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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 PGPB 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).

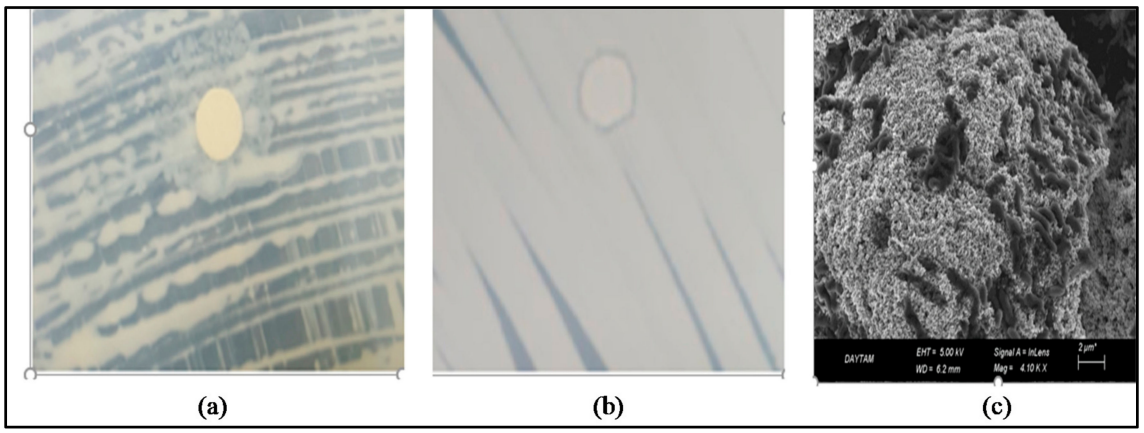

Figure 1. Testing mixture buffer impregnated to disks for 20 different PGPBs demonstrates (**a**) microorganisms that maintain their viability against nanoparticle and salt mixture in the selection; (**b**) microorganisms that do not maintain their viability against nanoparticle and salt mixture; and (**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 (1×109 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 $MgCl_2$, 10 mM dNTP mix, ddH_2O, 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	GAATCGGCCA
OPW-4	CAGAAGCGGA
OPW-5	GGCGGATAAG
OPY-8	AGGCAGAGCA
OPY-15	AGTCGCCCTT
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

Phonotypic 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:

$$GTS = (1 - a/n) \times 100 \quad (1)$$

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:

$$\text{Polymorphism} = (a/n) \times 100. \quad (2)$$

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^{-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 cf	0.18 ± 0.1 de
F value	17.70 **	29.38 **	9.31 **	24.00 **	14.20 **	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 $p \leq 0.01$. ** 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.

