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Application of *Trichoderma viride* and *Bacillus subtilis* Modulates Antioxidant System in Mustard (*Brassica juncea*) under Water-deficit Stress

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Water-deficit stress is an important concern worldwide that reduces crop yield and quality. Mustard is an important oilseed crop of India which is adversely affected by water-deficit stress in terms of growth and yield. Tolerance to water-deficit stress is correlated with the redox regulatory and antioxidant system. To mitigate negative effect of water-deficit stress, field experiment was conducted at Dholi (Muzaffarpur), Bihar during 2019-20 with an aim to study the effect of microbes on antioxidant systems in mustard grown under water-deficit stress vis-à-vis normal irrigated conditions. Pre-screened contrasting genotypes (tolerant 'NPJ 214' and sensitive 'TM 179') were sown in the experimental farm using factorial experiment in randomized block design with three replications. The treatments (12) comprised of two factors *viz.,* genotypes (2) and microbial inoculants along with control (3) sown under normal 'irrigated' and 'water-deficit stress' conditions. Soil inoculation microbes *Bacillus subtilis* and *Trichoderma viride* was done 35 days after sowing and was compared with non-inoculated control. Results revealed that the activity of antioxidative enzymes *viz.,* catalase and peroxidase increased under water-deficit stress; the increase was reduced by the application of *B. subtilis* and *T. viride* in both tolerant and sensitive genotypes, and more pronounced in the sensitive genotypes. Similar results were recorded with respect to lipid

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peroxidation and proline content. Increase in concentration of stress-induced metabolites was less in colonized plants of mustard indicating modulation of antioxidant system. The maximum ameliorating effect was observed with application of *T. viride* which could be an important tool in alleviating the adverse effects of water-deficit stress in mustard.

Keywords: Mustard; drought; antioxidant system; reactive oxygen species; Trichoderma; Bacillus.

1. INTRODUCTION

Mustard [*Brassica juncea* (L.) Czern and Coss] is an important oilseed crop belonging to the family Brassicaceae. India is the largest producer of rapeseed and mustard in the world. In India, rapeseed and mustard is cultivated in about 6.23 m ha with total production of about 72.42 million tonnes and an average productivity of 1980 kg ha^{-1} [1]. Numerous environmental factors such as drought, salinity, high and low temperatures influence plants grown in the field. Drought or water-deficit stress affects several morpho-physiological and biochemical parameters such as leaf wilting, reduction in leaf area, chlorophyll content, root elongation and production of reactive oxygen species (ROS) [2]. The ROS *viz.,* hydroxyl radical, singlet oxygen, hydrogen peroxide (H_2O_2) and superoxide are very damaging for cell, as they are robust oxidizing agents. In water-deficit stress situations, plant may initiate generation of various kinds of enzymatic and non-enzymatic antioxidants to dismiss the oxidative stress. ROS play a dual role in plant acting on the one hand as important signal transduction molecules and on the other as toxic by-products that accumulate in cells during stress conditions. Inoculation of plants with microbes such as *Trichoderma* spp. and *Bacillus* spp. can enhance plant growth under water-deficit stress conditions, providing an eco-friendly approach to sustainable agriculture. Alleviation of ROS in plants under water-deficit stress is an important mechanism employed by the fungi. *Trichoderma* spp. has been found to be associated with almost all natural ecosystems. They exist either in the rhizosphere or on plant roots. They form a symbiotic relationship with the host plant. In the existence of the relationship, the proteome and transcriptome of plants change as a consequence of the interaction of *Trichoderma* metabolites or plant colonization. Chepsergon et al. [3] reported an increment in the activities of antioxidant enzyme when *Trichoderma* were injected to the plants. During abiotic stress, *Bacillus* spp. has been reported to produce indole-3-acetic acid, gibberellic acid and 1-aminocyclopropane-1-carboxylate (ACC) deaminase that helps in regulating the intracellular phytohormone thereby initiating the antioxidant and defence systems and increases plant stress tolerance [4]. Therefore, the present investigation was carried out to study effect of microbial application *viz.*, *Trichoderma viride* and *Bacillus subtilis* on antioxidant enzymes, proline, and lipid peroxidation in contrasting mustard genotypes grown under water-deficit stress vis-à-vis normal irrigated conditions.

