g/plant), and root dry weight (RDW) (g/plant) were measured using a ruler and precision scales. Fresh leaves were collected prior to the flowering stage and stored at −80 °C until further analysis.
2.3. Genotypic Assay

2.3.1. Isolation of Genomic DNA

Genomic DNA was extracted based on a method described elsewhere [21]. The concentration of the extracted DNA was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and its quality was evaluated by 1.5% agarose gel electrophoresis.

2.3.2. Random Amplification of Polymorphic DNA and Coupled Restriction Enzyme Digestion-Random Amplification assays

First, 15 RAPD primers (Operon Technologies Inc., Alameda, CA, USA) were tested for their polymorphism values (Table 1). The PCR amplifications were performed in a mixture containing 20 mL 10× PCR buffer, 25 mM MgCl₂, 10 mM dNTP mix, ddH₂O, 10 pmol random primer, 1 U Taq DNA polymerase, and 50 ng mL⁻¹ template DNA. Amplifications were performed at 95 °C for 5 min, followed by 40 cycles of denaturing at 95 °C for 1 min, primer annealing at 35 °C for 1 min, and primer extension at 72 °C for 2 min. The final extension was 10 min at 72 °C.

Table 1. Primers used in RAPD and CRED-RA assays.

| Primer Name | Primer Sequence (5’→3’) |
|-------------|-------------------------|
| OPA-13      | CAGCACCCAC               |
| OPB-8       | GTCCACACGG               |
| OPB-10      | CTGCTGGGAC               |
| OPH-17      | CACTCTCCTC              |
| OPH-18      | GAATCGGGCA               |
| OPW-4       | CAGAAGCGGA               |
| OPW-5       | GCGGGATAAG               |
| OPY-8       | AGGCAGCGGA               |
| OPY-15      | AGTCGGCCCTT              |
| OPY-16      | GGGCCAATGT              |

For CRED-RA analysis, 1 mg of template DNA from each treatment was separately digested with 1 µL (1 FDU) HpaII and 1 µL (1 FDU) MspI (Thermo Scientific) endonucleases at 37 °C for 2 h according to the manufacturer’s guidelines. Digested DNA for each endonuclease was added to a PCR mix instead of nondigested gDNA. PCR amplifications were performed as described for amplification with RAPD markers.

2.3.3. Electrophoresis

All PCR products were separated with a 1.5% agarose gel at 100 V for 90 min and visualization was performed with ethidium bromide. To estimate the molecular weight of the fragments, a 100–1000 bp DNA ladder (Sigma Aldrich, # P1473-1VL) was used. The gels were photographed under UV light using a Universal Hood II (Bio-Rad, Hercules, CA, USA).

2.3.4. Statistical Analysis

Phenotypic Analysis

Analysis of variance (ANOVA) was performed using the general linear model (GLM) procedure in SPSS version 20 (SPSS, Chicago, IL, USA). Each pot was considered as an experimental unit. In each pot, three plants were selected to measure all variables. Treatment means were compared using Duncan’s test.
Genetic Analysis

The RAPD and CRED-RA banding patterns were analyzed using TotalLab TL120 software (Nonlinear Dynamics Ltd. Newcastle, UK). Polymorphism in the RAPD profiles was expressed as the disappearance of a normal band and the appearance of a new band relative to the control. The average polymorphism was calculated for each experimental group (NaCl treatment with ZnO-NP and PGPB applications), and changes in these values were calculated as a percentage of their value in the control (set to 100%) [19]. The genomic template stability (GTS), which is a quantitative measurement, was calculated for RAPD according to the following formula:

\[
GTS = (1 - \frac{a}{n}) \times 100
\]  

where ‘a’ is the average number of polymorphic bands found in each treated template, and n is the number of total bands in the control [4].

For CRED-RA analysis, the average values of polymorphism (%) were calculated for each concentration using the following formula:

\[
\text{Polymorphism} = \left( \frac{a}{n} \right) \times 100.
\]

3. Results

3.1. Phenotypic Analysis

Variance analysis on experimental data revealed significant differences in flag leaf width (FLW), plant height (PH), stem diameter (SD), leaf fresh weight (LFW), leaf dry weight (LDW), root fresh weight (RFW), and root dry weight (RDW) (p < 0.01) (Table 2). The highest mean FLW, PH, SD, LFW, LDW, RFW, and RDW were obtained in salinity stress + Lactobacillus casei + 40 mg L\(^{-1}\) ZnO-NPs (28 cm, 39.50 cm, 6.12 mm, 24.67 g/plant, 3.45 g/plant, 3.19 g/plant, and 0.31 g/plant, respectively) and the lowest mean values were observed under 250 mM NaCl stress (5 cm, 12 cm, 1.78 mm, 3.19 g/plant, 0.49 g/plant, 0.41 g/plant, and 0.08 g/plant, respectively) (Table 2; Figure 2).

Figure 2. Treated tomato seedlings (a)-Control, (b)-ZnO-NPs, (c)-Bacillus pumilis (N1), (d)-Bacillus pumilis (N1) + 20 mg L\(^{-1}\) ZnO-NPs, (e)-Bacillus pumilis (N1) + 40 mg L\(^{-1}\) ZnO-NPs.
Table 2. Mean comparison of different morphological traits after application of different PGPB and ZnO-NP concentrations in the tomato under salinity stress conditions.