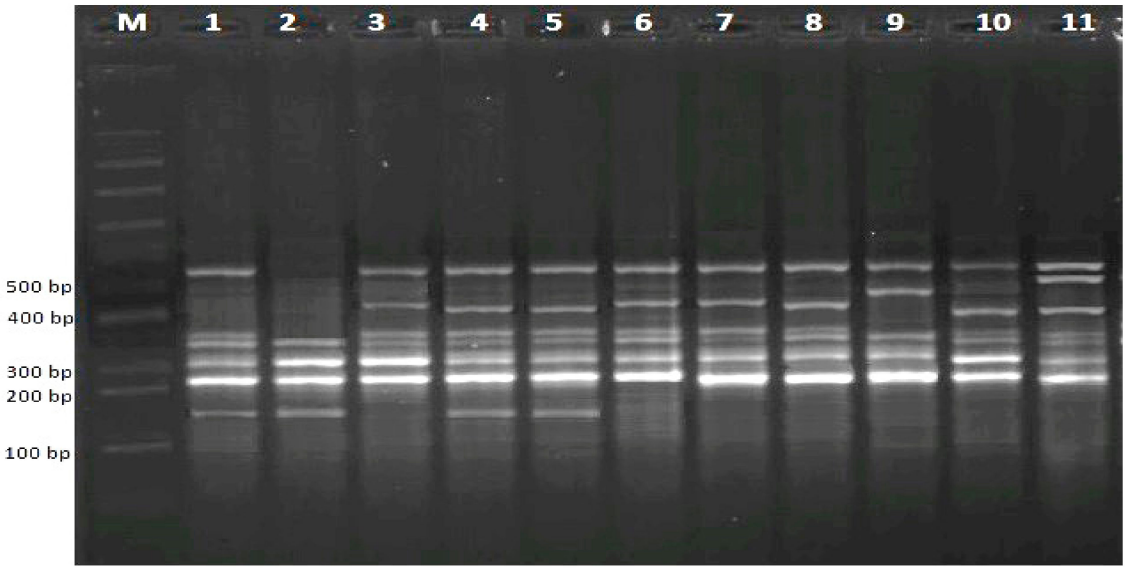

Figure 3. RAPD profiles for various experimental groups with OPH-17 primers in tomato. 1; 100–1000 bp DNA ladder, 2; Control, 3; 250 mM NaCl, 4; Salinity stress + *Bacillus pumilus* + 0 mg L^{-1} ZnO-NPs, 5; Salinity stress + *Bacillus pumilus* + 20 mg L^{-1} ZnO-NPs, 6; Salinity stress + *Bacillus pumilus* + 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.

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	Experimental Group								
			250 mM NaCl	Salinity Stress + *Bacillus pumilus*			Salinity Stress + *Lactobacillus Casei*			Salinity Stress + *Bacillus subtilis*	
				0 mgL^{-1} ZnO-NPs	20 mgL^{-1} ZnO-NPs	40 mgL^{-1} ZnO-NPs	0 mgL^{-1} ZnO-NPs	20 mgL^{-1} ZnO-NPs	40 mgL^{-1} ZnO-NPs	0 mgL^{-1} ZnO-NP	20 mgL^{-1} ZnO-NPs	40 mgL^{-1} ZnO-NPs
OPA-13	+	11	520; 140	-	518; 434	-	487; 175	500	-	175	-	-
	–		635; 396	665	-	665	665; 587	665	665; 587	665; 587; 539	665; 202; 110	665; 587
OPB-8	+	7	635	537; 274	534	547	366; 300	500	-	534	491	-
	–		390	173	377	306	-	377	-	-	-	-
OPB-10	+	5	375	348	-	-	-	-	-	-	-	-
	–		443; 325; 278	-	-	-	-	-	-	274	-	-
OPH-17	+	6	-	414	400	400	414	414	406	428	400	510; 410
	–		530; 340	147	-	-	147	147	147	340; 147	147	147
OPH-18	+	7	372	-	-	-	664	355	-	-	-	-
	–		420; 392; 125	328; 291	291	-	-	-	-	709; 451	-	-
OPW-4	+	5	337	317; 192	693; 400; 271; 163	427; 311	486; 200	422	490	486	427; 176	486; 264
	–		585; 395	585	-	-	-	-	-	633; 536	536	633
OPW-5	+	7	665; 428	464; 167	748; 152	484; 143	571; 109	484; 134	-	588; 148	420; 143	148
	–		120	258	-	-	-	-	-	-	258	-
OPY-8	+	5	-	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
	–		445; 372	-	-	580	-	-	-	-	-	-
OPY-15	+	9	639	-	554	588; 193; 147	-	558; 152	-	660	617	617
	–		410; 322	400; 359	359; 119	359	446; 359	359	446; 359	400; 227; 119	537	169; 119
OPY-16	+	9	428	215; 149	164	-	-	215	416	-	-	-
	–		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

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.