2. MATERIALS AND METHODS

2.1 Plant Material and Experimental Conditions

The field experiment was conducted at Research Farm, Tirhut College of Agriculture, Dholi (25.59° N, 85.75° E, 52.18 m asl) during 2019-2020 and samples were analysed for different parameters in the laboratory of Department of Botany, Plant Physiology and Biochemistry, College of Basic Sciences and Humanities, Dr. Rajendra Prasad Central Agricultural University, Pusa, Samastipur (Bihar). Pre-screened contrasting genotypes (tolerant 'NPJ 214' and sensitive 'TM 179') were sown in the experimental farm using factorial experiment in randomized block design (RBD) with three replications. The treatments (12) comprised of two factors *viz.,* genotypes and microbial treatments. Microbial treatment combination comprised of- i) Control: first irrigation, 35 days after sowing (DAS) and the second at 60 DAS, ii) Control + *Bacillus* (Soil application at 35 DAS), iii) Control + *Trichoderma* (Soil application 35 DAS), iv) Water-deficit stress (no irrigation), v) Water-deficit stress + *Bacillus* (Soil application at 35 DAS) and vi) Water-deficit stress + *Trichoderma* (Soil application at 35 DAS). Plot size was 2.5 m × 1.5 m and spacing was 30 cm × 10 cm. Seeds were sown in field with no pre-sowing irrigation.

2.2 Application of Microbial Inoculants

Talc formulation of *Trichoderma viride* (Strain NRCL-T-1) was obtained from ICAR- National Research Centre on Litchi, Muzaffarpur, Bihar and liquid formulation of *Bacillus subtilis* was

obtained from the Department of Microbiology, College of Basic Sciences & Humanities, RPCAU, Pusa, Bihar. Trichoderma (*T. viride*) was applied as soil application @ 5.0 kg/ha mixed with 100 kg farm yard manure (FYM). Similarly, Bacillus *(B. subtilis)* was applied as soil application @ 2.0 L/ ha with 100 kg FYM.

2.3 Sampling and Assay for Enzymes Activity, Lipid Peroxidation and Proline Content

Leaf samples were taken at 40 DAS during flowering stage. Assay was done for catalase enzyme activity, peroxidase enzyme activity, lipid peroxidation and content of proline. For the extraction of enzyme, 0.5 g of fresh leaf sample was triturated in 3 mL of pre-chilled extraction buffer (0.1 M phosphate buffer, pH 6.7) followed by centrifugation at 10000 × g for ten minutes. The supernatant was collected and used for the assays of enzymatic activities. All steps in the preparation of the enzyme extract were carried out at 4°C.

Catalase activity was determined by consumption of H_2O_2 using the method of Dhindsa et al. [5]. Peroxidase activity was determined spectrophotometrically using the method of Amako et al. [6] and Salama et al. [7]. The amount of lipid peroxidation was determined in terms of malondialdehyde (MDA) content, a product of lipid peroxidation measured by thiobarbituric acid reaction [8].

Proline content was determined in fresh leaf material, according to Bates et al. [9]. Extraction of proline was done by homogenizing 0.1 g of leaf sample in 10 mL of 3% sulfosalicylic acid. The reaction mixture was centrifuged for 10 minutes at 10000 × g. Supernatant was collected for the estimation of proline. The quantity of proline was calculated using standard curve which was prepared by taking 10-50 µg proline from the stock solution (10 mg/ 100 mL) of L-proline dissolved in water.

2.4 Statistical Analysis

Data of three separate replications were reported as the mean ± SD. The data were subjected to analysis of variance (ANOVA) using statistical computing software. The F value, least significant differences (LSD) between means at 5 % level of significance (P = 0.05) and the standard error (SE) of means were calculated. Microsoft Excel program was used to present the figures.