| Experimental Group | FLW (cm) ¹ | PH (cm) | SD (mm) | LFW (g/plant) | LDW (g/plant) | RFW (g/plant) | RDW (g/plant) |
|--------------------|-----------|--------|---------|---------------|---------------|---------------|---------------|
| Control            | 8.5 ± 1.2 e ² | 17.25 ± 1.6 f | 2.19 ± 0.42 d | 5.72 ± 1.8 ef | 0.68 ± 0.24 ab | 0.64 ± 0.09 ed | 0.1 ± 0.08 e  |
| 250 mM NaCl        | 5 ± 0.5 e   | 12 ± 2 f   | 1.78 ± 0.4 e  | 3.19 ± 0.8 f  | 0.49 ± 0.9 f  | 0.41 ± 0.15 d | 0.08 ± 0.02 e |
| Salinity stress + Bacillus pumilus + 0 mg L⁻¹ ZnO-NPs | 21 ± 1.5 abc | 24.3 ± 1.7 de | 4.45 ± 0.23 c | 15.46 ± 5.9 d | 1.68 ± 1.2 de | 1.57 ± 0.7 ef | 0.17 ± 0.12 cde |
| Salinity stress + Bacillus pumilus + 20 mg L⁻¹ ZnO-NPs | 20 ± 3 c    | 28.45 ± 0.7 bcd | 4.95 ± 0.23 a | 18.43 ± 3.9 c | 2.78 ± 1.2 bc | 2.17 ± 0.8 be | 0.21 ± 0.81 bc |
| Salinity stress + Bacillus pumilus + 40 mg L⁻¹ ZnO-NPs | 22.1 ± 2.0 bc | 29.75 ± 1.5 cde | 5.37 ± 1.13 a | 20.43 ± 3.1 bc | 3.20 ± 1.7 b | 2.58 ± 1.4 bc | 0.25 ± 0.13 ab |
| Salinity stress + Lactobacillus casei + 0 mg L⁻¹ ZnO-NPs | 24.3 ± 1.2 bc | 33.0 ± 1.3 abc | 4.83 ± 0.93 bc | 21.38 ± 4.5 bc | 2.30 ± 0.6 cd | 3.01 ± 1.2 b | 0.27 ± 0.80 bc |
| Salinity stress + Lactobacillus casei + 20 mg L⁻¹ ZnO-NPs | 26 ± 0.7 b  | 32.53 ± 0.76 ab | 5.90 ± 0.42 a | 22.34 ± 3.4 b | 3.25 ± 1.4 ab | 3.10 ± 1.7 ab | 0.29 ± 0.12 ab |
| Salinity stress + Lactobacillus casei + 40 mg L⁻¹ ZnO-NPs | 28 ± 0.9 a  | 39.50 ± 0.45 a | 6.12 ± 0.76 a | 24.67 ± 2.7 a | 3.45 ± 0.9 a | 3.19 ± 1.7 a | 0.31 ± 0.7 a  |
| Salinity stress + Bacillus subtilis + 0 mg L⁻¹ ZnO-NPs | 11 ± 1.5 d  | 18.25 ± 1.8 ef | 3.11 ± 0.29 d | 10.05 ± 1.4 f | 1.34 ± 1.1 def| 1.09 ± 0.2 ed | 0.08 ± 0.05 e  |
| Salinity stress + Bacillus subtilis + 20 mg L⁻¹ ZnO-NPs | 13 ± 5 dc   | 18.6 ± 1.8 def | 3.6 ± 0.31 bc | 13.50 ± 3.3 de | 1.24 ± 0.14 ef| 1.26 ± 0.4 def| 0.15 ± 0.03 de |
| Salinity stress + Bacillus subtilis + 40 mg L⁻¹ ZnO-NPs | 18.5 ± 2.2 c | 26.5 ± 1.4 de | 4.41 ± 0.94 c | 14.95 ± 2.3 d | 1.45 ± 0.3 def | 1.28 ± 0.2 ef | 0.18 ± 0.1 de  |
| F value            | 17.70 **   | 29.38 ** | 9.31 ** | 24.00 **      | 11.06 **      | 8.85 **       |

¹ FLW: Flag leaf width, PH: plant height, SD: stem diameter, LFW: leaf fresh weight, LDW: leaf dry weight, RFW: root fresh weight, and RDW: root dry weight. ² Means with various letters in each column are significantly different at p ≤ 0.01.  ** Significant at the 0.01 probability level.
3.2. Genetic Analysis

3.2.1. RAPD Analysis

RAPD analysis was performed to determine the effects of co-application of PGPB and ZnO-NP treatments on tomato gDNA. Our results indicated that only 10 primers (OPA-13, OPB-8, OPB-10, OPH-17, OPH-18, OPW-4, OPW-5, OPY-8, OPY-15, and OPY-16) revealed sufficient polymorphism, namely specific and stable band profiles in all treatments. As shown in Table 3, a total of 71 bands were observed with control treatment. The highest number of bands were recorded for OPA-13 (11 bands), and the lowest for OPB-10, OPW-4, and OPY-8 (5 bands).

The molecular sizes of polymorphic bands ranged from 109 (OPW-5) to 693 bp (OPW-4). There were significant differences between RAPD profiles in the control, 250 mM NaCl treatment, and PGPB with ZnO-NP treatment. These differences were assessed as appearance (+) or disappearance (-) of the bands (shown as ± in Table 3 and Figure 3). Compared with the control, 114 new bands appeared while 89 bands disappeared in experimental groups.

![Figure 3. RAPD profiles for various experimental groups with OPH-17 primers in tomato. 1: 100–1000 bp DNA ladder, 2: Control, 3: 250 mM NaCl, 4: Salinity stress + Bacillus pumilus + 0 mg L\(^{-1}\) ZnO-NPs, 5: Salinity stress + Bacillus pumilus + 20 mg L\(^{-1}\) ZnO-NPs, 6: Salinity stress + Bacillus pumilus + 40 mg L\(^{-1}\) ZnO-NPs, 7: Salinity stress + Lactobacillus casei + 0 mg L\(^{-1}\) ZnO-NPs, 8: Salinity stress + Lactobacillus casei + 20 mg L\(^{-1}\) ZnO-NPs, 9: Salinity stress + Lactobacillus casei + 40 mg L\(^{-1}\) ZnO-NPs, 10: Salinity stress + Bacillus subtilis + 0 mg L\(^{-1}\) ZnO-NPs, 11: Salinity stress + Bacillus subtilis + 20 mg L\(^{-1}\) ZnO-NPs, 12: Salinity stress + Bacillus subtilis + 40 mg L\(^{-1}\) ZnO-NPs.](image)

The rate of polymorphism for salinity stress treatment was 42.26%. Each PGPB had a different response to the polymorphism rate with different concentrations of ZnO-NPs. Indeed, there is a direct association between polymorphism rate and ZnO-NP concentrations. The positive effect of PGPBs with ZnO-NPs in coping with salinity stress was detected as polymorphism rates of 32.39%, 29.58%, and 25.35% for Bacillus pumilus with 0, 20, and 40 mg L\(^{-1}\) ZnO-NPs application, respectively. Treatment with Lactobacillus casei with 0, 20, and 40 mg L\(^{-1}\) ZnO-NPs application enhanced the polymorphism rate to 26.76%, 25.35%, and 19.72%, respectively. Combination of Bacillus subtilis with 0, 20, and 40 mg L\(^{-1}\) ZnO-NPs enhanced the polymorphism rate to 36.62%, 25.35%, and 22.54%, respectively. Moreover, the different concentrations of ZnO-NPs yielded different responses in GTS value. There was a clear inverse trend in the GTS value with increasing concentrations of ZnO-NPs in all PGPB applications. The lowest GTS value (57.74%) was observed in salinity stress, while the highest GTS values were observed with PGPB with ZnO-NP treatments.
Table 3. Molecular sizes (bp) of appearing and disappearing bands in RAPD profiles after the application of different PGPB and ZnO-NP concentrations in the tomato under salinity stress conditions.

