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Genotype-Dependent Antioxidative Response of Four Sweet Pepper Cultivars to Water Deficiency as Affected by Drought-Tolerant *Bacillus safensis* SS-2.7 and *Bacillus thuringiensis* SS-29.2 Strains

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Abstract: We examined the effect of drought-tolerant *Bacillus safensis* SS-2.7 and *B. thuringiensis* SS-29.2 strains on the response of four (133, 274, California Wonder—CalW, and Matica) sweet pepper genotypes to water deficiency conditions. Pepper seeds were sown in pots with (treated) and without (control) bacterial strain inoculation. After four weeks of growth under controlled conditions and regular watering, drought was imposed by completely withholding watering for seven days. Under conditions of normal watering, genotype 274 showed better seedling establishment than genotype 133 and CalW, while the slowest was genotype Matica. Antioxidant enzyme activity under drought conditions was genotype and bacterial treatment-dependent. The best response to bacterial treatment in order to cope with severe drought was found in the CalW genotype, while in genotype 133, we determined even faster plant decay during water deficiency in treated seeds. Inoculated seeds of the Matica genotype did not show different antioxidant enzyme activity under normal and drought conditions. According to the obtained results, we concluded that under drought conditions, the most susceptible was genotype 274, moderate susceptibility was detected in genotype 133, and CalW and Matica were the most tolerant genotypes. Our study demonstrates (1) that drought-tolerant *Bacillus* strains showed a plant growth-promoting effect on some selected pepper genotypes; (2) that there were genotype-dependent antioxidant enzyme activities under drought conditions in response to treatment with a particular bacterial strain; and (3) that we could expect a genotype-dependent response during biostimulant application, especially under stress conditions.

Keywords: pepper genotypes; antioxidant enzyme; drought; *Bacillus*; abiotic factors

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1. Introduction

Plant growth-promoting bacteria (PGPB) with *Bacillus* and *Pseudomonas* as the most dominant representatives are known for their ability to colonize plant roots, causing direct or indirect effects. Mechanisms of PGPB activity include hormone synthesis, phosphate solubilization, nitrogen fixation, and siderophore and antimicrobial compound production [1]. Plants attract microorganisms by production of root exudates rich in various nutrients essential for rhizobacteria growth [2]. As sessile organisms, plants suffer from numerous biotic and abiotic stressors. Osmotic stress usually occurs as a result of a lack of water due to drought and is the most devastating type of abiotic stresses for plants. Under drought conditions, the production of reactive oxygen species (ROS) increases [3]. One of the major causes of loss of crop and vegetable productivity worldwide is accumulation of ROS as a result of various environmental stresses (temperature extremes, drought, high soil salinity,

toxic metals, pathogen attacks, etc.) [3]. In response to the intensive production of ROS, a significant increase in the activity of key antioxidant enzymes was observed in many plants. The importance of this type of response in the development of plant tolerance to osmotic stress is also confirmed on transgenic plants that overexpress antioxidant enzymes [4]. However, plants can use other mechanisms to overcome osmotic stress besides antioxidant ones, which may be the reason for the observed reduced activity of antioxidant enzymes under such conditions [5]. Plant species and/or genotypes can have different antioxidant capacities and consequently different ability to tolerate oxidative stress under drought conditions. In hot pepper (*Capsicum chinense* Jacq.) hybrids, significant differences of capsaicinoid production were observed in studying genotype–environment and cultivar–environment interactions [6]. Eight pepper genotypes analyzed by Sensoy and coauthors [7] showed a different response to colonization, relative mycorrhizal dependency, and seedling traits in seeds inoculated with two different arbuscular mycorrhizal fungi.

The importance of evaluating the effect of inoculation with PGPB was demonstrated for *Bacillus amyloliquefaciens* BBC047 strain that increased content of vitamin C and antioxidant capacity of sweet pepper plants [8]. Moreover, PGPB *B. amyloliquefaciens* rhizobacterium protected *Capsicum annuum* cv. *Geumsugangsan* from drought, salinity, and heavy metal stresses [9]. Some of the mechanisms involved in PGPB-mediated drought tolerance in plants have been identified. *Pseudomonas putida* GAP-P45 rhizobacteria modulate proline metabolisms in inoculated *Arabidopsis thaliana* under drought conditions by upregulation of gene expression involved in proline biosynthesis and catabolism [10]. Additionally, this strain also modulates polyamine biosynthesis in *A. thaliana* under normal and water efficiency conditions [11].

With huge diversity and wide cultivation, pepper (*Capsicum annuum* L.) is one of the most important of all cultivated crops. Although it is possible to grow pepper in different environments, irrigation often becomes a limiting factor for pepper cultivation that can result in reduction of the yield. Several reports have been published about the response of different pepper genotypes to various growth conditions, but there is still limited information concerning the use of bacterial inoculants to overcome drought stress during pepper growth and response of different genotypes to bacterial inoculum. Our hypothesis was that treatment of seeds with selected drought-tolerant bacteria could improve both the growth and drought tolerance of sweet pepper. We also hypothesized that this response would depend on the specific genotype–bacteria interactions. In the present study, the *Bacillus safensis* SS-2.7 and *B. thuringiensis* SS-29.2 strains were used to evaluate the effect of their inoculation on four different sweet pepper genotypes to overcome conditions of water deficiency. Parameters of drought stress as well as the antioxidant enzyme activity of pepper genotypes subjected to normal watering and drought were measured to elucidate the impact of the conducted bacterial inoculation.