3. RESULTS AND DISCUSSION

3.1 Catalase Activity

Catalase activity was enhanced when plants were exposed to water-deficit stress but the microbial treatments reduced it both under normal irrigated and water-deficit stress conditions compared to their controls (Table 1). Catalase activity was enhanced from 15.26 to 32.84 µ mol H_2O_2 min^{-1} g^{-1} fresh weight under water-deficit stress conditions in case of tolerant genotype 'NPJ 214' and from 16.20 to 40.14 µ mol H_2O_2 min^{-1} g^{-1} fresh weight in sensitive genotype 'TM 179'. With application of *B. subtilis*, the decrease in catalase activity was 24.44 % in the sensitive genotype under water deficit stress conditions while with the application of *T. viride* it was 39.01 %. Both genotypes revealed statistically significant treatment difference in irrigated and water-deficit stress conditions. The interaction effect among them was also found to be statistically significant. The findings of the study thus demonstrated that water-deficit stress induced plants produced higher levels of catalase than the non-stressed plants, but *T. viride* reduced these values probably due to the lower stress level in inoculated plants. This corroborates with findings of Guler et al. [10] in maize. Similarly, application of *B. subtilis* reduced catalase activity which is similar to the findings of Saad and Abo-Koura [11] in sorghum and Vardharajula et al. [12] in maize.

3.2 Peroxidase Activity

Water-deficit stress increased the activity of peroxidase (POD) in both tolerant and sensitive genotypes whereas the plants inoculated with microbes showed decrease in POD activity over control. POD activity increased from 12.91 in control to 25.23 units mg^{-1} fresh weight under water-deficit stress in the genotype 'NPJ 214' and from 13.78 to 33.65 units mg^{-1} fresh weight, respectively in genotype 'TM 179' (Table 2). Inoculation of microbes reduced POD activity, reduction being higher in case of *T. viride* compared to that of *B. subtilis* however under normal irrigated condition effect of both the microbes were statistically at par. The reduction percent in tolerant genotype 'NPJ 214' under irrigated and water-deficit stress were 14.95 % and 16.41 %, respectively when treated with Trichoderma whereas, in susceptible genotype 'TM 179' decrease was found to be 19.45 % and 25.41 %, respectively under irrigated and water-deficit stress conditions. The value of interaction between treatment and soil condition in tolerant

genotype was found to be non-significant while it was significant in susceptible genotype. In the present study, higher activity of peroxidase in susceptible genotype shows that they require more enzyme activity to breakdown H_2O_2 which is generated by superoxide dismutase thus provides protection against the oxidative stress**.** Application of *B. subtilis* reduced peroxidase activity under both irrigated and water-deficit stress conditions effect being more pronounced under water-deficit stress. These findings are consistent with previous studies which reported that bacterial inoculation reduced peroxidase activity in leaves of maize to a large extent in comparison to control under non-stressed conditions [13]. Similarly, the findings of Guler et al. [10] in maize and Scudeletti et al. [14] in sugarcane are corroborating with our result that *T. viride* application decreased peroxidase activity.

3.3 Lipid Peroxidation

The data revealed that lipid peroxidation increased under water-deficit stress conditions. A higher increase (105.10 %) in susceptible genotype 'TM 179' (3.53 to 7.24 nmol MDA g^{-1} fresh weight) was recorded compared to tolerant genotype 'NPJ 214' (43.70 %) under control conditions (Table 3). Use of the microbes resulted in decrease in lipid peroxidation for both the genotypes of mustard. The maximum percent decrease in lipid peroxidation (34.81 %) was observed with application of *Trichoderma viride* in 'TM 179' under water-deficit stress. In both the genotypes treatment difference was found to be statistically significant whereas the interactions among them were found to be statistically non-significant.