| Primer | ± Control | 250 mM NaCl | Salinity Stress + *Bacillus pumilus* | Experimental Group |
|--------|-----------|--------------|-------------------------------------|--------------------|
|        |           | 20 mgL⁻¹ | 40 mgL⁻¹ | 0 mgL⁻¹ | 40 mgL⁻¹ | 20 mgL⁻¹ | 40 mgL⁻¹ | 0 mgL⁻¹ | 40 mgL⁻¹ | 20 mgL⁻¹ | 40 mgL⁻¹ |
|        |           | ZnO-NPs | ZnO-NPs | ZnO-NPs | ZnO-NPs | ZnO-NPs | ZnO-NPs | ZnO-NPs | ZnO-NPs | ZnO-NPs | ZnO-NPs |
| OPA-13 | + 11      | 520; 140 | 518; 434 | 487; 175 | 500 | 175 | - | - | - | - | - |
|        | - 6       | 635; 396 | 665 | 665 | 665; 587 | 665; 665 | 665; 587; 539 | 665; 202; 110 | 665; 587 |
| OPB-8  | + 7       | 635 | 537; 274 | 534 | 547 | 366; 300 | 500 | - | 534 | 491 | - |
|        | - 7       | 390 | 173 | 377 | 306 | 377 | - | - | - | - | - |
| OPB-10 | + 5       | 375 | 348 | - | - | - | - | - | - | 274 | - |
|        | - 5       | 443; 325; 278 | 414 | 400 | 400 | 414 | 414 | 406 | 428 | 400 | 510; 410 |
| OPH-17 | + 6       | 530; 340 | 147 | - | - | 147 | 147 | 147 | 340; 147 | 147 | 147 |
|        | - 6       | 372 | 147 | - | - | - | - | - | - | 709; 451 | - |
| OPH-18 | + 7       | 420; 392; 125 | 328; 291 | 291 | 664 | 355 | - | - | - | - | - |
|        | - 7       | 585; 395 | 585 | - | - | - | - | - | 633; 536 | 536 | 633 |
| OPW-4  | + 5       | 337 | 317; 192 | 693; 400; 271; 163 | 427; 311 | 486; 200 | 422 | 490 | 486 | 427; 176 | 486; 264 |
|        | - 5       | 585; 395 | 585 | - | - | - | - | - | 633; 536 | 536 | 633 |
| OPW-5  | + 7       | 665; 428 | 120 | 258 | 264; 152 | 484; 434 | 571; 109 | 484; 134 | - | 588; 148 | 420; 143 | 148 |
|        | - 7       | 445; 272 | - | - | 269; 211; 145 | 426; 300; 206; 161 | 339; 291; 222; 100 | 434; 288; 195; 122 | 400; 291; 200 | 393; 278; 228; 167 | 300; 248; 195 | 282; 222; 183 | 274; 167 |
| OPY-8  | + 5       | 639 | 554 | 588; 193; 147 | - | 558; 152 | - | 660 | 617 | 617 |
|        | - 5       | 410; 322 | 400; 359 | 359; 119 | 359 | 446; 359 | 359 | 446; 359 | 400; 227; 119 | 537 | 169; 119 |
| OPY-15 | + 9       | 428 | 215; 149 | 164 | - | - | 215 | 416 | - | - | - |
|        | - 9       | 395; 252 | 525 | 368 | 292 | - | 368 | 586; 118 | 586; 525; 246 | 586 | 586; 525 |
| Total band | 71 | 30 | 23 | 21 | 18 | 19 | 18 | 14 | 26 | 18 | 16 |
| Polymorphism (%) | 42.26 | 32.39 | 29.58 | 25.35 | 26.76 | 25.35 | 19.72 | 36.62 | 25.35 | 22.54 |
| GTS    | 57.74 | 67.61 | 70.42 | 74.65 | 73.24 | 74.65 | 80.28 | 63.38 | 74.65 | 77.46 | 80.28 |
GTS values for *Bacillus pumilus* with 0, 20, and 40 mg L\(^{-1}\) ZnO-NPs application were 67.61%, 70.42%, and 74.65%, respectively, while a combination of *Lactobacillus casei* with 0, 20, and 40 mg L\(^{-1}\) ZnO-NPs resulted in GTS values of 73.24%, 74.65%, and 80.28%, respectively. *Bacillus subtilis* with concentrations of 0, 20, and 40 mg L\(^{-1}\) ZnO-NPs resulted in values of 63.38%, 74.65%, and 77.46%, respectively (Table 3).

### 3.2.2. CRED-RA Analysis

Among the 15 tested RAPD primers, 10 generated acceptably specific and stable bands and were used for CRED-RA analysis (Figure 4 and Table 4). CRED-RA analysis enabled observation of any possible cytosine methylation caused by salinity stress and enhancement in cytosine methylation due to treatment of PGPBs with ZnO-NPs. The results of the CRED-RA analysis as the average polymorphism proportion with respect to HpaII and MspI digestions to determine cytosine methylation for experimental groups are presented in Table 4. A total of 78 and 86 fragments were observed for the MspI- and HpaII-digested control treatments, respectively.