2. Materials and Methods

2.1. Bacterial Strain Selection and Characterization for Plant-Growth Promoting (PGP) Treats

Bacterial strains *Bacillus safensis* SS-2.7 (Bs) and *B. thuringiensis* SS-29.2 (Bt) are part of the Laboratory Collection from Chair of Microbiology, Faculty of Biology, University of Belgrade, Serbia. Among other strains, this collection harbors 220 natural bacterial isolates from the genus *Bacillus* spp. isolated from different localities in Serbia. The sources of their isolation were soil, hay, and manure. Strains' characterization and identification were performed previously [12,13]. The potential of 220 strains for their ability to tolerate heat stress (50, 60, 70 °C); tolerance to salinity by observing the growth on medium amended with various concentration of NaCl (5, 7, 13, 15, 17, 20, 22, and 25 % (*w*/*v*)); and drought tolerance obtained by adding 5, 10, 15, 20, 30, and 40% of poly(ethylene) glycol 6000 (PEG6000) in medium was tested. From 220 natural bacterial isolates tested preliminarily, we selected six strains: *Bacillus safensis* SS-2.7, *B. velezensis* SS-6.4 and SS-8.2.2, *B. thuringiensis* SS-29.2, *B. licheniformis* SS-30.3, and *B. paralicheniformis* SS-37.5 that are able to grow at 50 °C in Luria–Bertani (LB) medium with 7% NaCl and in LB medium with 30% PEG6000. These

strains were used for pepper seed treatment; after sterilization, seed (20 per tray) were imbibed in the bacterial suspension (overnight culture in the LB medium during 16 h at 30 °C and diluted to a final concentration of OD600 = 1) by using the same volume (20 mL) of each culture for 1 h (for control treatment, seeds were imbibed in the same volume of sterile water). Imbibed seeds were allowed to dry under sterile conditions before being placed on sterile filter paper moistened with sterile distilled water in plastic trays. Twenty seeds per tray of each genotype were germinated in triplicate. Then, they were placed in the growth chamber under controlled laboratory conditions: 25 ± 1 °C incubation, with a light intensity of 80 μmol ms^{-1}, photoperiod of 16 h/8 h day/night cycle, relative humidity 40–45%, and watered regularly with the same quantity of water (10 mL per tray). When the seedlings were 3 weeks old, shoot and root length, total weight, and percentage of seed germination were measured. Vigor index (VI) was determined: VI = [(RL + SL)*G], where RL is root length, SL is shoot lent, and G is percentage of germination.

Strains selected from the previous test were analyzed further for their ability to solubilize phosphate (using Pikovskaya's medium) and produce indole-3-acetic acid (IAA) using the spectrophotometric method based on the growth of the isolate in the presence of 2 mg mL^{-1} L-tryptophan reaction with Salkowski's reagent; IAA production was detected as the development of a pink-red color, and the absorbance was measured at 530 nm. 1-Aminocyclopropane-1-carboxylate deaminase (ACC)-deaminase activity was determined by a qualitative method developed by [14]; determining change of color from yellow to brown was considered as positive. Biofilm formation assay was performed as described by Stepanović et al. [15].

2.2. Plant Material and Overall Experimental Design

The seeds of four different sweet pepper (*Capsicum annuum* L.) genotypes: 133, 274, Matica, and California wonder (CalW) were obtained from the Institute of Field and Vegetable Crops, Novi Sad, Serbia. Genotype 133 is the inbred line of variety Una, and genotype 274 is the inbred line of variety Amfora (Supplementary Materials). The seeds were washed with sterile water and ethanol solution (70%) for 30 s and were surface-sterilized using sodium hypochlorite solution (0.8%) for 15 min. After sterilization, seeds were washed with sterile distilled water three times. Bacterial strains *B. safensis* SS-2.7 (Bs) and *B. thuringiensis* SS-29.2 (Bt) were cultivated in the LB medium for 16 h at 30 °C and diluted to a final concentration of OD_{600} = 1. Sterilized seeds (10 per pot) were imbibed in the bacterial suspension by using the same volume (20 mL) of each culture for 1 h (for control treatment, seeds were imbibed in the same volume of sterile water). Imbibed seeds were allowed to dry under sterile conditions before being placed into plastic pots (65 × 65 × 60 mm) filled with soil (Plagron light mix substrate fertilized—black and white peat moss, perlite, N (180 g m^{3-1}), NO_3 (105 g m^{3-1}), NH_4 (75 g m^{3-1}), P_2O_3 (210 g m^{3-1}), P_2O_5 (210 g m^{3-1}), K_2O (360 g m^{3-1})). Pots were placed in a growth chamber under 25 ± 1 °C incubation with a light intensity of 80 μmol ms^{-1}, photoperiod of 16 h/8 h day/night cycle, and relative humidity 40–45%, and were watered regularly with the same quantity of water (50 mL per pot). All experiments were repeated 3 times with 6 pots per replication, with four genotypes, two strains, and the control in a total of 72 pots. After 4 weeks, pots were divided into 2 groups: the drought-stressed group and the irrigated group. For the drought stress treatments, plants were subjected to progressive drought by withholding water for 7 days. At the end of the experiment, the leaves were harvested from three different pots in each replication, immediately frozen in liquid nitrogen, and stored at −80 °C for further experiments. Additional leaves were collected for relative water content (RWC) determination.

2.3. Antioxidant Enzymes Activity Assays

The protein extract used for determination of enzyme activities was obtained by grinding plant leaves in liquid nitrogen, followed by homogenization in cold extraction buffer containing 50 mM potassium phosphate buffer (pH 7) and 0.1 mM EDTA. The

obtained homogenates were centrifuged for 30 min at 13,000 rpm (Hettich, Rotina 380R, Germany) at 4 °C. Protein content in samples was evaluated spectrophotometrically [16]. Obtained protein extracts were stored at −80 °C until further use.

Superoxide dismutase (SOD) activity was determined by measuring the photochemical reduction of NBT [17]. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 2 µM riboflavin, 75 µM NBT, and 30 µL extract in 1 mL volume. The reaction tubes were exposed to the fluorescent light for 15 min and absorbance was detected at 560 nm. One unit of SOD activity was defined as the amount of enzyme that inhibited the rate of NBT photochemical reduction by 50%. SOD activity was expressed as U mg^{-1} of protein.

Ascorbate peroxidase (APX) activity was determined in 1 mL reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 0.1 mM H_2O_2, and 25 µL extract. The APX activity was measured by monitoring the decrease in absorbance at 290 nm [18] as a consequence of hydrogen peroxide-dependent oxidation of ascorbate. The activity of APX was expressed as U mg^{-1} of protein.

Total soluble peroxidase (POD) activity was determined by monitoring the absorbance increase at 620 nm due to the formation of colored product [19]. The reaction mixture contained 675 µL 10 mM H_2O_2, 50 µL 2 mM α-naphthol, and 25 µL of protein extract. POD activity was expressed as U mg^{-1} of protein.