Table 1. Effect of plant growth promoting microbes on catalase activity (μ mol H_2O_2 $min^{-1}g^{-1}$ fresh weight) of mustard genotypes at flowering under irrigated and water-deficit stress conditions

Treatments (T)	Genotypes (G)					
	NPJ 214			TM 179		
	Soil condition (C)					
	IR	WS	Mean	IR	WS	Mean
Control	15.26	32.84 (115.20)	24.05	16.20	40.14 (47.78)	28.17
Bacillus (B)	13.20	26.90 (103.79)	20.05	13.96	30.33 (117.26)	22.15
% decrease	-13.50	-18.09		-13.83	-24.44	
Trichoderma (TR)	11.46	22.10 (92.84)	16.78	12.06	24.48 (102.99)	18.27
% decrease	-24.90	-32.70		-25.56	-39.01	
Mean	13.31	27.28		14.07	31.65	
Factors	C	T	C×T	C	T	C×T
LSD (p=0.05)	1.16	1.33	2.71	1.03	2.49	2.82
SEm±	0.09	0.16	0.84	0.33	0.38	0.70

Figures in parentheses indicate percent increase over irrigated. IR=Irrigated, WS=Water-deficit stress

Table 2. Effect of plant growth promoting microbes on peroxidase activity (Units mg^{-1} fresh weight) of mustard genotypes at flowering under irrigated and water-deficit stress conditions

Treatments (T)	Genotypes (G)					
	NPJ 214			TM 179		
	Soil condition (C)					
	IR	WS	Mean	IR	WS	Mean
Control	12.91	25.23 (95.43)	19.07	13.78	33.65 (144.19)	23.72
Bacillus (B)	11.77	23.31 (98.05)	17.54	12.33	28.12 (128.06)	20.33
% decrease	-8.83	-7.61		-10.52	-16.43	
Trichoderma (TR)	10.98	21.09 (92.08)	16.04	11.10	25.10 (126.13)	18.10
% decrease	-14.95	-16.41		-19.45	-25.41	
Mean	11.89	23.21		12.40	28.96	
Factors	C	T	C×T	C	T	C×T
LSD (p=0.05)	0.78	1.14	NS	1.11	1.26	2.49
SEm±	0.06	0.42	0.59	0.13	0.19	0.78

Figures in parentheses indicate percent increase over irrigated. IR=Irrigated, WS=Water-deficit stress

Peroxidation of membrane lipids leads to the loss of integrity of cell membrane and also alters some other macromolecules. Thus, quantification of lipid peroxide content is an effective indicator for oxidative damage due to abiotic factors [15]. In current study, water-deficit stress showed an increase in lipid peroxidation in both mustard genotypes, the increase being more prominent in susceptible genotype (Table 3). The amount of malondealdehyde is an indicator of oxidative stress. Free radical–induced oxidative damage in the cell is reflected by the peroxidation of lipids in plant cell membranes under drought. Drought hampers the fatty acid composition (polyunsaturated), which leads to dysfunction of the membrane [10]. Water-deficit stress led to an increase in lipid peroxidation but application of *T. viride* decreased lipid peroxidation. Similar finding was reported by Guler et al. [10] in maize and Shukla et al [16] in rice. Degree of accumulation of MDA content has been confirmed to be a benchmark for the rate of lipid peroxidation due to drought stress. Kumar et al. [17] have reported that the accumulation of malondialdehyde content was lower in treatment of Bacillus (*B. altitudinis)* revealing that accumulation of lipid peroxides was reduced during drought stress by this treatment.

3.4 Proline Content

It was observed that water-deficit stress led to increase in proline content in both tolerant and sensitive genotypes but reduced with the application of microbes. Under irrigated conditions genotype 'NPJ 214' and 'TM 179' recorded a proline content of 0.86 and 0.89 mg g^{-1} fresh weight, respectively which increased to 1.48 and 1.69 mg g^{-1} fresh weight, respectively under water-deficit stress (Table 4). With application of *B. subtilis* it decreased by 4.65 % and with application of *T. viride* decreased by

Table 3. Effect of plant growth promoting microbes on lipid peroxidation (nmol MDA g^{-1} fresh weight) of mustard genotypes at flowering under irrigated and water-deficit stress conditions

