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Novel Mechanism of Modulating Natural Antioxidants in Functional Foods: Involvement of Plant Growth Promoting Rhizobacteria NRRL B-30488

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The significance of plant growth-promoting rhizobacteria (PGPR) mediated increase in antioxidant potential in vegetables is yet unknown. The plant growth-promoting bacterium *Bacillus lentimorbus* NRRL B-30488 (B-30488) mediated induction of dietary antioxidant in vegetables (*Trigonella foenum-graecum*, *Lactuca sativa*, *Spinacia oleracea*, and *Daucus carota*) and fruit (*Citrus sinensis*) after minimal processing (fresh, boiled, and frozen) was tested by estimating the total phenol content, level of antioxidant enzymes, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide scavenging activities along with integral radical scavenging capacity by photochemiluminescence assay and inhibition of lipid peroxidation. Minimal processing of vegetables showed that *T. foenum-graecum* had the highest phenol content in B-30488-treated plants followed by *L. sativa*, *D. carota*, and *S. oleracea*. Thermally treated vegetables *T. foenum-graecum* (26−114.5 GAE μg mg^{-1}) had an exceptionally high total phenolic content, followed by *D. carota* (25.27−101.32 GAE μg mg^{-1}), *L. sativa* (23.22−101.10 GAE μg mg^{-1}), and *S. oleracea* (21.87−87.57 GAE μg mg^{-1}). Among the vegetables and fruit used in this study for enzymatic estimation, induction of antioxidant enzymes, namely, polyphenol oxidase (PPO), ascorbate peroxidase (APX), catalase (CAT), and superoxidase dismutase (SOD), was observed in edible parts of *T. foenum-graecum*, *L. sativa*, *S. oleracea*, and *D. carota,* after inoculation with B-30488. The scavenging capacity of the vegetables treated with B-30488 against DPPH and superoxide anion radical activity was found to be significantly high as compared to nontreated control. Mild food processing had no adverse effect on radical scavenging capacity. Photochemiluminescence also ascertains the above findings. The ability of the plant extracts to protect against lipid peroxidation and its ability to prevent oxidation of reduced glutathione (GSH) was measured in rat liver homogenate, and the results suggested that the inoculated plant exhibited better activity in all of the screened plants. Significant increases in shoot length, root length, and dry weight, averaging 164, 132, and 135% in *T. foenum-graecum*, 174, 141, and 156% in *L. sativa*, 129, 141, and 59%, in *S. oleracea*, and 125, 146, and 42% in *D. carota*, respectively, over untreated controls, were attained in greenhouse trials. To the best of the authors' knowledge, this is the first report of PGPR-mediated induction of antioxidant enzyme activity (PPO, APX, CAT, and SOD) along with the antioxidant activity of the extracts in both in vitro (DPPH radical scavenging and superoxide scavenging) and ex vivo conditions using the rat liver tissue (percent inhibition of lipid peroxidation and prevention of oxidation of GSH) and phenolic content. The results demonstrate the PGPR-mediated induction of antioxidant level in vegetables and fruit controls oxidative damage even after minimal processing and thus is indicative of its potential as a viable substitute of synthetic antioxidants.

KEYWORDS: Plant growth-promoting rhizobacteria (PGPR); vegetables; phenolics; antioxidant enzymes; lipid peroxidation; GSH oxidation; hotochemiluminescence; DPPH and superoxide anion radical activity

INTRODUCTION

In recent years, increasing attention has been paid to the role of diet in human health. Several epidemiological studies have indicated that a high intake of plant products is associated with a reduced risk of a number of chronic diseases, such as atherosclerosis and cancer (*1*). It is increasingly being realized that a majority of the present day diseases are due to the shift in the balance of the pro-oxidant and antioxidant homeostatic phenomena in the body. Pro-oxidant conditions dominate either due to the increased generation of the free radicals caused by excessive oxidative stress of the current life or due to the poor

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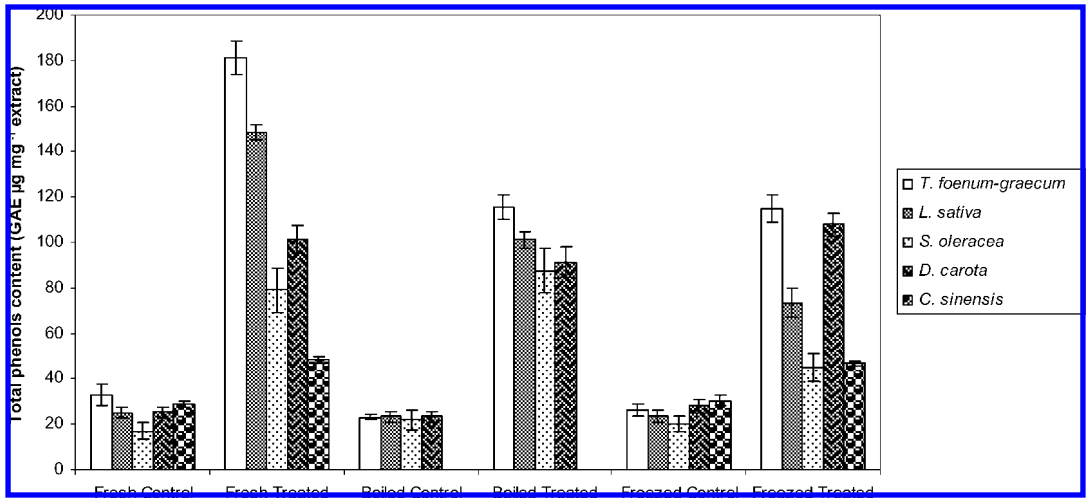

Figure 1. Total phenols in *T. foenum-graecum*, *L. sativa*, *S. oleracea*, *D. carota*, and *C. sinensis* 90 days after inoculation with *B. lentimorbus* B-30488. Each column represents the mean of four replicates ± SE.