Glutathione reductase (GR) activity was determined by measuring the oxidation of NADPH to NADP+ as a decrease in absorbance at 340 nm [20]. The reaction mixture (1 mL) contained 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.1 mM NADPH, 2.5 mM GSSG, and 25 µL extract. The reaction was started by the addition of NADPH. GR activity was expressed as U mg^{-1} of protein.

2.4. Determination of Relative Water Content—RWC

The relative water content (RWC) in leaf samples (three per pot) was determined using the following formula: RWC (%) = [(FW − DW)/(WT − DW)] × 100, where FW is the weight of fresh leaf measured immediately after sampling, DW is the weight of leaf material dried in the oven at 90 °C until constant mass, and WT is the saturated weight measured after incubation of the leaves on the heavily moistened filter paper (24 h at room temperature) [21].

2.5. Determination of Lipid Peroxidation and Hydrogen Peroxide Levels

The level of lipid peroxidation was determined indirectly by measuring the amount of malondialdehyde (MDA) produced by the thiobarbituric acid (TBA) reaction [22]. The MDA content was calculated using the extinction coefficient of 155 $mM^{-1}cm^{-1}$ and expressed as nmol MDA g^{-1} DW. Hydrogen peroxide content was determined using a spectrophotometric test on the basis of the oxidation of iodide by H_2O_2 to iodine in an acidic medium [23]. The amount of H_2O_2 was determined using the standard curve and expressed as nmol/g DW.

2.6. Statistical Analysis

All assays were performed with three biological replicates (three plants per treatment) and data are expressed by the mean ± standard errors (mean ± SE). Data were analyzed by one-way analysis of variance (ANOVA), and Duncan's multiple-range test at significance at $p < 0.05$ was performed to separate the means. Pearson correlation coefficients were determined at the 0.05 and 0.01 probability levels. Principal components for principal component analysis (PCA) have been extracted until the eigenvalue >1. The first three components explain the maximum variance that was selected for the ordination analysis, and the correlation between the original traits and the respective principal component was calculated. Traits with a correlation above 0.6 were considered relevant for that component [24]. Mean values per genotype were used for principal component analysis.

Software package Statistica for Windows ver. 13.2 (Dell Inc., Aliso Viejo, CA, USA) was used for all statistical analyses.

3. Results

3.1. Strain Selection and Plant Growth Promotion (PGP) Trait Analysis

The effects of treatment with five drought-tolerant strains on the establishment of four pepper seedlings was assayed (data not shown), and the obtained results pointed out two bacterial strains *Bacillus safensis* SS-2.7 (Bs) and *B. thuringiensis* SS-29.2 (Bt) as the most promising candidates for PGP. These two strains had different effects on four sweet pepper genotypes; however, most of the measured results in treated plants were significantly higher than those of untreated plants (C—control) (Table 1).

Table 1. Duncan's test of pepper growth parameters treated with *Bacillus safensis* SS-2.7 (Bs) and *B. thuringiensis* SS-29.2 (Bt) strains.

Genotype	RL (cm)	SL (cm)	TW (cm)	G (%)	V.I.
133C	1.418 ± 0.056^{e}	1.245 ± 0.099^{e}	0.29 ± 0.086^{c}	80 ± 4.082^{def}	212.8 ± 8.914^{g}
133Bs	1.295 ± 0.045^{e}	1.44 ± 0.059^{de}	0.322 ± 0.043^{c}	95 ± 4.082^{ab}	259.84 ± 11.725^{fg}
133Bt	1.36 ± 0.069^{e}	2.508 ± 0.465^{b}	0.282 ± 0.025^{c}	96.25 ± 4.787^{ab}	371.79 ± 39.513^{de}
274C	2.55 ± 0.244^{c}	2.488 ± 0.172^{b}	0.508 ± 0.065^{b}	82.5 ± 13.229^{cde}	414.46 ± 66.879^{cd}
274Bs	3.87 ± 0.243^{a}	3.56 ± 0.422^{a}	0.81 ± 0.055^{a}	91.25 ± 4.787^{abc}	678.1 ± 65.946^{b}
274Bt	3.765 ± 0.261^{ab}	3.86 ± 0.214^{a}	0.872 ± 0.048^{a}	98.75 ± 2.5^{a}	753.34 ± 57.587^{a}
CalWC	1.55 ± 0.191^{e}	1.15 ± 0.058^{e}	0.525 ± 0.096^{b}	83.75 ± 7.5^{cde}	225.62 ± 15.445^{g}
CalWBs	3.475 ± 0.33^{b}	1.9 ± 0.141^{c}	0.9 ± 0.141^{a}	87.5 ± 6.455^{bcd}	470.62 ± 57.098^{c}
CalWBt	3 ± 0.182^{c}	1.75 ± 0.129^{cd}	0.85 ± 0.058^{a}	75 ± 12.247^{ef}	354.38 ± 45.668^{de}
Matica C	1.525 ± 0.263^{e}	0.75 ± 0.129^{f}	0.4 ± 0.141^{bc}	55 ± 4.082^{g}	125.75 ± 28.619^{h}
Matica Bs	3.6 ± 0.258^{ab}	1.625 ± 0.126^{cd}	0.9 ± 0.141^{a}	71.25 ± 4.787^{f}	373 ± 39.151^{de}
Matica Bt	3.465 ± 0.299^{b}	1.7 ± 0.081^{cd}	0.775 ± 0.096^{a}	60 ± 7.071^{g}	307.25 ± 38.413^{ef}

RL—root length, SL—shoot length, TW—total weight, G—germination, V.I.—vigor index, C—control; different letters show significant differences between genotypes ($p < 0.05$) according to Duncan's test.