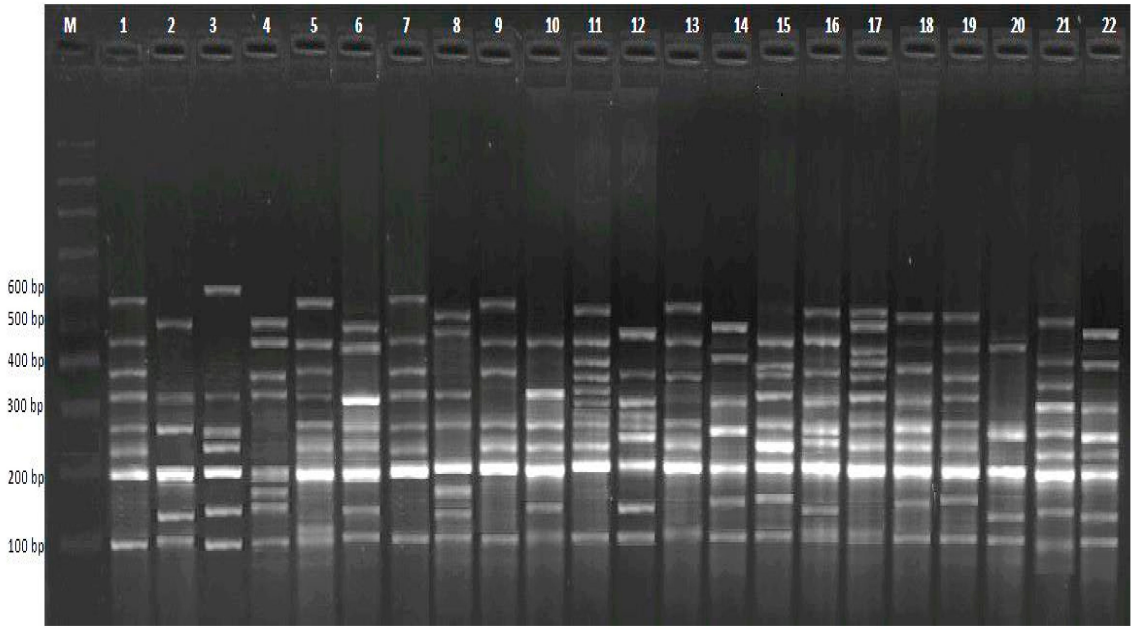

Figure 4. CRED-RA profiles for various experimental groups with OPH-17 primers in tomato. 1; 100–1000 bp DNA ladder, 2; Control Hpa II, 3; Control Msp I, 4; 250 mM NaCl Hpa II, 5; 250 mM NaCl Msp I, 6; Salinity stress + *Bacillus pumilus* + 0 mg L^{-1} ZnO-NPs Hpa II, 7; Salinity stress + *Bacillus pumilus* + 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.

Table 4. Results of CRED-RA analysis, molecular size of bands, polymorphism percentage, and type of methylation.

Primer	M/H [1]	±	Control [2]	Experimental Group								
				250 mM NaCl	Salinity Stress + *Bacillus pumilus*			Salinity Stress + *Lactobacillus casei*			Salinity Stress + *Bacillus subtilis*		
					0 mgL^{-1} ZnO-NPs	20 mgL^{-1} ZnO-NPs	40 mgL^{-1} ZnO-NPs	0 mgL^{-1} ZnO-NPs	20 mgL^{-1} ZnO-NPs	40 mgL^{-1} ZnO-NPs	0 mgL^{-1} ZnO-NP	20 mgL^{-1} ZnO-NPs	40 mgL^{-1} ZnO-NPs
OPA-13	M	+	8	552; 356; 252	-	-	-	394; 238	574;414	-	-	-	100, 190
		–		325; 232	146	464; 115	464; 115	464	-	464; 432	464	473; 329	464
	H	+	9	225; 182	209; 146	-	-	190	-	-	-	-	120
		–		585; 382	473; 439	473; 439	473; 329; 126	473	473; 429	473; 329	473; 386	464; 432	473; 429; 386
	Class			Class II	Class I	Class I	Class I	Class II	Class II	Class I	Class I	Class I	Class II
OPB-8	M	+	7	285; 220	-	211	194	-	-	138	-	-	-
		–		650	685	-	-	-	685; 592	-	685	669; 126	685; 150
	H	+	8	522	367	-	353	339	-	367	-	-	-
		–		750; 652; 225	150; 126	669; 600; 150	200	150; 126	126	150	600; 126	-	669; 126
	Class			Class II	Class I	Class II	Class II	Class I	Class I	Class III	Class I	Class I	Class I
OPB-10	M	+	6	485; 335	481; 435	674; 600; 459	623; 476	817; 687; 527; 431	600; 445	487	762; 661; 578; 440	674; 454; 427	578; 435
		–		204	144	144	223; 144	223; 144	223; 144	144	223; 144	-	223; 144
	H	+	5	628; 324	427	762; 600; 427	611; 260	661; 500; 272	440	623; 389	674; 379	635; 431; 372	266
		–		-	-	-	-	-	319	-	-	223; 144	-
	Class			Class IV	Class IV	Class IV	Class III	Class IV	Class I	Class III	Class IV	Class IV	Class III
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
		–		285	-	-	-	-	-	-	-	320	-
	H	+	8	580; 150	129	-	-	400; 300	-	407; 180	531; 415	170	150
		–		545; 450; 354	-	-	320	-	320	-	-	-	-
	Class			Class II	Class IV	Class II	Class II	Class IV	Class II	Class IV	Class IV	Class III	Class IV
OPH-18	M	+	12	423; 378; 310	223	472; 294	328; 287: 223	142	217; 120	462; 229	558; 467	431; 316; 205	216; 116
		–		628; 487; 300	-	-	517	-	-	-	-	628	-
	H	+	12	776; 505; 319; 257; 157	379; 316; 216; 157; 122	311; 211	305; 242; 205	359; 120	728; 323; 130	350; 229	242; 194	536; 223	200; 130
		–		309; 116	-	629	589; 528	-	-	589	589	-	528
	Class			Class III	Class IV	Class IV	Class IV	Class IV	Class IV	Class IV	Class IV	Class IV	Class IV
OPW-4	M	+	9	462; 476; 352	-	463; 304	454	561	459	250	504; 485	-	-
		–		525; 439	332	565	-	332	332	-	332	404; 284; 135	593; 227
	H	+	11	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			Class II	Class I	Class II	Class II	Class I	Class I	Class II	Class II	Class I	Class I