![Figure 4. CRED-RA profiles for various experimental groups with OPH-17 primers in tomato. 1; 100–1000 bp DNA ladder, 2; Control Hpa II, 3; Control Msp I, 4; 250 mM NaCl Hpa II, 5; 250 mM NaCl Msp I, 6; Salinity stress + *Bacillus pumilus* + 0 mg L\(^{-1}\) ZnO-NPs Hpa II, 7; Salinity stress + *Bacillus pumilus* + 0 mg L\(^{-1}\) ZnO-NPs Msp I, 8; Salinity stress + *Bacillus pumilus* + 20 mg L\(^{-1}\) ZnO-NPs Hpa II, 9; Salinity stress + *Bacillus pumilus* + 20 mg L\(^{-1}\) ZnO-NPs Msp I, 10; Salinity stress + *Bacillus pumilus* + 40 mg L\(^{-1}\) ZnO-NPs Hpa II, 11; Salinity stress + *Bacillus pumilus* + 40 mg L\(^{-1}\) ZnO-NPs Msp I, 12; Salinity stress + *Lactobacillus casei* + 0 mg L\(^{-1}\) ZnO-NPs Hpa II, 13; Salinity stress + *Lactobacillus casei* + 0 mg L\(^{-1}\) ZnO-NPs Msp I, 14; Salinity stress + *Lactobacillus casei* + 20 mg L\(^{-1}\) ZnO-NPs Hpa II, 15; Salinity stress + *Lactobacillus casei* + 20 mg L\(^{-1}\) ZnO-NPs Msp I, 16; Salinity stress + *Lactobacillus casei* + 40 mg L\(^{-1}\) ZnO-NPs Hpa II, 17; Salinity stress + *Lactobacillus casei* + 40 mg L\(^{-1}\) ZnO-NPs Msp I, 18; Salinity stress + *Bacillus subtilis* + 0 mg L\(^{-1}\) ZnO-NPs Hpa II, 19; Salinity stress + *Bacillus subtilis* + 0 mg L\(^{-1}\) ZnO-NPs Msp I, 20; Salinity stress + *Bacillus subtilis* + 20 mg L\(^{-1}\) ZnO-NPs Hpa II, 21; Salinity stress + *Bacillus subtilis* + 20 mg L\(^{-1}\) ZnO-NPs Msp I, 22; Salinity stress + *Bacillus subtilis* + 40 mg L\(^{-1}\) ZnO-NPs Hpa II, 23; Salinity stress + *Bacillus subtilis* + 40 mg L\(^{-1}\) ZnO-NPs Msp I.](image-url)
Table 4. Results of CRED-RA analysis, molecular size of bands, polymorphism percentage, and type of methylation.

| Primer | M/H | ± | Control | Experimental Group |
|--------|-----|---|---------|---------------------|
|        |     |   | 250 mM NaCl | Salinity Stress + Bacillus pumilis | Salinity Stress + Lactobacillus casei | Salinity Stress + Bacillus subtilis |
|        |     |   | 0 mgL⁻¹ | 20 mgL⁻¹ | 40 mgL⁻¹ | ZnO-NPs | ZnO-NPs | ZnO-NPs | 0 mgL⁻¹ | 20 mgL⁻¹ | 40 mgL⁻¹ | ZnO-NPs | ZnO-NPs | ZnO-NPs |
|        |     |   | 0 mgL⁻¹ | 20 mgL⁻¹ | 40 mgL⁻¹ | ZnO-NPs | ZnO-NPs | ZnO-NPs | 0 mgL⁻¹ | 20 mgL⁻¹ | 40 mgL⁻¹ | ZnO-NPs | ZnO-NPs | ZnO-NPs |
| OPA-13 | M  | + | 8  | 552; 356; 252 | - | - | 394; 238 | 574; 414 | - | - | - | 100; 190 | - | - |
|        | H  | + | 9  | 325; 232 | 146 | 464; 115 | 464; 115 | - | - | - | 464; 432 | 464 | 473; 329 | 464 |
|        |    |   | 225; 182 | 209; 146 | - | 190 | - | - | - | - | - | - | 120 |
|        | Class |   |    |    | 585; 382 | 473; 439 | 473; 439 | 473; 329; 126 | 473 | 473; 429 | 473; 329 | 473; 386 | 464 | 432 | 473; 429; 386 |
| OPB-8  | M  | + | 7  | 285; 220 | - | 211 | 194 | - | - | - | 138 | - | - |
|        | H  | + | 8  | 650 | 685 | - | - | - | - | - | - | - | - |
|        |    |   | 522 | 367 | - | 353 | 339 | - | - | - | 367 | - | - |
|        | Class |   |    |    | 750; 652; 225 | 150; 126 | 669; 600; 150 | 200 | 150; 126 | 126 | 150 | 600; 126 | - | 669; 126 |
| OPB-10 | M  | + | 6  | 465; 335 | 481; 435 | 674; 600; 459 | 623; 476 | 817; 687; 527; 435 | 600; 445 | 487 | 762; 661; 578; 440 | 674; 454; 427 | 578; 435 |
|        | H  | - | 5  | 204 | 144 | 144 | 223; 144 | 223; 144 | 223; 144 | 144 | 223; 144 | - | - | 223; 144 |
|        | Class |   |    |    | 628; 324 | 427 | 762; 600; 427 | 611; 260 | 661; 500; 272 | 440 | 623; 389 | 674; 379 | 635; 431; 372 | 266 |
| OPH-17 | M  | + | 6  | 424; 357; 189 | 431; 230 | 533; 167 | 230; 192 | 431; 300; 258 | 431 | 550; 400; 254 | 550; 330; 230 | - | 451; 220 |
|        | H  | - | 8  | 580; 150 | 129 | - | - | 400; 300 | 407; 180 | 531; 415 | 170 | 150 | - | - |
|        | Class |   |    |    | 545; 450; 354 | - | - | - | - | - | - | - | - |
| OPH-18 | M  | + | 12 | 423; 378; 310 | 472; 294 | 328; 287; 223 | 142 | 217; 120 | 462; 229 | 558; 467 | 431; 316; 205 | 216; 116 | - | - |
|        | H  | + | 12 | 379; 316; 216; 157 | 311; 211 | 305; 242; 205 | 359; 120 | 728; 323; 130 | 350; 229 | 242; 194 | 536; 223 | 200; 130 | - | - |
|        | Class |   |    |    | 309; 116 | - | 629 | 589; 328 | - | - | 589 | 589 | - | 528 |
| OPW-4  | M  | + | 9  | 462; 476; 352 | - | 463; 304 | 454 | 561 | 459 | 250 | 504; 485 | - | - |
|        | H  | + | 11 | 525; 439 | 332 | 565 | - | 332 | 332 | - | 332 | 404; 284; 135 | 593; 227 | - | - |
|        | Class |   |    |    | 641; 521; 402 | 528 | - | - | - | - | - | - | - |
|        |    |   | 505; 450; 310 | 345; 284 | 284 | 284; 135 | 505; 284 | 474; 345; 284 | 345; 284; 135 | 345; 284 | 622; 505; 284 | - | - |
|        | Class |   |    |    | 750; 652; 225 | 150; 126 | 669; 600; 150 | 200 | 150; 126 | 126 | 150 | 600; 126 | - | 669; 126 |
Table 4. Cont.