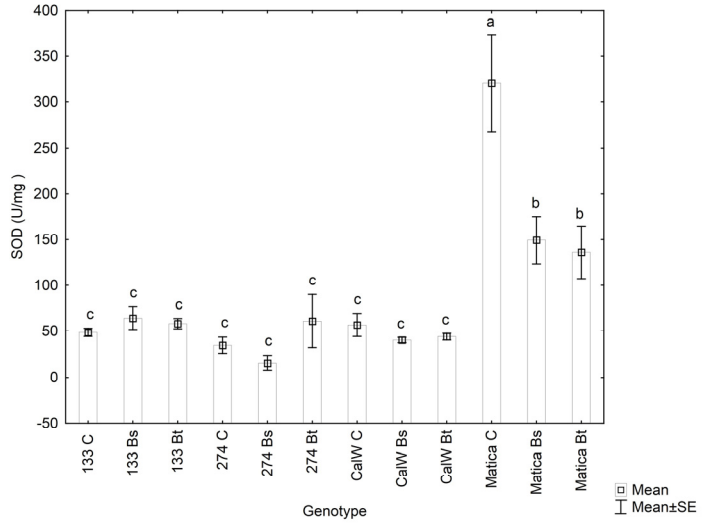
3.2. Antioxidant Enzyme Activities

The effects of treatment with *Bacillus safensis* SS-2.7 (Bs) and *B. thuringiensis* SS-29.2 (Bt) bacterial strains on the activities of four antioxidant enzymes in pepper leaves under drought conditions were assayed. The activity of SOD and GR enzyme was found to be increased in 133 genotypes after treatment with Bt strain (Figures 1 and 2). Treatment of the Matica genotype with both strains decreased both SOD and GR activity under drought conditions, while SOD was also decreased under well-watered conditions (Figure 1).

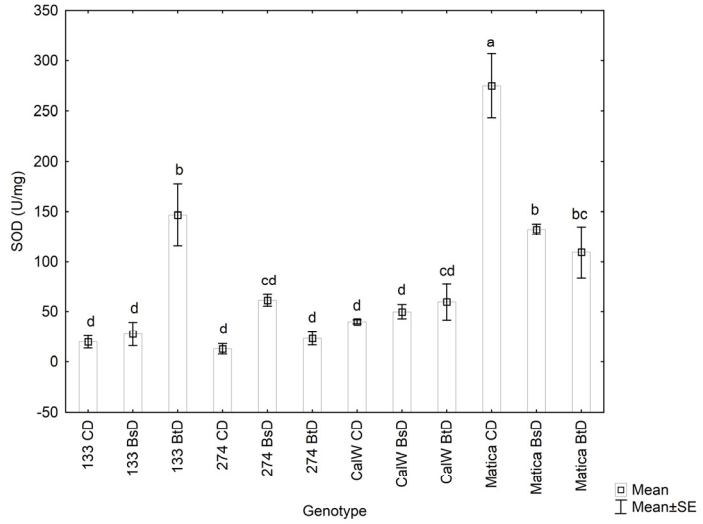
At the same time, the detected activity of APX after treatment with the Bt strain under the same conditions was increased in CalW and 274 genotypes, while both strains increased APX activity in Matica genotype under drought conditions. Genotype 133 showed increased APX activity after treatment with Bs strain alone in both control and drought conditions (Table 2). Treatment with both Bs and Bt strains in genotype 133 led to increase of POD activity in both conditions analyzed, while Bs strain alone increased the POD activity in CalW during drought. Matica was the only genotype where POD activity was not detected (Table 2).

3.3. Effect of Bacterial Inoculation on Plant Hydrogen Peroxide Levels, Lipid Peroxidation under Drought Conditions, and Relative Water Contnt

Results of hydrogen peroxide (H_2O_2) level in pepper leaves showed significant changes, depending on both the genotype and the bacterial strains used for treatment. Under drought conditions, as compared to non-inoculated plants, inoculated plants exhibited significantly lower values for H_2O_2 content in genotype 274 after treatment with both strains under drought conditions. Decrease of H_2O_2 was also detected for the CalW and Matica genotypes after treatment with the Bs and Bt strains, respectively (Figure 3).



(**A**)

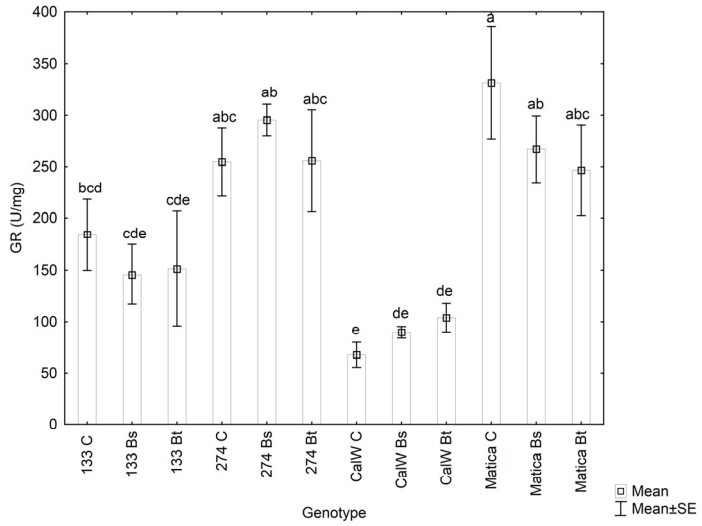


(**B**)

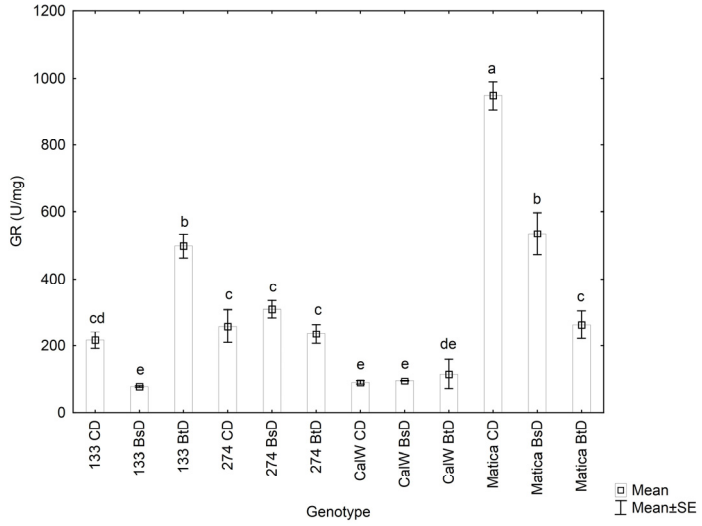
Figure 1. The activity of superoxide dismutase (SOD) in leaves of four pepper genotypes in (**A**) control (C) and (**B**) drought (D) conditions treated with two bacterial strains, *B. safensis* SS-2.7—Bs and *B. thuringiensis* SS-29.2—Bt. Different letters show significant differences between genotypes ($p < 0.05$) according to Duncan's test.