Table 4. *Cont.*

Primer	M/H [1]	±	Control [2]	Experimental Group								
				250 mM NaCl	Salinity Stress + *Bacillus pumilus*			Salinity Stress + *Lactobacillus casei*			Salinity Stress + *Bacillus subtilis*		
					0 mgL^{-1} ZnO-NPs	20 mgL^{-1} ZnO-NPs	40 mgL^{-1} ZnO-NPs	0 mgL^{-1} ZnO-NPs	20 mgL^{-1} ZnO-NPs	40 mgL^{-1} ZnO-NPs	0 mgL^{-1} ZnO-NP	20 mgL^{-1} ZnO-NPs	40 mgL^{-1} ZnO-NPs
OPW-5	M	+	12	562; 424; 325; 282; 171	-	529; 171	656	-	-	680	-	-	-
		–		-	557; 368	395; 303	512; 253	614; 557; 395; 230	614; 368	303	557; 395; 230	614; 557; 475; 409	614; 557; 395; 368
	H	+	13	203	-	249	-	-	-	-	-	-	-
		–		585; 553; 475; 347; 255; 154	553; 347; 230	679; 553; 525; 409	679; 553	679; 614; 525; 492; 475; 409	492; 475; 409	679; 614; 475; 409; 347	679; 614; 492; 347	230	679; 614; 553; 525; 475
	Class			Class II	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	-
		–		342	-	381; 164	-	323	-	164	-	-	657; 562; 492
	H	+	9	441; 414; 278;189	419; 281	-	687; 500	476; 143	-	300	508	693; 358; 281	-
		–		-	-	164	-	606	-	719; 164	-	-	719; 651; 606
	Class			Class IV	Class IV	Class I	Class IV	Class IV	Class I	Class III	Class IV	Class IV	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
		–		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
		–		579; 180	184	513	-	513; 184	184	-	-	-	-
	Class			Class I	Class IV	Class II	Class IV	Class I	Class IV	Class I	Class IV	Class IV	Class II
OPY-16	M	+	6	-	668; 400	280; 247	529; 405; 239	669	704	641; 474; 264	665; 509; 280; 247	509; 452; 224	-
		–		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
		–		223	339	260	-	422; 339; 260	260	339	339	-	485; 422; 339
	Class			Class III	Class IV	Class IV	Class IV	Class I	Class II	Class IV	Class IV	Class IV	Class I
Polymorphism %			M	53.88	39.03	38.98	32.73	36.79	35.42	30.46	40.70	36.12	33.78
			H	51.53	37.31	30.53	30.11	35.91	29.19	30.37	34.01	29.60	33.32

[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
		Restriction Pattern		
		HpaII	MspI	
Class I	$\underline{\text{CCGG}}$ $\underline{\text{CCGG}}$ GGCC GGCC	Active	Active	35.00%
Class II	$\underline{\text{CCGG}}$ GGCC	Active	Inactive	20.00%
Class III	CCGG $\underline{\text{GGCC}}$	Inactive	Active	8.00%
Class IV	CCGG GGCC	Inactive	Inactive	37.00%

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 (*Bacillus subtilis*, *Lactobacillus casei*, and *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 un-methylated) 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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