| Primer | M/H | ± | Control | 250 mM NaCl |
|--------|-----|---|---------|-------------|
|        |     |   |         | Salinity Stress + Bacillus pumilus | Salinity Stress + Lactobacillus casei | Salinity Stress + Bacillus subtilis |
|        |     |   |         | 0 mL L⁻¹ | 20 mL L⁻¹ | 40 mL L⁻¹ | 0 mL L⁻¹ | 20 mL L⁻¹ | 40 mL L⁻¹ | 0 mL L⁻¹ | 20 mL L⁻¹ | 40 mL L⁻¹ |
|        |     |   |         | ZnO-NPs | ZnO-NPs | ZnO-NPs | ZnO-NPs | ZnO-NPs | ZnO-NPs | ZnO-NPs | ZnO-NPs | ZnO-NPs |
| OPW-5  | M + | 12|         | 562; 424; 325; 282; 171 | 529; 171 | 656 | - | - | - | - | - | - |
|        | M - |   |         | - | 557; 368 | 395; 303 | 512; 253 | 614; 557; 395; 230 | 614; 368 | 303 | 557; 395; 230 | 614; 557; 395; 409 | 614; 557; 395; 409 |
|        | H + | 13|         | 203 | 585; 553; 475; 347; 255; 154 | 249 | - | - | - | - | - | - |
|        | H - |   |         | - | 553; 347; 230 | 679; 533; 525; 409 | 679; 533 | 679; 614; 525; 492; 475; 409 | 679; 614; 492; 347 | 230 | 679; 614; 553; 525; 475 |
| Class  | Class I | Class I | Class I | Class I | Class I | Class I | Class I | Class I | Class I | Class I | Class I |
| OPY-8  | M + | 7 |         | 487; 215 | 706; 607 525; 281 | 613; 255 | 766; 281 | 693; 234; 100 | 712; 268; 214 | 600; 281 | 700; 274 | 500 | - |
|        | M - |   |         | - | 342 | - | 381; 164 | - | 322 | - | 164 | - | 657; 562; 492 |
|        | H + | 9 |         | 441; 414; 278; 189 | 419; 281 | - | 687; 500 | 476; 143 | - | 300 | 508 | 693; 358; 281 | - |
|        | H - |   |         | - | - | 164 | - | 606 | - | 719; 164 | - | - | 719; 651; 606 |
| Class  | Class IV | Class IV | Class IV | Class IV | Class IV | Class I | Class III | Class IV | Class IV | Class I | Class I |
| OPY-15 | M + | 5 |         | 550 | 433; 315 | 480; 414; 323 | 386; 358 | 344 | 364; 321 | 413; 344; 233 | 354; 307; 270 | 433; 347; 239 | 331 |
|        | M - |   |         | 512; 386 | 542 | - | - | 491; 195 | 542 | - | - | 542 |
|        | H + | 4 |         | 420; 358; 262 | 240 | 350; 243 | 427 | 413; 336 | 413; 355; 232 | 463; 413; 315; 232 | 362; 307; 206 | 354; 307 |
|        | H - |   |         | 579; 180 | 184 | 513 | - | 513; 184 | 184 | - | - | - |
| Class  | Class I | Class II | Class IV | Class II | Class IV | Class I | Class I | Class IV | Class I | Class IV | Class I |
| OPY-16 | M + | 6 |         | 668; 400 | 280; 247 | 529; 405; 239 | 669 | 704 | 641; 474; 264 | 665; 509; 280; 247 | 509; 452; 224 | - |
|        | M - |   |         | 519; 422; 375; 227 | 569 | 449 | - | 449 | - | - | 375 | 569 |
|        | H + | 7 |         | 503; 454; 221 | 669; 517; 500 | 546; 506 | 557; 465; 351 | - | - | 466; 264 | 646; 448 | 641; 227 | 679 |
|        | H - |   |         | 223 | 339 | 260 | - | 422; 339; 260 | 260 | 339 | 339 | - | 485; 422; 339 |
| Class  | Class III | Class IV | Class IV | Class IV | Class IV | Class I | Class IV | Class IV | Class I | Class IV | Class I |
| Polymorphism % | M | 53.88 | 39.03 | 38.98 | 32.73 | 36.79 | 35.42 | 30.46 | 30.37 | 34.01 | 29.60 | 33.32 |
|        | H | 51.53 | 37.31 | 30.53 | 30.11 | 35.91 | 29.19 | 29.46 | 30.70 | 29.60 | 29.46 | 33.78 |

1 M- Msp I, H- Hpa II, 2 Control (without bacteria and ZnO-NPs).
Under salinity treatment and PGPB with ZnO-NP treatments, MspI had a higher polymorphism value than HpaII. While a 53.88% MspI polymorphism proportion was observed in 250 mM NaCl stress, this value decreased and ranged between 30.46% to 40.70% with the application of PGPBs with different concentrations of ZnO-NPs. Polymorphism values detected by HpaII digestion ranged from 29.19% (at different application levels of PGPB and ZnO-NP concentrations) to 51.53% (under salinity stress) (Table 4). Thus, salinity-stressed plants showed high polymorphism values in both MspI- and HpaII-digested CRED-RA assays. In contrast, application of PGPBs with different concentrations of ZnO-NPs under salinity stress resulted in decreased polymorphism in both HpaII and MspI digestions. The results therefore indicate that the 50-mM NaCl treatment had an impact on cytosine methylation status and can be classified as hypermethylation when the average polymorphism percentage for MspI digestion is considered. When different PGPB with various concentrations of ZnO-NPs were applied with 250 mM NaCl, a clear decrease in the average polymorphism percentage and methylation status was observed, which indicates that PGPB with various concentrations of ZnO-NPs have a protective role under salinity stress conditions. The polymorphism percentage gradually decreased with applications of PGPB with ZnO-NP treatment when compared with 50-mM NaCl treatment (Table 4). This status can be described as a hypomethylation phenomenon.

Based on the presence or absence of amplified fragments, the methylation patterns grouped into four classes (Table 5). A possible explanation for the protective role may be that the application of PGPB with different concentrations of ZnO-NPs leads to demethylation of one of the DNA strands or transfer of a methyl group from the outer to the inner cytosine (Table 5). Experimental groups were divided into four classes. The average rate of methylation for classes I, II, III, and IV was 35%, 20%, 8%, and 37%, respectively. Overall, classes I and IV were determined as the classes with the highest DNA methylation, occurring with treatments of PGPB with various ZnO-NP concentrations into the root rhizosphere of tomato.