The amount of MDA accumulation in plant tissues was used to estimate the level of lipid peroxidation. Lowering of MDA content was detected in genotype 274 and Matica treated with the Bs and Bt strains, respectively, under conditions of water deficiency. Additionally, treatment with both strains lowered MDA content in the CalW genotype under drought conditions. Interestingly, in Matica and CalW genotypes, MDA levels decreased after treatment with Bt strain, even under well-watered conditions (Figure 4).

Measured values of RWC indicated treatment with the Bt strain to be important only for genotype 133 under drought conditions. However, treatment with the Bs strain in all genotypes except 133 led to a lowering of RWC under drought conditions (Figure 5). Interestingly, treatment of 274 genotype with Bs strain in well-watered conditions increased RWC, while in drought conditions, it had the opposite effect.



(A)



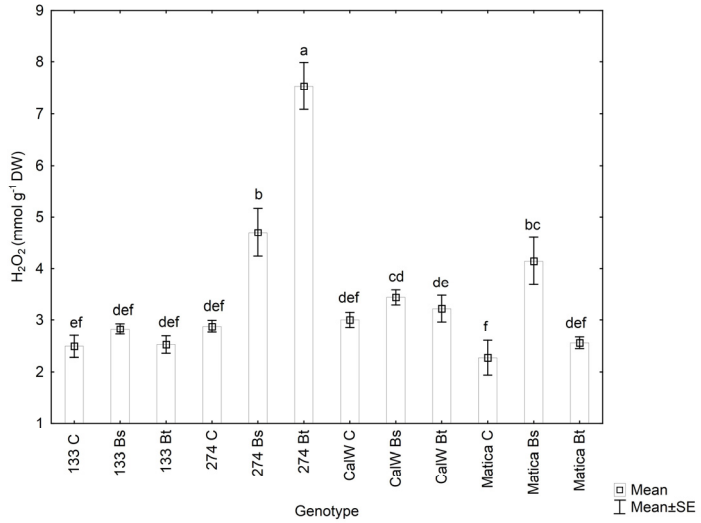
(B)

Figure 2. The activity of glutathione reductase (GR) in leaves of four pepper genotypes in (**A**) control (C) and (**B**) drought (D) conditions treated with two bacterial strains, *B. safensis* SS-2.7—Bs and *B. thuringiensis* SS-29.2—Bt. Different letters show significant differences between genotypes ($p < 0.05$) according to Duncan's test.

Table 2. Activity of ascorbate peroxidase (APX) and total soluble peroxidase (POD) in leaves of four pepper genotypes in well-watered (C) and drought (D) conditions with and without treatment with two bacterial strains (*B. safensis* SS-2.7—Bs) and (*B. thuringiensis* SS-29.2—Bt).

Genotype	**133C**	**133Bs**	**133Bt**	**133CD**	**133BsD**	**133BtD**	**CalWC**	**CalWBs**	**CalWBt**	**CalWCD**	**CalWBsD**	**CalWBtD**
APX	78.828 ± 3.165 ^{j}	97.134 ± 4.367 ^{ij}	101.334 ± 8.118 ^{i}	257.015 ± 5.288 ^{d}	766.799 ± 11.141 ^{a}	183.64 ± 10.254 ^{ef}	198.965 ± 17.505 ^{e}	158.312 ± 5.816 ^{gh}	81.087 ± 8.353 ^{j}	151.613 ± 16.995 ^{h}	108.082 ± 3.776 ^{i}	328.368 ± 13.999 ^{c}
POD	14.552 ± 1.487 ^{g}	19.238 ± 1.546 ^{g}	39.118 ± 2.931 ^{d}	50.296 ± 5.006 ^{c}	114.565 ± 3.413 ^{a}	79.33 ± 7.401 ^{b}	18.835 ± 1.747 ^{g}	13.884 ± 0.051 ^{g}	14.351 ± 1.952 ^{g}	18.388 ± 1.675 ^{g}	32.411 ± 3.625 ^{e}	16.333 ± 2.833 ^{g}
Genotype	274C	274Bs	274Bt	274CD	274BsD	274BtD	MaticaC	MaticaBs	MaticaBt	MaticaCD	MaticaBsD	MaticaBtD
APX	251.913 ± 12.5 ^{d}	241.743 ± 14.565 ^{d}	248.444 ± 30.719 ^{d}	175.355 ± 2.028 ^{fg}	141.605 ± 10.789 ^{h}	392.05 ± 20.501 ^{b}	3.682 ± 0.935 ^{k}	0.579 ± 0.104 ^{k}	0.637 ± 0.214 ^{k}	2.451 ± 0.138 ^{k}	3.38 ± 0.238 ^{k}	6.189 ± 0.293 ^{k}
POD	25.923 ± 4.373 ^{f}	26.788 ± 4.31 ^{f}	24.786 ± 2.516 ^{f}	53.281 ± 3.474 ^{c}	50.119 ± 6.727 ^{c}	38.76 ± 2.841 ^{d}	0 ± 0 ^{h}	0 ± 0 ^{h}	0 ± 0 ^{h}	0 ± 0 ^{h}	0 ± 0 ^{h}	0 ± 0 ^{h}

Different letters show significant differences between genotypes ($p < 0.05$) according to Duncan's test.



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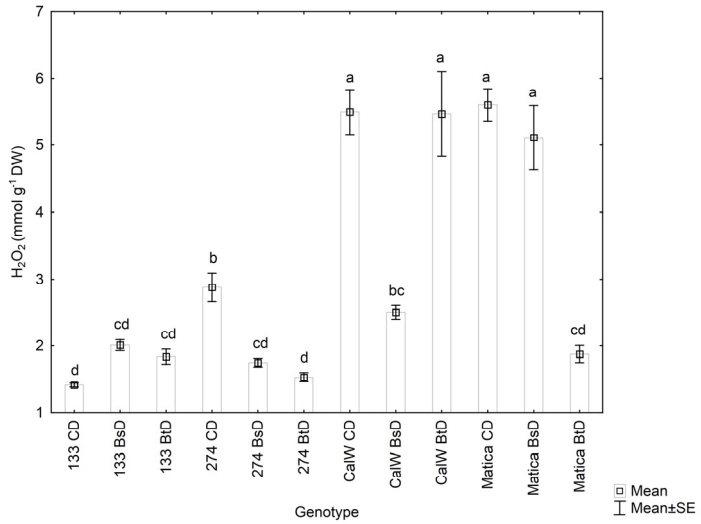


(**B**)

Figure 3. H_2O_2 content in leaves of 133, 274, CalW, and Matica genotypes in (**A**) control (C) and (**B**) drought (D) conditions treated with two bacterial strains, *B. safensis* SS-2.7—Bs and *B. thuringiensis* SS-29.2—Bt. Different letters show significant differences between genotypes ($p < 0.05$) according to Duncan's test.