scavenging/quenching in the body caused by depletion of the dietary antioxidants (*2*). The major antioxidants of vegetables are vitamins C and E, which contribute to the protective effect of many fruits and vegetables. Apart from these, many constituents such as carotenoids and plant phenols have been proven to be involved in antioxidants in vitro (*3*). The flavonoids and other dietary polyphenols contribute to a sizable part of the antioxidant defense system of the organism against oxidative stress. These antioxidants scavenge radicals, inhibit chain initiation, or break chain propagation (the second defense line). Nutrients and antioxidants may act together to reduce reactive oxygen species level more effectively than single dietary antioxidants, because they can function as synergists (*4, 5*). Combinations of α-tocopherol or vitamin C plus phenolic compounds also provided synergistic effects in human erythrocyte membrane ghosts and phosphatidylcholine liposome systems (*6*). In addition to antioxidant vitamins, carotenoids, and polyphenols, vegetables provide a large group of glucosinolates, which according to Plumb et al. (*7*) possess rather low antioxidant activity, but the products of their hydrolysis can protect against cancer (*8*). Variation in the antioxidant contents of vegetables is caused by many factors: variety, maturity at harvest, growing condition, soil state, and condition of postharvest storage (*9*). Green leafy vegetables may be eaten raw as an excellent component of salads. Industrial processing cooking of vegetables such as blanching, canning, sterilization, and freezing, as well as domestic cooking, affects the content, composition, antioxidant activity, and bioavailability of antioxidants. The losses during vegetable processing need to be taken into account in the calculation of the dietary intake of dietary antioxidants from processed food.

Therefore, we compared changes in antioxidant potential and phenolics in vegetable systems even after minimal processing induced by either plant growth-promoting rhizobacteria (PGPR) treated fresh or processed vegetables and fruits to explore whether PGPR might be acting through this system. In the present study we explore the induction of antioxidant potential in vegetable and fruit systems and describe for the first time the ability of *Bacillus lentimorbus* B-30488 (B-30488) to induce antioxidant potential in functional food. The objectives of this study were to determine (1) the potential of B-30488-mediated induction of antioxidant enzyme activities of polyphenol oxidase (PPO), ascorbate peroxidase (APX), catalase (CAT), and superoxidase dismutase (SOD) and (2) the antioxidant potential of the hydroalcohalic extracts in in vitro [1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, superoxide scavenging, and photochemiluminescence] and using the rat liver tissue [percent inhibition of lipid peroxidation and prevention of oxidation of reduced glutathione (GSH)] and phenolic content in B-30488-treated fresh or processed vegetables and fruits.

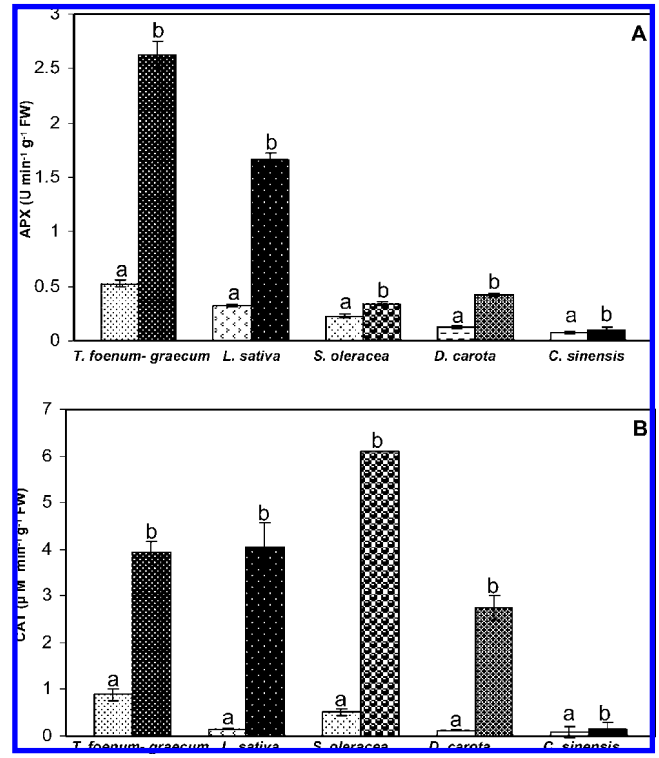

Figure 2. CAT (**A**) and APX (**B**) activity in *T. foenum-graecum*, *L. sativa*, *S. oleracea*, *D. carota*, and *C. sinensis* 90 days after inoculation with *B. lentimorbus* B-30488. Uninoculated controls (a); inoculated (b). Each bar represents the mean of four replicates ± SE.

MATERIALS AND METHODS

Greenhouse Experiment. The plant experiments were conducted in greenhouse conditions at the National Botanical Research Institute, Lucknow, India (latitude/longitude 11° 24′ N/79° 044′ E). Four replicates of each treatment with eight plants in each replicate were maintained, as described earlier (*10*). A bacterial cell suspension (9.0 $\log_{10}$ CFU mL^{-1}) was then inoculated in autoclaved soil in about 23 cm diameter earthen pots to obtain a final moisture level of 20%. This moisture level in the soil was maintained throughout the growing season