Table 5. Methylation sensitivity and restriction pattern of isoschizomers and the average (%) rates of methylation class occurrences of CRED-RA bands in treated experimental groups.

| Type   | Methylation Status | Digestibility by Enzymes | Average (%) Rate of Methylation Class |
|--------|--------------------|--------------------------|---------------------------------------|
|        |                    | HpaII       | MspI       |                                        |
| Class I| CCGG CCGG          | Active      | Active     | 35.00%                                |
|        | GCCC GCCC          |             |            |                                        |
| Class II| CCGG CCGG        | Active      | Inactive   | 20.00%                                |
|        | GCCC GCCC          |             |            |                                        |
| Class III| CCGG CCGG    | Inactive    | Active     | 8.00%                                 |
|        | GCCC GCCC          |             |            |                                        |
| Class IV| CCGG CCGG        | Inactive    | Inactive   | 37.00%                                |
|        | GCCC GCCC          |             |            |                                        |

Underlined cytosine is methylated.

4. Discussion

Salinity is a significant problem that limits plant growth and development and dramatically reduces crop production [22]. High salt accumulation first causes an ion imbalance, leading to the production of ROS that can alter cellular metabolism [23]. In addition, salinity stress causes nucleus deformation [24].

In this study, morphologic traits differed among experimental groups. Various studies have shown that morphological traits in tomato plants are negatively affected by NaCl stress [25]. This decrease may be driven by changes in the expression of SOD and GPX encoding genes under salinity stress, as reduced expression has been reported under these conditions [7]. In the present study, tomato plants treated with ZnO-NPs at both levels (20 and 40 mg L\(^{-1}\)), with different PGPBs (\textit{Bacillus subtilis}, \textit{Lactobacillus casei}, and \textit{Bacillus pumilus}), under NaCl stress, showed improved morphological
characteristics (Table 2). It appears that the presence of a combination of ZnO-NPs with PGPB under salinity stress could affect morphological traits of tomato plants and alleviate the detrimental effects of salinity stress. This theory is supported by previous results indicating that a low dose of ZnO-NPs has a positive impact on plant metabolism, enhancing absorption of essential nutrients, such as nitrogen, which then affects ion homeostasis, osmolytic biosynthesis, protein content, and toxic radical scavenging [26].

In our study, we observed changes in genetic template stability and cytosine methylation caused by salinity stress in tomato. In addition, we observed that plants treated with different PGPBs at different concentrations of ZnO-NPs in the rhizosphere significantly reduced molecular disorders caused by salinity stress (Table 3). Currently, nanotechnology can be efficiently used in various fields of science. The interaction between salinity stress and the micronutrient composition in plants is poorly understood. However, micronutrients are affected by salinity stress [27]. Among plant micronutrients, Zn has important roles in plant growth and metabolism processes [28]. This micronutrient is essential for activating different enzymes such as aldolases, dehydrogenases, transphosphorylases, isomerases, and RNA and DNA polymerases. Zn is also required for tryptophan synthesis, cell division, maintenance of membrane structure, and photosynthesis [29]. It also has a crucial role in producing natural auxin (IAA) [30], decreasing uptake of excess Na⁺ and Cl⁻ [31], and regulation of protein synthesis [32]. ZnO-NPs have significant electrical and optical characteristics that can be employed in different areas of biological research such as coatings for removal of biological substances and toxic chemicals that contain heavy metals [33].

PGPBs are microorganisms that have positive effects on plant growth via a variety of mechanisms [34]. These effects include increased availability of nutrients and fixation of biological nitrogen [35], solubilization of phosphate and mineralization [36], and synthesis of plant hormones such as indole, gibberellins, or cytokinins [37]. Many researchers have reported that the use of PGPBs alleviates plant stress caused by salinity [9]. In the present study, we observed a similar result that the use of PGPB with ZnO-NPs decreased the adverse effects of salinity stress. In addition, this study revealed different perspectives with respect to reducing cytosine hypermethylation and improving genomic template stability. Our results show that salinity stress decreased the GTS value, indicating that NaCl had genotoxic effects on the tomato genome based on RAPD profiles (Table 3). The RAPD technique is known to be sensitive enough to detect DNA damage [38]. Any changes in RAPD profiles compared with profiles obtained from control samples were considered reductions in GTS [39,40]. DNA methylation is one of several epigenetic mechanisms that cells use to control gene expression. Plants under salinity stress can reprogram their gene expression through methylation and demethylation [7,41,42]. Methylation distinguishes between normal plants and plants under stress in terms of RAPD band profiling. In the present study, the CRED-RA technique was employed to investigate how the tomato genome alters its cytosine methylation status in response to salinity stress and any enhancement in DNA methylation status against salinity after PGPB with ZnO-NPs treatment. In this respect, under salinity stress conditions the tomato plants exhibited significant changes in cytosine methylation status that can be referred to as hyper methylation. Likewise, several studies have demonstrated abiotic stresses, such as chromium nitrate, zinc, arsenic, and lead sulfate stress/toxicity in maize [17,43] and aluminum chlorite stress/toxicity in wheat [18].

Our results also indicated that the application of PGPBs with ZnO-NPs significantly improved cytosine methylation status. This result suggests a hypomethylation status under salinity stress (Table 4). Indeed, hypermethylation is associated with gene silencing, while hypomethylation is related to active transcription [44]. Based on the classification of amplified fragments, methylation patterns mostly fell into classes I (MspI and HpaII are active only if both cytosines are unmethylated) and IV (MspI and HpaII are inactive if both cytosines are methylated) [45,46] (Table 4). A previous investigation into DNA methylation levels with the CRED-RA technique in the sunflower genome against zinc stress concluded that the highest methylation type was class IV [47].
5. Conclusions

Under salinity stress conditions, tomato plants often display phenotypic variations due to epigenetic polymorphisms at the cytosine methylation level. Our results revealed the protective role of ZnO-NPs and PGPBs against the negative effects of salinity stress on DNA damage and DNA hypermethylation in tomato (Solanum lycopersicum L. ‘Linda’). The results obtained through the application of different PGPBs and various concentrations of ZnO-NPs suggest an inverse relationship between the level of cytosine methylation and salinity tolerance. We propose that the salinity-mediated genotoxic effect leading to DNA hypermethylation in tomato plants could be mitigated by the application of PGPBs and ZnO-NPs.