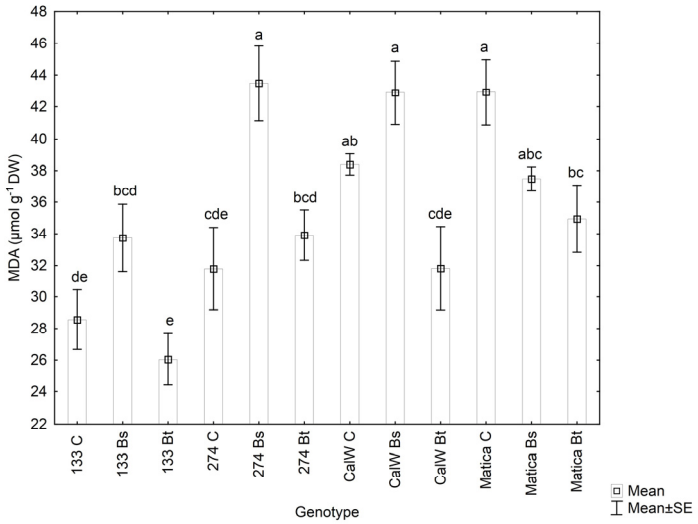
When drought was initiated by completely withholding water after the 28th day of well-watered conditions, genotype 274 was the first to show changes by wilting of the lower leaves. This genotype decreased the most severely at the end of the experiment (Table 3). On the other hand, genotype 274 exhibited a faster seedling establishment ability in comparison with other genotypes, regardless of bacterial treatment. The slowest was the Matica genotype (Table 3), but at the same time, Matica was the most tolerant genotype to drought. However, changes caused by the bacterial treatment were clearly detected in plant physiology and biochemistry than in visible phenotype assessments.

3.4. Principal Component Analysis—PCA

Principal component analysis (PCA) indicated that the first three components explain 84.11% of the total variance (Table 4).

Since the first three principal components (PC) were over eigenvalue 1, they were the only ones interpreted. The most important positive traits in the first PC were RWC, SOD,

and H_2O_2, while APX and POD were negative. MDA was the most important negative trait in the second PC. According to PCA, GR and SOD were close to each other on the biplot, which indicates positive correlations between them (Figure 6).



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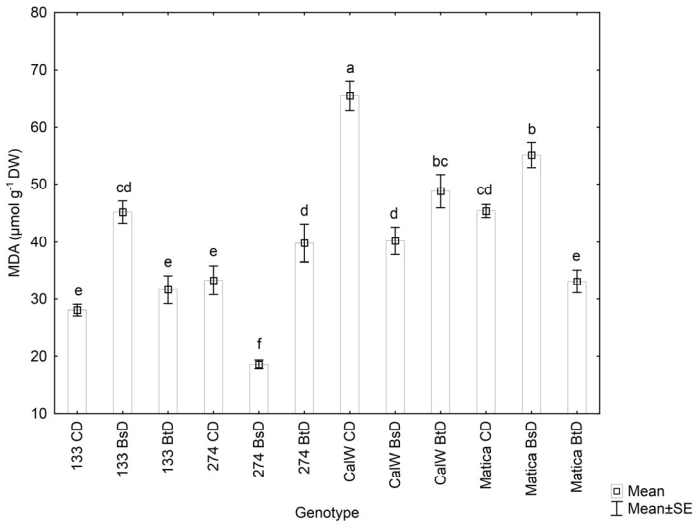

(**B**)

Figure 4. MDA content in leaves of 133, 274, CalW, and Matica genotypes in (**A**) control (C) and (**B**) drought (D) conditions treated with two bacterial strains, *B. safensis* SS-2.7—Bs and *B. thuringiensis* SS-29.2—Bt. Different letters show significant differences between genotypes ($p < 0.05$) according to Duncan's test.

A similar positive correlation with the first PC was noted between RWC, H_2O_2, and MDA. APX and POD were on the opposite left side of the biplot, which indicates a negative correlation with GR and SOD. Pearson coefficients also revealed a correlation between drought tolerance traits (MDA, RWC, and H_2O_2) and antioxidant enzyme (SOD, GR) activities (Table 5).

The obtained results showed that increase in H_2O_2 content leads to a consequent increase of MDA. The same results were obtained for the relationship between activities of two antioxidant enzymes, viz., SOD and GR. Further PCA analysis of the four genotypes under two types of conditions with and without bacterial treatment revealed that all variants of the Matica variety (5/6) except Matica BsD were in the first quadrant (Figure 6) with GR and SOD traits, indicating higher values of these traits in the Matica variety.