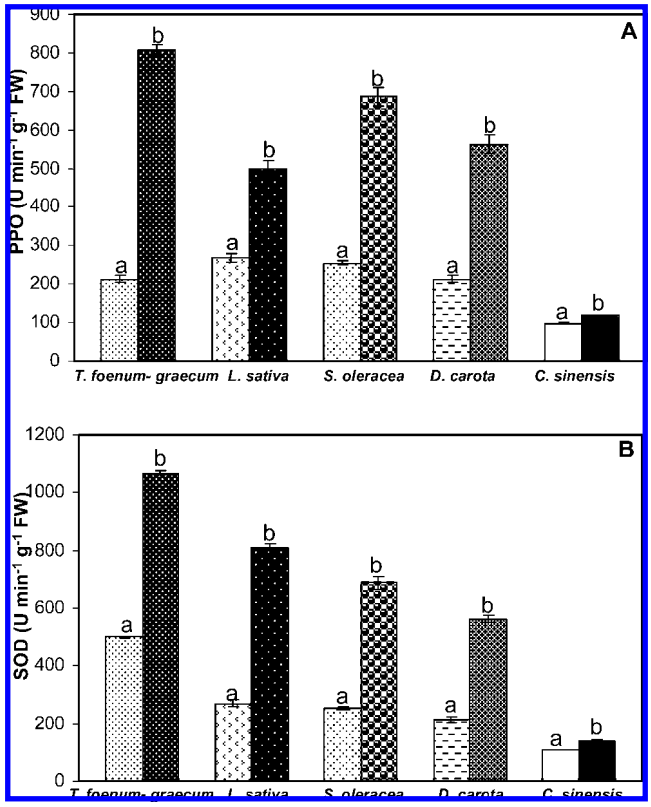

Figure 3. Activities of polyphenol oxidase (PPO; **A**) superoxide dismutase (SOD; **B**) and in *T. foenum-graecum*, *L. sativa*, *S. oleracea*, *D. carota*, and *C. sinensis* 90 days after inoculation with *B. lentimorbus* B-30488. Uninoculated controls (a); inoculated (b). Each column represents the mean of four replicates ± SE.

by irrigating the crops as and when required. Data on the shoot length, root length, total dry weight and total microbial population were determined after 90 days, when the *Trigonella foenum-graecum*, *Lactuca sativa*, *Spinacia oleracea*, and *Daucus carota* crops were harvested, using general methods as described earlier, for other plants (*10*).

Microbial Analysis. Microflora associated with soil and rhizosphere was determined by the culture enrichment technique (*11*). The total microbial counts were determined as follows: nutrient agar for heterogeneous bacteria, soil sample treated at 80 °C for 30 min for total *Bacillus* counts; isolation agar with 0.15% (w/v) sodium deoxycholate for *Pseudomonas* spp. Kenknight and Munaier's medium for *Actinomycetes* spp.; and Rose Bengal chloramphenicol agar for selective isolation of fungi (from HI-MEDIA Laboratories Pvt. Ltd., Bombay, India). Microbial rhizosphere colonization was determined at the time of harvesting from four replications of three plants each. B-30488 has been deposited under the Budapest treaty in the ARS Patent culture collection, U.S. Department of Agriculture, Illinois, as described earlier (*10*).

Plant Material and Extraction. Edible parts of *T. foenum-graecum*, *L. sativa*, *S. oleracea*, and *D. carota* were harvested during the month of November 2005. Plants were cultivated in greenhouse conditions of the National Botanical Research Institute. One hundred grams of plant material was extracted with 50% aqueous alcohol three or four times (exhaustive extraction) and then filtered. The extract was concentrated under reduced pressure and lyophilized to get dry residue (400 mg).

Preparation of Vegetable Samples. Vegetables were washed with tap water after manual removal of inedible parts with a sharp knife. Vegetables were dried on paper towels and cut into almost equal small pieces or slices and mixed well; 300 g was taken and divided into three portions (100 g for each application). One portion was retained as fresh, the second one was cooked, and rest was deep-frozen.

Minimal Processing of Vegetables for Antioxidant Responses. For fresh samples, 100 g of plant material was extracted with 50% aqueous alcohol immediately after harvesting as described by Turkmen et al. (*12*). For boiled samples, 100 g of vegetable was added to 150 mL of water that had just reached the boil in a stainless steel pan and cooked for 5 min. The water was drained off, and samples were cooled rapidly on ice as described by Turkmen et al. (*12*). For frozen samples 100 g of vegetable was stored at −20 °C and time on the antioxidant capacity of harvested vegetables kept in polypropylene bags. For antioxidant potential determination, samples were taken out on 30th day of storage as described by Viña and Chaves (*13*).

Table 1. Radical Scavenging Capacity of Hydroalcoholic Extract of *T. foenum-graecum*, *L. sativa*, *S. oleracea*, *D. carota*, and *C. sinensis* 90 Days after Inoculation with *B. lentimorbus* B-30488[a]

treatment	DPPH scavenging (%)	superoxide scavenging (%)	photochemiluminescence (nM ascorbic acid g^{-1} of equiv)
control			
		T. foenum-graecum	
fresh, control	47.97 ± 1.8	62.39 ± 2.7	1.58 ± 0.17
fresh, treated	66.87 ± 2.2	86.02 ± 1.3	3.55 ± 0.7
boiled, control	40.45 ± 1.9	36.10 ± 1.9	1.29 ± 0.21
boiled, treated	59.15 ± 2.4	72.37 ± 2.7	3.29 ± 0.17
frozen, control	46.34 ± 1.7	48.08 ± 1.3	1.22 ± 0.02
frozen, treated	62.60 ± 2.7	78.70 ± 1.9	3.05 ± 0.18
		L. sativa	
fresh, control	36.38 ± 1.9	48.08 ± 1.9	1.10 ± 0.11
fresh, treated	58.54 ± 1.9	76.70 ± 2.7	3.02 ± 0.16
boiled, control	32.52 ± 1.3	32.44 ± 1.3	1.02 ± 0.1
boiled, treated	47.36 ± 1.8	70.38 ± 1.9	2.88 ± 0.19
frozen, control	35.16 ± 1.7	42.09 ± 1.1	0.98 ± 0.13
frozen, treated	50.81 ± 1.9	76.03 ± 2.7	2.46 ± 0.14
		S. oleracea	
fresh, control	34.15 ± 1.8	42.42 ± 1.1	0.91 ± 0.17
fresh, treated	56.91 ± 1.4	79.03 ± 1.9	2.29 ± 0.13
boiled, control	29.07 ± 2.1	30.44 ± 2.7	0.87 ± 0.02
boiled, treated	42.89 ± 0.9	28.15 ± 1.3	2.76 ± 0.13
frozen, control	31.50 ± 1.4	40.09 ± 1.1	0.84 ± 0.09
frozen, treated	48.78 ± 1.5	73.37 ± 1.3	2.54 ± 0.23
		D. carota	
fresh, control	26.63 ± 2.0	19.80 ± 1.1	0.66 ± 0.02
fresh, treated	46.54 ± 1.1	68.38 ± 2.7	2.00 ± 0.21
boiled, control	32.11 ± 1.3	32.11 ± 1.1	0.78 ± 0.05
boiled, treated	49.59 ± 1.7	75.37 ± 1.9	2.22 ± 0.16
frozen, control	45.53 ± 1.5	34.77 ± 1.3	0.79 ± 0.04
frozen, treated	53.05 ± 1.4	76.37 ± 2.7	2.30 ± 0.17
		C. sinensis	
fresh, control	63.97 ± 2.3	71.39 ± 2.9	2.63 ± 0.21
fresh, treated	76.87 ± 2.7	82.21 ± 1.6	3.49 ± 0.22

[a] Values are expressed as means of six replicates ± SE.