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References

1. Shrivastava, P.; Kumar, R. Soil salinity: A serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. Saudi J. Biol. Sci. 2015, 22, 123–131. [CrossRef]
2. Belhadj Slimen, I.; Najar, T.; Ghram, A.; Dabbebi, H.; Ben Mrad, M.; Abdarabh, M. Reactive oxygen species, heat stress and oxidative-induced mitochondrial damage. A review. Int. J. Hyperth. 2014, 30, 513–523. [CrossRef]
3. Chen, L.-T.; Luo, M.; Wang, Y.-Y.; Wu, K. Involvement of Arabidopsis histone deacetylase HDA6 in ABA and salt stress response. J. Exp. Bot. 2010, 61, 3345–3353. [CrossRef] [PubMed]
4. Sigmaz, B.; Agar, G.; Arslan, E.; Aydin, M.; Taspinar, M.S. The role of putrescine against the long terminal repeat (LTR) retrotransposon polymorphisms induced by salinity stress in Triticum aestivum. Acta Physiol. Plant. 2015, 37, 251. [CrossRef]
5. Kumar, S.; Beena, A.S.; Awana, M.; Singh, A. Salt-induced tissue-specific cytosine methylation downregulates expression of HKT genes in contrasting wheat (Triticum aestivum L.) genotypes. DNA Cell Biol. 2017, 36, 283–294. [CrossRef] [PubMed]
6. Rinaldi, M.; Garofalo, P.; Rubino, P.; Steduto, P. Processing tomatoes under different irrigation regimes in Southern Italy: Agronomic and economic assessments in a simulation case study. J. Agrometeorol. 2011, 3, 39–56.
7. Wang, Y.; Wisniewski, M.; Meilan, R.; Uratsu, S.L.; Cui, M.; Dandekar, A.; Fuchigami, L. Ectopic expression of Mn-SOD in Lycopersicon esculentum leads to enhanced tolerance to salt and oxidative stress. J. Appl. Hortic. 2007, 9, 3–8. [CrossRef]
8. Dimkpa, C.; Weinand, T.; Asch, F. Plant–rhizobacteria interactions alleviate abiotic stress conditions. Plant Cell Environ. 2009, 32, 1682–1694. [CrossRef]
9. Shilev, S.; Sancho, E.D.; Benlloch-González, M. Rhizospheric bacteria alleviate salt-produced stress in sunflower. J. Environ. Monit. 2012, 15, 537–541. [CrossRef]
10. Nair, R.; Varghese, S.H.; Nair, B.G.; Maekawa, T.; Yoshida, Y.; Kumar, D.S. Nanoparticulate material delivery to plants. Plant Sci. 2010, 179, 154–163. [CrossRef]
11. Shykh, B.N.; Hasanazadeh, G.T.A.; Baghestani, M.M.; Zand, B. Study the effect of zinc foliar application on the quantitative and qualitative yield of grain corn under water stress. Electron. J. Crop Prod. 2009, 2, 59–73.
12. Prasad, T.; Sudhakar, P.; Sreenivasulu, Y.; Latha, P.; Munaswamy, V.; Reddy, K.R.; Sreeprasad, T.; Sajanlal, P.; Pradeep, T. Effect of nanoscale zinc oxide particles on the germination, growth and yield of peanut. J. Plant Nutr. 2012, 35, 905–927. [CrossRef]
13. Murali Achary, V.M.; Panda, B.B. Aluminium-induced DNA damage and adaptive response to genotoxic stress in plant cells are mediated through reactive oxygen intermediates. *Mutagenesis* **2009**, *25*, 201–209. [CrossRef] [PubMed]

14. Nardemir, G.; Agar, G.; Arslan, E.; Erturk, F.A. Determination of genetic and epigenetic effects of glyphosate on *Triticum aestivum* with RAPD and CRED-RA techniques. *Theor. Exp. Plant Physiol.* **2015**, *27*, 131–139. [CrossRef]

15. Saleh, B. DNA changes in cotton (*Gossypium hirsutum* L.) under salt stress as revealed by RAPD marker. *Adv. Hortic. Sci.* **2016**, *30*, 13–21.

16. Zhang, Q.; Liu, H.; Hu, H.; Li, S.; Ying, Y.; Wu, J. Analysis of the DNA methylation on *Camptotheca acuminate* decene plants growing in vitro in response to sodium chloride stress. *Propag. Ornam. Plants* **2014**, *14*, 76–83.

17. Erturk, F.A.; Agar, G.; Arslan, E.; Nardemir, G. Analysis of genetic and epigenetic effects of maize seeds in response to heavy metal (Zn) stress. *Environ. Sci. Pollut. Res.* **2015**, *22*, 10291–10297. [CrossRef]

18. Hossein-Pour, A.; Ozkan, G.; Nalci, O.B.; Haliloglu, K. Estimation of genomic instability and DNA methylation due to aluminum (Al) stress in wheat (*Triticum aestivum* L.) using iPBS and CRED-iPBS analyses. *Turk. J. Bot.* **2019**, *43*, 27–37. [CrossRef]

19. Taspinar, M.S.; Aydin, M.; Arslan, E.; Sigmaz, B.; Agar, G. Salinity and putrescine effects on DNA methylation changes in *Triticum aestivum* L. *J. Biotechnol.* **2017**, *256*, S101. [CrossRef]

20. Yildirim, N.; Agar, G.; Taspinar, M.S.; Turan, M.; Aydin, M.; Arslan, E. Protective role of humic acids against dicamba-induced genotoxicity and DNA methylation in *Phaseolus vulgaris* L. *Acta Agric. Scand. Sect. B Soil Plant Sci.* **2014**, *64*, 141–148.

21. Zeinalzadehtabrizi, H.; Hosseinpour, A.; Aydin, M.; Haliloglu, K. A modified genomic DNA extraction method from leaves of sunflower for PCR based analyzes. *J. Biodivers. Environ. Sci.* **2015**, *7*, 222–225.