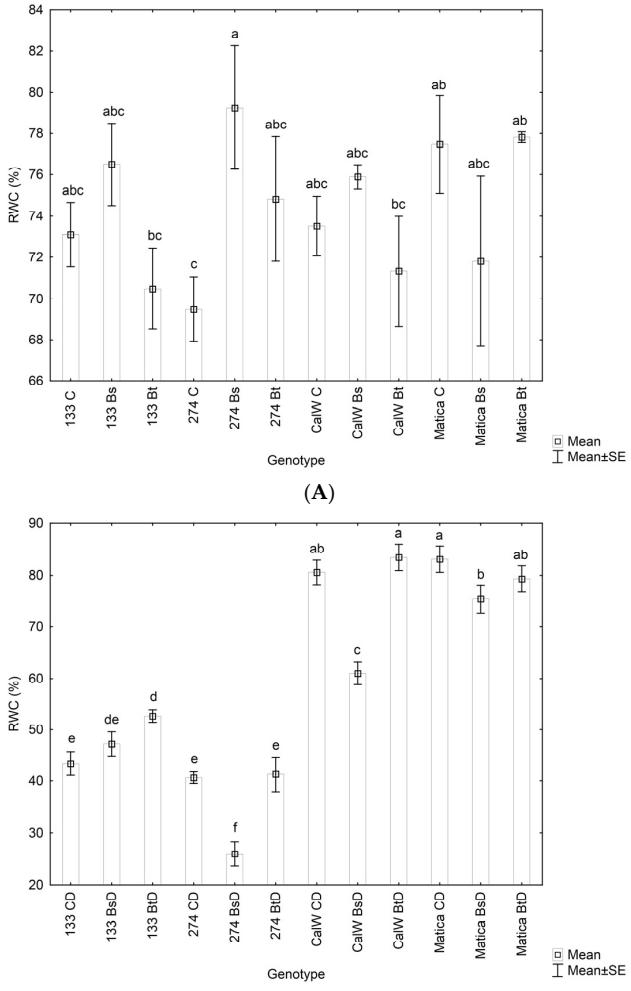

Figure 5. RWC (relative water content, %) in leaves of 133, 274, CalW, and Matica genotypes in (**A**) control (C) and (**B**) drought (D) conditions treated with two bacterial strains, *B. safensis* SS-2.7—Bs and *B. thuringiensis* SS-29.2—Bt. Different letters show significant differences between genotypes ($p < 0.05$) according to Duncan's test.

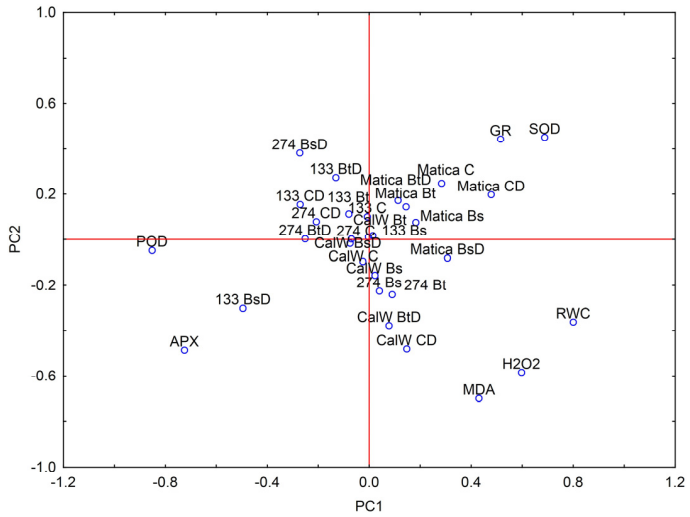

Figure 6. Principal component analysis (PCA) biplot of evaluated traits among four pepper genotypes in control (C) and drought (D) conditions treated with two bacterial strains, *B. safensis* SS-2.7—Bs and *B. thuringiensis* SS-29.2—Bt.

Table 3. Description of visual assessment of seedling establishment, cotyledon and leaf development, and decay after drought stress application.

Genotype	6th Day	9th Day	14th Day	17th Day	19th Day	22nd Day	28th Day *	30th Day	33rd Day	35th Day
133CD	-	+	++	++	+++	+++	++++/4	3	2	1
133BsD	-	+	++	++	+++	+++	++++/4	3	2	1
133BtD	-	+	++	++	+++	+++	++++/4	3	2	1
274CD	+	+	++	++	+++	++++	++++/4	2	1	0
274BsD	+	+	++	++	+++	++++	++++/4	2	1	0
274BtD	+	+	++	++	+++	++++	++++/4	2	1	0
CalWCD	-	-	+	+	++	+++	++++/4	3	2	2
CalWBsD	-	-	+	+	++	+++	++++/4	3	2	2
CalWBtD	-	-	+	+	++	+++	++++/4	3	2	2
Matica CD	-	-	-	+	++	+++	++++/4	3	2	2
Matica BsD	-	-	-	+	++	+++	++++/4	3	2	2
Matica BtD	-	-	-	+	++	+++	++++/4	3	2	2

4—no visible symptoms, 3—wilting of lower leaves, 2—rolling of upper leaves and wilting at whole plant level, 1—heavy wilting and yellowing visible necrotic areas on the leaves, 0—entire plant collapsed, —-no seedling establishment, +—seedling establishment started, ++—cotyledon formed, +++—first leaves formed, ++++—first true leaves formed, *—drought started on the 28th day.

Table 4. Correlation between original variables and the first three principal components (PC), eigenvalues, and total variance in the evaluated pepper genotypes treated with two bacterial strains.

Trait	PC 1	PC 2	PC 3
SOD	**0.683**	0.467	−0.359
GR	0.509	0.468	**−0.647**
MDA	0.437	**−0.686**	−0.348
H_2O_2	**0.603**	−0.574	−0.205
RWC	**0.804**	−0.363	0.245
APX	**−0.721**	−0.480	−0.386
POD	**−0.852**	−0.040	−0.440
Eigenvalue	3.173	1.601	1.114
Total variance %	45.323	22.870	15.917
Cumulative variance %	45.323	68.193	84.110

Table 5. Correlation between drought tolerance traits (MDA, RWC, and H_2O_2) and antioxidant enzyme (SOD and GR).

Variable	H_2O_2	RWC	SOD	GR
MDA	0.518 **	0.466 **	0.142	0.057
H_2O_2		0.580 **	0.098	0.194
RWC			0.312 **	0.058
SOD				0.621 **

** significant at $p < 0.01$.

This is not surprising since the Matica genotype under both normal and drought conditions showed different behavior from that of the other genotypes tested. Variants of genotypes 133 and 274 were dominant in the second biplot quadrant. POD and APX were arranged in the third quadrant and with 133 BsD variant were most distinct from the other treatments and genotypes. The opposite direction of APX and POD with GR and SOD indicates a negative correlation.