Estimation of Total Phenol. The amount of total phenolic was determined using Folin−Ciocalteu's reagent, as described by Malick and Singh (*14*). Hydroalcoholic extracts were analyzed for determination of phenolic content. In a test tube 500 μL of the sample was pipetted out, and the volume was made to 3 mL with water. Five hundred microliters of Folin−Ciocalteu's phenol reagent was added, followed by the addition of 2 mL of a 20% Na_2CO_3 solution after 3 min, and mixed thoroughly. The tubes were placed in boiling water for exactly 1 min and cooled, and the absorbance was measured at 650 nm against a reagent blank. A standard curve was prepared using different concentrations of gallic acid.

Enzyme Assay. Protein concentrations in the enzyme extract were determined according to the method of Bradford (*15*) using bovine serum albumin (BSA) as a standard.

Polyphenol Oxidase (PPO; EC 1.14.18.1). For the assay of PPO, fresh leaves (1 g) were homogenized in 5 mL of Tris-HCl buffer (50 mM, pH 7.2) containing 0.4 M sorbitol and 10 mM NaCl in chilled pestle and mortar. The homogenate was centrifuged at 20000*g* for 20 min. The supernatant was collected and used to assay PPO (*16*). The reaction mixture consisted of 2.5 mL of 0.1 M phosphate buffer (pH 6.5) and 0.300 mL of catechol solution (0.01 M). To start the reaction, 200 μL of the enzyme extract was added, and the rate of increase in

Table 2. Effect of PGPR *B. lentimorbus* B-30488 on Lipid Peroxidation and Activity of Glutathione Oxidase in the Rat Liver[a]

treatment	TBARS (nM mg^{-1} of protein)	glutathione (nM DTNB mg^{-1} of protein)
control	1.53 ± 0.09	5.143 ± 0.21
	T. foenum-graecum	
fresh, control	0.386 ± 0.18	2.593 ± 0.09
fresh, treated	0.159 ± 0.09	2.307 ± 0.14
boiled, control	0.710 ± 0.14	3.046 ± 0.18
boiled, treated	0.412 ± 0.21	2.498 ± 0.09
frozen, control	0.889 ± 0.09	3.261 ± 0.14
frozen, treated	0.507 ± 0.18	2.641 ± 0.18
	L. sativa	
fresh, control	0.662 ± 0.14	3.046 ± 0.21
fresh, treated	0.400 ± 0.21	2.569 ± 0.14
boiled, control	0.746 ± 0.18	3.271 ± 0.18
boiled, treated	0.460 ± 0.09	2.700 ± 0.09
frozen, control	0.889 ± 0.14	3.475 ± 0.21
frozen, treated	4.738 ± 0.21	2.855 ± 0.18
	S. oleracea	
fresh, control	0.936 ± 0.12	3.630 ± 0.14
fresh, treated	0.531 ± 0.18	2.903 ± 0.18
boiled, control	1.044 ± 0.09	3.833 ± 0.09
boiled, treated	0.591 ± 0.14	3.320 ± 0.14
frozen, control	1.079 ± 0.18	4.059 ± 0.18
frozen, treated	0.626 ± 0.09	3.511 ± 0.21
	D. carota	
fresh, control	1.222 ± 0.14	4.095 ± 0.18
fresh, treated	1.222 ± 0.21	3.475 ± 0.14
boiled, control	1.032 ± 0.18	3.833 ± 0.18
boiled, treated	0.579 ± 0.09	3.332 ± 0.18
frozen, control	0.996 ± 0.08	3.761 ± 0.09
frozen, treated	0.555 ± 0.04	3.308 ± 0.18
	C. sinensis	
fresh, control	0.73 ± 0.05	1.930 ± 0.07
fresh, treated	0.41 ± 0.07	1.714 ± 0.06

[a] Values are expressed as means of six replicates ± SE. $p < 0.001$ with respect to the control and $p < 0.05$ with respect to the control (ANOVA with Nueman−Kuels post hoc).

absorbance at 495 nm was measured at every 30 s up to 5 min. The activity was expressed as change in the absorbance of reaction mixture per minute per gram of protein.

Ascorbate Peroxidase (APX; EC 1.11.1.11). APX was assayed as described by Nakano and Asada (*17*). For the assay of APX, fresh leaves (1 g) were homogenized in 2 mL of 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM ascorbic acid, 2% (w/v) PVP, and 0.05% (w/v) Triton X-100 by grinding in chilled pestle and mortar. The homogenate was centrifuged at 20000*g* for 20 min. The supernatant was collected and used to assay APX. The reaction contained 50 mM potassium phosphate buffer (pH 7.0), 500 mM L^{-1} ascorbic acid, 0.2 mM EDTA, and 0.25 mL L^{-1} H_2O_2. The reaction was started at 25 °C by the enzyme extract containing 50 μg of protein. The decrease in absorbance at 290 nm for 1 min was recorded, and the amount of ascorbate oxidized was calculated from the extinction coefficient (ϵ = 2.8 mM L^{-1} cm^{-1}).