22. Ahmadi, J.; Pour-Aboughadareh, A.; Fabriki-Ourang, S.; Mehrabi, A.-A.; Siddique, K.H. Screening wild progenitors of wheat for salinity stress at early stages of plant growth: Insight into potential sources of variability for salinity adaptation in wheat. *Crop Pasture Sci.* **2018**, *69*, 649–658. [CrossRef]

23. Ahmadi, J.; Pour-Aboughadareh, A.; Ourang, S.F.; Mehrabi, A.A.; Siddique, K.H. Wild relatives of wheat—*Aegilops*–*Triticum* accessions disclose differential antioxidative and physiological responses to water stress. *Acta Physiol. Plant.* **2018**, *40*, 90. [CrossRef]

24. Katsuhara, M.; Kawasaki, T. Salt stress induced nuclear and DNA degradation in meristematic cells of barley roots. *Plant Cell Physiol.* **1996**, *37*, 169–173. [CrossRef]

25. Nouck, A.E.; Taoufou, V.D.; Tsota, E.; Dibong, D.S.; Nguemezi, S.T.; Gouado, I.; Youmbi, E. Growth, biochemical constituents, micronutrient uptake and yield response of six tomato (*Lycopersicon esculentum* L.) cultivars grown under salinity stress. *Agron. J.* **2016**, *15*, 58–67.

26. Laware, S.; Raskar, S. Influence of zinc oxide nanoparticles on growth, flowering and seed productivity in onion. *Int. J. Curr. Microbiol. Appl. Sci.* **2014**, *3*, 874–881.

27. Falhi, A.; Zahedi, M.; Torabian, S.; Khoshgoftar, A. Response of wheat genotypes to foliar spray of ZnO and Fe2O3 nanoparticles under salt stress. *J. Plant Nutr.* **2017**, *40*, 1376–1385. [CrossRef]

28. Ojeda-Barrios, D.L.; Perea-Portillo, E.; Hernández-Rodriguez, O.A.; Ávila-Quezada, G.; Abadía, J.; Lombardini, L. Foliar fertilization with zinc in pecan trees. *Hort Sci.* **2014**, *49*, 562–566. [CrossRef]

29. Cakmak, I. Enrichment of cereal grains with zinc: Agronomic or genetic biofortification. *Plant Soil* **2008**, *302*, 1–17. [CrossRef]

30. Jiang, W.; Sun, X.; Xu, H.; Mantri, N.; Lu, H. Optimal concentration of zinc sulfate in foliar spray to alleviate salinity stress in Glycine soja. *J. Agric. Sci. Technol.* **2014**, *16*, 445–460.

31. Marschner, P. Rhizosphere biology. In Marschner’s *Mineral Nutrition of Higher Plants*, 3rd ed.; Elsevier: London, UK, 2012; pp. 369–388.

32. Ali, E.; Mahmoud, A.M. Effect of foliar spray by different salicylic acid and zinc concentrations on seed yield and yield components of mungbean in sandy soil. *Asian J. Crop Sci.* **2013**, *5*, 33–40. [CrossRef]

33. Behnajady, M.; Modirshahla, N.; Hamzavi, R. Kinetic study on photocatalytic degradation of CI Acid Yellow 23 by ZnO photocatalyst. *J. Hazard. Mater.* **2006**, *133*, 226–232. [CrossRef]

34. Vessey, J.K. Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil* **2003**, *255*, 571–586. [CrossRef]

35. Graham, P.; Vance, C. Nitrogen fixation in perspective: An overview of research and extension needs. *Field Crop. Res.* **2000**, *65*, 93–106. [CrossRef]
36. Rodríguez, H.; Fraga, R.; Gonzalez, T.; Bashan, Y. Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. *Plant Soil* **2006**, *287*, 15–21. [CrossRef]

37. Costacurta, A.; Vanderleyden, J. Synthesis of phytohormones by plant-associated bacteria. *Crit. Rev. Microbiol.* **1995**, *21*, 1–18. [CrossRef]

38. Citterio, S.; Aina, R.; Labra, M.; Ghiani, A.; Fumagalli, P.; Sgorbati, S.; Santagostino, A. Soil genotoxicity assessment: A new strategy based on biomolecular tools and plant bioindicators. *Environ. Sci. Technol.* **2002**, *36*, 2748–2753. [CrossRef] [PubMed]

39. Aly, A.A. Application of DNA (RAPD) and ultrastructure to detect the effect of cadmium stress in Egyptian clover and Sudan grass plantlets. *J. Stress Physiol. Biochem.* **2012**, *8*, 241–257.

40. Tanee, T.; Chadmuk, P.; Sudmoon, R.; Chaveerach, A.; Noikotr, K. Genetic analysis for identification, genomic template stability in hybrids and barcodes of the Vanda species (Orchidaceae) of Thailand. *Afr. J. Biotechnol.* **2012**, *11*, 11772–11781.

41. Peng, H.; Zhang, J. Plant genomic DNA methylation in response to stresses: Potential applications and challenges in plant breeding. *Prog. Nat. Sci.* **2009**, *19*, 1037–1045. [CrossRef]

42. Zhong, L.; Wang, J.-B. The Role of DNA Hypermethylation in Salt Resistance of *Triticum aestivum* L. *J. Wuhan Bot. Res.* **2007**, *1*, 19.

43. Erturk, F.A.; Aydin, M.; Sigmaz, B.; Taspinar, M.S.; Arslan, E.; Agar, G.; Yagci, S. Effects of As$_2$O$_3$ on DNA methylation, genomic instability, and LTR retrotransposon polymorphism in *Zea mays*. *Environ. Sci. Pollut. Res.* **2015**, *22*, 18601–18606. [CrossRef]

44. Steward, N.; Ito, M.; Yamaguchi, Y.; Koizumi, N.; Sano, H. Periodic DNA methylation in maize nucleosomes and demethylation by environmental stress. *J. Biol. Chem.* **2002**, *277*, 37741–37746. [CrossRef]

45. Lu, Y.; Rong, T.; Cao, M. Analysis of DNA methylation in different maize tissues. *J. Genet. Genom.* **2008**, *35*, 41–48. [CrossRef]

46. Zhang, C.; Shi, W.; Ma, K.; Li, H.; Zhang, F. EGTA, a calcium chelator, affects cell cycle and increases DNA methylation in root types of *Triticum aestivum* L. *Acta Soc. Bot. Pol.* **2016**, *85*, 3502. [CrossRef]

47. Bolukbasi, E.; Aras, E. Determination of DNA Methylation Levels with CRED-RA Technique in the Genome of Sunflower Seedlings (*Helianthus annuus* L.) Subjected to Zinc Stress. *Int. J. Environ. Agric. Biotechnol.* **2016**, *1*, 438–444. [CrossRef]

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