4. Discussion

Increase in average annual temperature and longer sunny periods lead to a decrease in rainfall, lower humidity, and drying of the soil, converting normal to semi-arid soil and semi-arid soil to desert [25]. These changes result not only in the occurrence of drought, but also in an increase in the concentration of various substances in the soil, mainly salt,

pesticides, herbicides, and toxic metals. Our primary hypothesis was that treating sweet pepper seeds with bacterial strains able to grow at high temperatures and under drought conditions might help plants to cope with drought. A preliminary experiment of ours showed their plant growth-promoting (PGP) effects on seedling establishment and root and shoot development (Table 1). We also determined the ability of the *Bacillus safensis* SS-2.7 (Bs) and *B. thuringiensis* SS-29.2 (Bt) strains to produce indole-3-acetic acid and 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which can be directly connected with their positive effect on plant development, since plant hormones [26] and enzymes such as ACC deaminase [27] produced by microbes could affect plant physiology. The use of microorganisms, primarily rhizobacteria, is well documented in the context of PGP and protection [28,29]. More specifically, inoculation of green pepper seeds with *B. amyloliquefaciens* BBC047 increases the content of bioactive substances such as Ca, Fe, crude proteins, vitamin C, and total phenolic compounds, in addition to which it enhances antioxidant capacity via 2,2-diphenyl-1-picrylhydrazyl (DPPH) and by oxygen radical absorbance capacity (ORAC) [8]. In a study of Kazerooni and co-authors [9], treatment of pepper seeds with PGP rhizobacteria *B. amyloliquefaciens* improved the survival of plants under drought, salinity, and heavy metal stress.

In response to drought, various mechanisms of defense are triggered in plants, both activation of antioxidant enzymes and non-enzymatic defense systems [3,30,31]. The analyzed genotypes differed with respect to starting growth parameters, germination, and plant development, regardless of treatment during the well-watered period. Nevertheless, our results showed the relationship between bacterial treatment and drought as an abiotic factor, as well as a relationship with different genotype-dependent plant responses. Although the genotype 274 had faster seedling establishment and the best plant development, it collapsed the fastest under drought conditions. This finding also corresponds with the lowest RWC results obtained for this genotype. The result correspondingly documented that treatment helps to reduce the concentration of H_2O_2, probably with an increase of APX enzyme activity and decrease in the concentration of malondialdehyde (MDA) as parameters of lipid peroxidation. However, this was not enough for the plants to overcome water deficiency, and their deterioration was severe. The genotype 133 looked slightly better than genotype 274 in the presence of drought, which can be attributed to the activation of all the analyzed antioxidant enzymes after bacterial treatment. An even better relationship between beneficial bacteria and abiotic factors can be seen in the CalW genotype. These results are consistent with H_2O_2 content determined in the CalW genotype and confirm the positive effect of bacterial strains on management of drought conditions through the activation of antioxidant enzymes from the peroxidase family in order to eliminate H_2O_2 and cope with drought. Bacterial treatment led to an increase in the activity of the enzymes ascorbate peroxidase (APX) and total soluble peroxidase (POD), as well as to a decrease in the concentration of H_2O_2 and MDA. As a result, we could see moderate traces of deterioration on the plants during the period of water deficiency.

The genotype most tolerant to water deficiency was Matica. After seven days of complete withholding of water, the plants were still only at the beginning of their deterioration. With regard to the least significant damage to the Matica genotype under drought conditions, we can conclude that since the antioxidant enzymes analyzed in this study were not activated, treatment with drought-tolerant bacteria enabled plants to overcome water deficiency conditions by activation of some other drought protection mechanisms leading to high relative water content (RWC) and low levels of H_2O_2 and MDA. Plants are known to upregulate antioxidant enzymes in response to adverse abiotic and biotic stressors or an ROS itself [32], the resulting activation of its antioxidant enzymes being the plant's answer to higher H_2O_2 content. However, drought protection can be achieved by synthesis of other osmoprotectants or xeroprotectants, which may account for Matica's drought tolerance. Production of proline and polyamine was modulated after treatment with *Pseudomonas putida* GAP-P45 rhizobacteria in *Arabidopsis thaliana* [10,11]. A contribution of bacteria in protecting plants from drought stress has been reported for *Mycobacterium* sp. 31. This strain

improves the tolerance of pepper and tomato to drought by modulation of the plant's glutamine and α-ketoglutarate production [28]. Different responses of genotypes to drought stress were confirmed in chili pepper leaves, and in principal component analysis (PCA), 20 analyzed genotypes were segregated into three clusters, viz., tolerant, susceptible, and moderately tolerant [33]. The classification was based on results of determining antioxidant enzyme activity. Influence of bacterial treatment on plant physiology in normal watering conditions could modulate their response on drought [29]. Our study is an example of these previous results adding another variable to the equation, which is the plant genotype.

5. Conclusions

The ability of plants to survive drought depends on numerous factors, one of them being the plant genotype. However, as with all other living organisms, the expression of a particular phenotype depends not only on the genotype, but also on some environmental factors, such as the presence of microorganisms that interact with the plant. Our results showed that treatment with Bs and/or Bt strains able to grow under drought conditions improves the response of some sweet pepper cultivars to drought by enhancing the activity of antioxidant enzymes that reduce the content of H_2O_2 and MDA. However, this response was genotype-dependent. The CalW genotype confirms this statement by showing the best response to bacterial treatment, which helps it to survive drought with moderate damage. Genotypes 274 and 133, although more advanced under normal conditions, deteriorate the fastest in drought. Although the bacterial treatment provokes a certain response in antioxidant protection, this was not sufficient in terms of removing the effects of stress conditions, indicating that these are drought-susceptible genotypes. The most tolerant genotype on drought was Matica, where we observed almost no effect of bacteria treatment on the activity of the analyzed enzymes, both in normal and water-stressed conditions. On the basis of our findings, we hypothesize that Matica's behavior is a result of the activity of other enzymes involved in plant protection that were not analyzed in this study or in the synthesis of certain non-enzymatic factors that make it drought-tolerant. Our study showed that the use of biostimulants containing microorganisms must be tested in advance on several different genotypes, not only to show their positive action, but also to avoid potentially negative effects, especially when the action of an abiotic factor occurs. To the best of our knowledge, this is the first study comparing different sweet pepper genotypes under conditions of normal watering and drought with and without bacterial treatment in order to determine the relationship between treatment with beneficial bacteria and abiotic factors. Further investigation of Bs and Bt strains for their effect on Matica (as the most drought-tolerant) and 274 (as the most drought-sensitive) genotypes during water deficiency will provide greater insight into the specific role of each strain under particular conditions and, even more importantly, into the genotype-dependent response of sweet pepper plants.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae8030236/s1, Genotype information.

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