Catalase (CAT; EC 1.11.1.6). CAT activity was determined spectrophotometrically by measuring the rate of H_2O_2 disappearance at 240 nm, taking $\Delta\epsilon$ at 240 nm as 43.6 M L^{-1} cm^{-1} (*18*). To assay of CAT, fresh leaves (1 g) were homogenized in 2 mL of 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM ascorbic acid, 2% (w/v) PVP, and 0.05% (w/v) Triton X-100 by grinding in a chilled pestle and mortar. The homogenate was centrifuged at 20000*g* for 20 min. The reaction mixture contained 50 mM potassium phosphate (pH 7.0) and 10.5 mM L^{-1} H_2O_2 (*19*). The reaction was run at 25 °C for 2 min, after addition of the enzyme extract containing 20 μg of protein, and the initial linear rate of decrease in absorbance at 240 nm was used to calculate the activity.

Superoxide Dismutase (SOD; EC 1.15.1.1). For the assay of SOD, fresh leaves (1 g) were homogenized in 8 mL of potassium phosphate

Table 3. Greenhouse Evaluation of PGPR on Plant Growth Promotion, 90 Days after Inoculation

	T. foenum-graecum				*L. sativa*				*S. oleracea*				*D. carota*			
			CD[a] at				CD at				CD at				CD at	
parameter	C[b]	T[b]	1%	5%	C	T	1%	5%	C	T	1%	5%	C	T	1%	5%
root length (cm)	5.83 ± 0.40	9.00 ± 0.57	1.910	1.630	4.83 ± 0.16	5.83 ± 0.30	0.669	0.481	4.16 ± 0.16	4.83 ± 0.40	1.035	0.745	5.83 ± 0.30	5.50 ± 0.34	1.158	0.833
shoot length (cm)	18.33 ± 0.76	23.00 ± 0.68	5.710	4.020	8.83 ± 0.47	17.66 ± 1.60	5.380	3.070	10.16 ± 0.83	11.66 ± 0.76	6.970	5.010	16.83 ± 0.98	21.00 ± 0.96	2.390	1.47
root/ shoot ratio	0.31 ± 0.01	0.39 ± 0.03	0.006	0.004	0.55 ± 0.02	0.34 ± 0.03	0.010	0.007	0.42 ± 0.03	0.41 ± 0.02	0.109	0.078	0.35 ± 0.02	0.26 ± 0.01	0.003	0.002
dry weight (g)	0.16 ± 0.01	0.36 ± 0.02	0.004	0.003	0.16 ± 0.02	0.41 ± 0.03	0.008	0.002	0.22 ± 0.02	0.35 ± 0.01	0.003	0.002	0.26 ± 0.01	0.37 ± 0.01	0.005	0.004

[a] Critical difference. [b] Control (C); treated (T). Mean values of four replicates ± SE of eight plants each.

Table 4. Effect of (6.0 $\log_{10}$ CFU g^{-1}) on Microbial Population of *T. foenum-graecum*, *L. sativa*, *S. oleracea*, and *D. carota* Grown in a Greenhouse for 90 Days

	log $_{10}$ CFU[a] g^{-1}															
	T. foenum-graecum				*L. sativa*				*S. oleracea*				*D. carota*			
	bulk soil		rhizosphere		bulk soil		rhizosphere		bulk soil		rhizosphere		bulk soil		rhizosphere	
microbe	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T
PGPR (Rif^r)	0.00	7.73	0.00	8.78	0.00	7.26	0.00	8.00	0.00	6.79	0.00	7.40	0.00	6.96	0.00	7.73
heterogenous population	6.56	5.90	7.14	6.58	6.80	6.10	7.10	7.28	6.82	7.80	7.32	7.00	6.54	6.80	6.37	6.60
Bacillus	3.18	3.26	0.00	0.00	4.56	4.30	2.10	0.00	4.80	4.20	3.19	2.20	5.60	5.11	3.60	2.60
Pseudomonas	5.15	5.17	5.00	4.80	5.15	4.80	4.70	3.20	4.20	3.80	3.20	2.80	4.50	4.00	4.00	3.10
fungi	2.34	1.1	1.34	0.00	1.56	0.00	1.20	0.00	1.21	0.00	1.20	0.00	3.20	1.20	2.10	1.10
actinomycetes	2.46	0.00	1.21	0.00	2.10	0.00	0.00	0.00	1.36	0.00	0.00	0.00	3.20	1.30	0.00	0.00

[a] Rhizosphere colonization (log $_{10}$ CFU g^{-1} root) was determined at the time of harvesting from four replications of three plants each. A ±SE of 0.25 log $_{10}$ CFU g^{-1} root was found for the viable cell counts. Control (C); treated (T).

buffer (50 mM, pH 7.8) containing 0.1 mM Na_2EDTA and 1% insoluble PVP in a chilled pestle and mortar. The homogenate was centrifuged at 20000*g* for 20 min. The supernatant was collected and used to assay SOD following the method of Beyer and Fridovich (*20*). The reaction mixture was prepared by mixing 27 mL of 50 mM potassium phosphate buffer (pH 7.8), 1.5 mL of L-methionine (300 mg 10 mL^{-1}), 1 mL of nitroblue tetrazolium salt (14.4 mg mL^{-1}), and 0.75 mL of Triton X-100. Aliquots (1.0 mL) of this mixture were delivered into small glass tubes, followed by 20 μL of enzyme extract and 10 μL of riboflavin (4.4 μg mL^{-1}). The cocktail was mixed and then illuminated for 7 min in an aluminum foil-lined test tube rack. A control tube in which the sample was replaced by 20 μL of buffer was run in parallel, and the A_{560} was measured in all tubes. The test tube containing the reaction was exposed to light for 7 min. The increase in absorbance due to formazan formation was read at 560 nm. Under the described conditions, the increase in absorbance without enzyme extract was taken as 100% and enzyme activity was calculated by determining the percentage inhibition per minute. Fifty percent inhibition was taken as equivalent to 1 unit of SOD activity.