Treatments (T)	Genotypes (G)					
	NPJ 214			TM 179		
	Soil condition (C)					
	IR	WS	Mean	IR	WS	Mean
Control	3.57	5.13 (43.70)	4.35	3.53	7.24(105.10)	5.39
Bacillus (B)	3.08	4.50 (46.10)	3.79	3.20	5.33(66.56)	4.27
% decrease	-13.73	-12.28		-9.35	-26.38	
Trichoderma (TR)	3.02	4.22 (39.74)	3.62	3.13	4.72(50.80)	3.93
% decrease	-15.41	-17.74		-11.33	-34.81	
Mean	3.22	4.62		3.29	5.76	
Factors	C	T	C×T	C	T	C×T
LSD (p=0.05)	0.13	0.21	NS	0.11	0.30	NS
SEm±	0.07	0.03	0.18	0.04	0.12	0.31

Figures in parentheses indicate percent increase over irrigated. IR= Irrigated, MS= Water-deficit stress

Table 4. Effect of plant growth promoting microbes on proline content (mg g^{-1} fresh weight) of mustard genotypes at flowering under irrigated and water-deficit stress conditions

Treatments (T)	Genotypes (G)					
	NPJ 214			TM 179		
	Soil condition (C)					
	IR	WS	Mean	IR	WS	Mean
Control	0.86	1.48 (72.09)	1.17	0.89	1.69(89.89)	1.29
Bacillus (B)	0.82	1.36 (65.85)	1.09	0.77	1.32(71.43)	1.05
% decrease	-4.65	-8.11		-13.48	-21.89	
Trichoderma (TR)	0.73	1.21 (65.75)	0.97	0.72	1.19 (65.28)	0.96
% decrease	-15.12	-18.24		-19.10	-29.59	
Mean	0.80	1.35		0.80	1.40	
Factors	C	T	C×T	C	T	C×T
LSD (p=0.05)	0.06	0.08	NS	0.09	0.11	NS
SEm±	0.02	0.03	1.09	0.03	0.02	0.05

Figures in parentheses indicate percent increase over irrigated. IR=Irrigated, WS=Water-deficit stress

13.48% under irrigated condition in genotype 'NPJ 214' and 'TM 179', respectively. Under water deficit stress, decrease in proline content in 'NPJ 214' was 8.11 % and 18.24 % by application of *B. subtilis* and *T. viride*, respectively while in 'TM 179' it was 21.89 % and 29.59 % respectively. Thus, it was evident that decrease was higher in susceptible genotype 'TM 179' than tolerant 'NPJ 214' and application of Trichoderma showed the best ameliorative effect against water-deficit stress. Both the genotypes revealed statistically significant treatment difference with respect to proline content in irrigated and water-deficit stress conditions. However, the interaction effect of treatments was found to be statistically non-significant. Thus, studies showed that the amount of proline in leaves of mustard genotypes enhanced when there was exposure to water-deficit stress which is in consonance with the findings of Rawat et al. [18] in wheat. Proline concentration reduced with application of *T. viride* in both irrigated and water-deficit stress conditions which is consistent with the findings of Shukla et al. [16] in rice and Scudeletti et al. [14] in sugarcane.

4. CONCLUSION

Results revealed that the activity of antioxidative enzymes *viz.,* catalase and peroxidase decreased under both irrigated and water-deficit stress conditions compared to control when soil application of *Bacillus subtilis* and *Trichoderma viride* was done at 35 DAS in both tolerant and susceptible genotypes. Percent decrease in catalase and peroxidase activity in susceptible genotype (TM 179) was much higher than the tolerant genotype (NPJ 214). In case of water-deficit stress, lipid peroxidation increased commendably over control. Application of microbes reduced lipid peroxidation in the genotypes under both irrigated and water-deficit stress conditions and the minimum amount of MDA content was recorded in *Trichoderma viride* treatment while the maximum amount was found in control (without microbial application). Proline content showed a significant rise in both tolerant and in sensitive genotypes as a result of water-deficit stress which was decreased by microbial application and the maximum reduction was recorded in *Trichoderma viride.*

Water-deficit stress is one of the major environmental stress factors that cause biochemical alterations in plants. Increase in concentration of stress induced metabolites was less in colonized plants of mustard indicating modulation of antioxidant system. These findings can help in understanding the effects of microbial inoculation in alleviating the drought stress effects with respect to antioxidant metabolism. The outcome of the study reinforces that application of microbes especially Trichoderma being useful in ameliorating effect of water-deficit stress and can be applied under field conditions.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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