Radical Scavenging Activity. Radical scavenging activity was quantified in the presence of stable DPPH radical on the basis of Blois method (*21*) with slight modification based on that of Govindrajan et al. (*22*). The degree of discoloration indicates the scavenging efficacy of the extract at 517 nm. Ascorbic acid was used as standard. Superoxide scavenging activity was carried out according to an alkaline DMSO method as described by Govindrajan et al. (*22*) using Greiss reagent, and the absorbance was recorded at 560 nm against a control in which pure DMSO had been added instead of alkaline DMSO.

Lipid Peroxidation Inhibition. *Liver Homogenate.* Male Sprague−Dawley rats (160−180 g) were obtained from rhw animal house of the Central Drug Research Institute, Lucknow, India. These were kept in the departmental animal house at 26 ± 2 °C and relative humidity of 44−55% under light and dark cycles of 10 and 14 h, respectively, for 1 week before the experiment. Animals were provided with rodent diet (Amruth, India) and water ad libitum. Randomly selected male rats were fasted overnight and were sacrificed by cervical dislocation. The dissected abdominal cavity was perfused with 0.9% saline. Whole liver was taken out, and visible clots were removed and weighed; the amount of liver was processed to get 10% homogenate in cold phosphate-buffered saline (pH 7.4) using a glass Teflon homogenizer and filtered to get a clean homogenate.

Assay of Lipid Peroxidation and Glutathione. The degree of lipid peroxidation was assayed by estimating the thiobarbituric acid reactive substances (TBARS) according to the method of Govindrajan et al. (*22*). In brief, an extract, 250 μg mL^{-1}, in water was added to the liver homogenate. Lipid peroxidation was initiated by adding 100 μL of 15 mM $FeSO_4$ solution to 3 mL of liver homogenate (final concentration was 0.5 mM). After 30 min, 100 μL of this reaction mixture was taken in a tube containing 1.5 mL of 10% TCA. After 10 min, tubes were centrifuged and supernatant was separated and mixed with 1.5 mL of 0.67% TBA in 50% acetic acid. The mixture was heated in a water bath at 85 °C for 30 min to complete the reaction. The intensity of pink-colored complex formed was measured at 535 nm. The value of TBARS was calculated from a standard curve (absorption against concentration of tetraethoxypropane) and expressed as nanomolar per milligram of protein. The reduced glutathione was determined according to the Ellman method (*23*) using dithiobis.

Photochemiluminescence Assay. For the determination of the integral antioxidant capacity (AC) of the water-soluble substances in *T. foenum-graecum*, *L. sativa*, *S. oleracea*, and *D. carota* extracts, the method of photochemiluminescence (PCL) was used. The apparatus used was a Photochem with Standard Kit ACW (Analitik jena AG), where the luminol plays a double role as photosensitizer as well as radical-detecting agent. Lyophilized extract was measured at 10 μg mL^{-1} concentrations. A standard curve was plotted, and the results were calculated in ascorbic acid equivalents (μM g^{-1}).

Statistical Analysis. Data were recorded as means ± SE and analyzed by SPSS for Windows (ver. 10.1). One-way analysis of variance (ANOVA) was carried out to test level significance between fresh, cooked, and frozen vegetables. Correlations among variables were assessed with Pearson's correlation coefficient. Results were considered at $p < 0.05$ and <0.01 levels of significance.

RESULTS

Phenolics and Antioxidant Enzymes. The total phenolic content of the vegetable extracts is shown in **Figure 1**. Among all of the tested vegetables, *T. foenum-graecum* had the highest phenolic content in B-30488-treated plants (33.34−180.7 GAE μg mg^{-1} of vegetable extract), followed by *L. sativa* (25.07−148.30 GAE μg mg^{-1}), *D. carota* (27.9−107.8 GAE μg mg^{-1}), *S. oleracea* (16.95−79.0 GAE μg mg^{-1}), and *C. sinensis* (29.0−48.50 GAE μg mL^{-1}). For thermally treated vegetables *T. foenum-graecum* (26−114.5 GAE μg mg^{-1}) still had an exceptionally high total phenolic content, followed by *D. carota* (25.27−101.32 GAE μg mg^{-1}), *L. sativa* (23.22−101.10 GAE μg mg^{-1}), and *S. oleracea* (21.87−87.57 GAE μg mg^{-1}). *L. sativa* lost the highest amount of phenolic content (156.5%) after a 5 min cooking in boiling water followed by *T. foenum-graecum* (114.3%), *S. oleracea* (66%), and *D. carota* (9%). In frozen samples, the total phenolic content was significantly reduced in *D. carota* (14.2%), *T. foenum-graecum* (53.3%), *S. oleracea* (245%), and *L. sativa* (281%). The finding indicates that phenolic compounds were very sensitive to heat treatment even in a short period of cooking.

APX activity was found to increase in *T. foenum-graecum* (5-fold), *L. sativa* (5-fold), *S. oleracea* (1.5-fold), *D. carota* (3−4-fold), and *C. sinensis* (1.3-fold). APX activity was present in various amounts in all of the nontreated controls (**Figure 2B**). Contrary to the PPO and APX activities, the background CAT activity was found to be maximum in *T. foenum-graecum* (0.756 μmol min^{-1} g^{-1}) and minimum in *D. carota* and *L. sativa*. CAT activity was found to be 4−5-fold in PGPR-treated *T. foenum-graecum*. However, its induction was observed in *L. sativa* (25-fold), *D. carota* (21-fold), *S. oleracea* (12-fold), and

C. sinensis (0.64-fold) as compared with their respective controls (**Figure 2A**). The PPO activity was found to increase in inoculated *T. foenum-graecum* (278%), *L. sativa* (86.5%), *S. oleracea* (171%), *D. carota* (163%), and *C. sinensis* (22%). Induction of antioxidant enzymes, namely, PPO, APX, CAT, and SOD, was observed in edible parts of *T. foenum-graecum*, *L. sativa*, *S. oleracea*, and *D. carota* after 90 days of inoculation with PGPR. Significant variations were observed in the level of antioxidant enzymes between PGPR-treated and nontreated vegetables. PPO activity was found to be minimum in *T. foenum-graecum* as compared to *L. sativa*, *S. oleracea*, *D. carota*, and *C. sinensis* in nontreated control (**Figure 3A**). Similarly to CAT, SOD activity was found to be maximum in inoculated *L. sativa*. The increase in SOD activity was found to be 200% in *L. sativa*, 171% in *S. oleracea*, 163% in *D. carota*, 70% in *T. foenum-graecum*, and 27% in *C. sinensis*. The SOD activity in control condition was 1.5−2.0-fold higher in *T. foenum-graecum* as compared with *L. sativa*, *S. oleracea*, and *D. carota* (**Figure 3B**).

Radical Scavenging Capacity of Hydroalcoholic Extract. The scavenging capacity of the plants against DPPH and superoxide anion radical showed in general that the inoculated plants exhibited better activity in all four plants screened, with *T. foenum-graecum* exhibiting maximum scavenging capacity (**Table 1**).

Lipid Peroxidation and Activity of Glutathione Oxidase. The ability of the plant extract to protect against lipid peroxidation and its ability to prevent GSH oxidation were measured in rat liver homogenate, and the result suggested that the inoculated plant exhibited better activity in all four plants screened, with *T. foenum-graecum* exhibiting maximum scavenging potential (**Table 2**). For the determination of the integral antioxidant capacity (AC) of the water-soluble substances in *T. foenum-graecum*, *L. sativa*, *S. oleracea*, and *D. carota* extract, the method of photochemiluminescence (PCL) was used. The apparatus used was a Photochem with Standard Kit ACW (Analitik jena AG), where the luminol plays the double role of photosensitizer and radical-detecting agent. Lyophilized extract was measured at 10 μg mL^{-1} concentrations. A standard curve was plotted, and the results were calculated in ascorbic acid equivalents (μM g^{-1}) (**Table 2**).

Greenhouse Evaluation of B-30488. The effect of B-30488 was also studied on growth promotion of *T. foenum-graecum*, *L. sativa*, *S. oleracea*, and *D. carota*. Vegetables treated with PGPR showed a significantly higher biomass compared with uninoculated controls. Significant increases in shoot length, root length, and dry weight, averaging 164, 132, and 135% in *T. foenum-graecum*, 174, 141, and 156% in *L. sativa*, 129, 141, and 59% in *S. oleracea*, and 125, 146, and 42% in *D. carota*, respectively, over untreated controls, were attained in greenhouse trials (**Table 3**). As with PGPR, vegetable seed can support the prolonged survival and plant growth. Rhizosphere colonization by B-30488 persisted throughout the growing period of 90 days, and the population at the time of harvesting (after 90 days) ranged from 6.76 to 8.78 $\log_{10}$ CFU g^{-1}. Hetrogeneous counts indicate that natural bacterial populations can reach levels of 10^6-10^7 $\log_{10}$ CFU g^{-1} in rhizosphere as well as in bulk soils. No significant differences in the total viable counts of total heterogeneous microbial population, *Bacillus* and *Pseudomonas*, was observed in the rhizosphere and bulk soil of the nonbacterizied control and bacterizied 90-day-old *T. foenum-graecum*, *L. sativa*, *S. oleracea*, and *D. carota* (**Table 4**). On the contrary total viable counts of fungi and actinomycetes were in general lower in the bacterizied rhizosphere and bulk soil of 90-day-old *T. foenum-graecum*, *L. sativa*, *S. oleraces*, and *D. carota* as compared with nonbacterizied control (**Table 4**).

DISCUSSION

Phenolic compounds play an important role in plant resistance and defense against microbial infections, which are intimately connected with reactive oxygen species (ROS). These compounds contribute to the overall fitness of plants with ascertained multifold functions such as insect attraction by colors, protection against pathogens, and competitive neighbor plants (*24*). This study indicates that among the screened vegetables, *T. foenum-graecum* had the highest amount of phenolics. Mild food processing of PGPR-treated samples had no adverse effect on total phenolics. This might be considered as useful for health purposes. Phenolics are also able to act as radical scavengers or radical chain breakers, thus extinguishing strongly oxidative free radicals such as the hydroxyl radical. Antioxidant activity has been described for phenolic-rich beverages such as wine and teas and has led to the suggestion that some phenolic compounds may prevent oxidative damage in vivo and thus protect against the development of disease such as cardiac disease and cancer (*25, 26*).

PPO, APX, CAT, and SOD are the key antioxidant enzymes, which exert their effect through different pathways explained by different workers. In the present study we find an increase in antioxidant enzyme activity of all the selected vegetables, irrespective of the site of inoculation (soil inoculation), which shows that these enzymes are induced by PGPR. The increase in activity of APX could be due to activation of preexisting APX or synthesis of new APX by PGPR, which in turn may be associated with the deactivation of catalase or due to overproduction of H_2O_2. Antioxidant enzymes exist that convert ROS into less noxious compounds, for example, SOD, CAT, and APX. In plant systems, enzymes and redox metabolites act in synergy to carry out ROS detoxification. SOD catalyzes the dismutation of O_2^- to H_2O_2, CAT dismutates H_2O_2 to oxygen and water, and APX reduces H_2O_2 to water by utilizing ascorbate as a specific electron donor. These are considered to be the main enzymatic systems for protecting cells against oxidative damage (*27*). The balance between SOD and APX or CAT activities in cells is crucial for determining the steady-state level of O_2^- and H_2O_2. Collectively, these enzymes provide a first line of defense against superoxide and hydrogen peroxides. They are of enormous importance in limiting ROS-mediated damage to biological macromolecules, but they are not 100% effective because certain compounds generated by the interaction of ROS with macromolecules are highly reactive. It is then mandatory to detoxify these secondary products to prevent further intracellular damage, degradation of cell component, and eventual cell death. Our data suggest that PGPR induces an overproduction of O^{2-} in all of the tested vegetables, which is counterbalanced by increasing activity of SOD. Increased SOD activity induces an overproduction of H_2O_2, which is partially counterbalanced by APX activity. The increased level of antioxidant enzymes SOD and APX has been detected in rice under salt stress condition (*28*), and it has been reported that APX is essential for maintaining the redox state of ascorbate. PPO is copper-containing enzyme, which oxidizes phenolics to highly toxic quinine, and is involved in terminal oxidation, by which it is attributed a major role in disease resistance. The beneficial effects of polyphenols are mainly ascribed to their capacity to counteract conditions of oxidative stress. Polyphenols have antioxidant properties in vitro as they

can act as chain breakers or radical scavengers depending on their chemical structures, which also influence their antioxidant power. Many of these phenols exert powerful antioxidant effects in vitro by inhibiting LPO by acting as peroxy radical scavengers (*29*). The relative significance of PGPR-mediated increases in antioxidant potential in vegetables is yet unknown. Such studies demonstrate the PGPR-mediated induction of antioxidant level in vegetables to control the oxidative damage, and it can serve as a substitute for synthetic antioxidants.

DPPH reactivity has been widely used to test the ability of compounds/plant extracts to act as free radical scavengers (*21*). Superoxide radical O_2^- is a highly toxic species, which is generated by numerous biological and photochemical reactions. Both aerobic and anaerobic organisms possess superoxide dismutase enzymes, which catalyze the breakdown of superoxide radical. SOD is effective in reducing leukocyte adhesion in inflammatory conditions (*23*). The potassium superoxide assay was used to measure the superoxide dismutase activity of plant extracts. Potent scavenging of DPPH and superoxide anion radical was exhibited by the inoculated plants, thus showing that PGPR inoculation increased the radical scavenging capacity of all four vegetables.

Initiation of the lipid peroxidation by ferrous sulfate takes place either through the ferryl−perferryl complex or through OH radical by Fenton's reaction. Plant extracts inoculated with PGPR had better lipid peroxidation inhibition, compared with uninoculated plant extracts. The inhibition could be caused by the absence of the ferryl−perferryl complex, by scavenging the OH radical or the superoxide radicals, by changing the Fe^{3+}/Fe^{2+}, by reducing the rate of conversion of ferrous to ferric, or by chelating the iron itself. Iron catalyzes the generation of hydroxyl radicals from hydrogen peroxide and superoxide radicals. The hydroxyl radical is highly reactive and can damage biological molecules; when it reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids, lipid hydroperoxide is produced (*30*). Lipid hydroperoxide can be decomposed to produce alkoxy and peroxy radicals; they eventually yield numerous carbonyl products such as malondialdehyde (MDA). Thus, the decrease in the MDA level upon treatment with plant extracts indicates the role of the plants as an antioxidant. The importance of thiols especially of cysteine and glutathione to lymphocyte function has been known for many years. GSH is a nonenzymic mode of defense against free radicals. Glutathione is an intracellular thiol-rich tripeptide, which plays a major role in the protection of cells and tissue structures (*31*).

The results indicate that PGPR is potentially useful as a bioinoculant for vegetables as well as fruits. Recent surveys of both conventional and organic growers indicate an interest in using bioinoculant products (*32*), suggesting that the market potential of bioinoculant products will increase in the coming years. Additionally, induction of phenolics and dietary antioxidant in *T. foenum-graecum*, *L. sativa*, *D. carota*, *S. oleracea*. and *C. cinesis* following treatment with B-30488 and their probable role as an efficient tool in protecting against oxidative stress have been shown for the first time. The continued presence of B-30488 for 90 days in rhizosphere as well as bulk soil shows that it has reached homeostasis after undergoing exchange with indigenous microflora and is not affected by the active and passive processes restricting the soil community. This makes B-30488 ideally suited as a soil inoculant because of its potential for rapid and aggressive colonization. Besides a suitable rhizosphere environment, it is generally assumed that successful bioinoculant treatment requires root colonization by introduced bacterium (*10*).

The promotion of a high level of food safety is a major policy priority worldwide. The innocuousness of foodstuffs is a concept inherent in food safety and is related to many aspects of agrarian technologies as well as to food production and processing. The guarantee of food safety and quality along the food chain is a principal demand of consumers (*33*). Inoculation of seeds or soil with beneficial microorganisms for crop improvement has been practiced for a number of years. PGPR include but are not limited to *Rhizobium*, *Pseudomonas*, and *Bacillus*, etc. (*10, 34, 35*). Despite increasing interest in functional food, it is surprising that no information exists regarding the induction of antioxidant potential by PGPR. We have reported that B-30488 is a good competitor with the native microbes and has the ability to promote the growth of different plant species, including monocots, dicots, C3, C4, and legume plants and shows good promise as an inoculant for plant growth promotion (*10*). Vegetables represent a source of compounds with antioxidant activity, as they produce a lot of antioxidants to control the oxidative stress caused by oxygen apart from their nutritional value. Induction of antioxidant potential in vegetables is essential as they are the major dietary element. Regular consumption of dietary antioxidants may reduce the risk of several serious diseases such as cancer, cardiovascular disease, and macular degeneration. This study indicates that inoculation with B-30488 increases the total phenolic content and antioxidant enzyme level in all of the tested vegetables. Samples of fresh vegetables had substantially higher phenolic content as well as antioxidant enzyme level as compared to cooked and frozen ones. In addition, green leafy vegetables may be eaten raw as ingredients of salad. Carrot may be prepared in the same way as green leafy vegetables, and their major amounts are used as an excellent component of salad. Cooking and freezing had no deleterious effect on total phenolic content of vegetables with the exception of some losses of phenolics. The results obtained show that B-30488 can serve as a useful tool to control epidemiological disorders as well as improve nutritional quality through optimum methods of processing and thus contribute to an overall assessment of functional food. Our work is of significance especially because there is a lot of apprehension about the safety of genetically modified foods to deliver and/or increase the beneficial effects of foods (*33*). In this scenario we believe that PGPR has an important role to play in providing more nutritional value for the vegetables and fruits without compromising the safety aspects of food.

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