JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effects of Phosphorus Fertilizer Supplementation on Antioxidant Enzyme Activities in Tomato Fruits

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The effects of soil and foliar phosphorus supplementation on the activities and levels of superoxide dismutase (SOD), guaiacol peroxidase (POX), and ascorbate peroxidase (APX) in tomato fruits were evaluated by determining enzyme activities and isoenzyme analysis. Both protein levels and enzyme activities varied depending on the variety and season. In general, phosphorus supplementation did not alter SOD, POX, and APX activities significantly;however, some treatments showed season- and stage-specific enhancement in activities as noticed with hydrophos and seniphos supplementation. Three different SOD isozymes were observed, and these isozymes showed very similar staining intensities in response to P application and during the three developmental stages studied. Two major isozymes of POX and two different APX isozymes were observed at all the developmental stages. The results suggest that antioxidant enzyme activities may be influenced by the availability of phosphorus, but are subject to considerable variation depending on the developmental stage and the season.

KEYWORDS: Ascorbate peroxidase; fruit quality; *Lycopersicon esculentum*; peroxidase; superoxide dismutase

INTRODUCTION

During growth and development under field conditions, crop plants are exposed to several external stress factors that in turn may affect the quality of their produce. In general, active oxygen species (AOS) such as superoxide radicals, singlet oxygen, H_2O_2, and hydroxyl radicals are generated during growth and developmental processes under normal metabolic conditions and/or under the stress conditions (*1*−*4*). The generation of AOS is linked to various cellular deterioration including membrane lipid peroxidation, DNA mutation, protein denaturation, and enzyme inactivation (*5*). Furthermore, the role of AOS as signal molecules is becoming increasingly clear, and suggests that AOS are not only stress signal molecules but may also be an intrinsic part of signal transduction in plant growth and development (*6*).

Plants have several mechanisms to prevent or alleviate the damage that may occur from AOS. These mechanisms include scavenging the AOS by natural low molecular mass antioxidants such as ascorbate, glutathione, α-tocopherol, and carotenoids, and the use of an enzymatic antioxidant system that includes superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POX, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.1), catalase (CAT, EC 1.11.1.6), and glutathione reductase (GR EC 1.6.4.2) (*7*, *8*). Antioxidant enzymes are important because they prevent the initiation of oxidation by the removal of the oxygen species $O_2^{\bullet-}$ and H_2O_2, before they participate in reactions that form initiation species. Within the cell, SOD which converts $O_2^{\bullet-}$ to H_2O_2 is the first line of defense against AOS, and the level of H_2O_2 is controlled by POX and CAT localized in compartments of the plant cell. Main function of POX is the elimination of H_2O_2 formed by SOD activity. APXs are chloroplastic or cytosolic enzymes that act in tandem with SOD to scavenge H_2O_2 generated through SOD action, and APX also catalyzes the first step of the H_2O_2 scavenging pathway by oxidizing reduced ascorbate (ASA) (*2*, *8*−*10*).

Antioxidant enzymes have been studied in relation to their role under stress conditions and diseases because AOS are generated during pathogen attack and abiotic stress situations (*2*, *6*, *11*−*13*). In *Arabidopsis thaliana*, UV−B exposure enhanced activities of POX and APX, while O_3 exposure enhanced activities of SOD, POX, GR, and APX (*2*). However, water deficiency enhanced activities of dehydroascorbate reductase (DHAR) and POX (*12*), while chilling temperature increased activities of APX and GR, and decreased CAT activity (*14*). In addition, excess copper (Cu) or iron (Fe) affects certain antioxidant enzyme activities. In tomato, excess Cu increased POX activity, but neither APX nor CAT (*15*), and Fe increased SOD activity in *Hydrilla verticillata* (*16*). Changes in the activity of antioxidant enzymes have been accomplished by genetic manipulation (*17*), and plant growth regulator treatment (pa-

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10.1021/jf040248y CCC: $30.25
Published on Web 02/08/2005

clobutrazol) caused an increase in the antioxidant enzyme activities in corn seedlings (*3*, *18*).

Although there are a number of reports on antioxidant enzymes in plants, little is known about the effects of nutrients such as phosphorus (P), nitrogen (N), and potassium (K) on the expression and activities of antioxidant enzymes. Recent studies have shown that chloroplasts of coffee plants grown under conditions of high N availability possessed increased activities of Cu,Zn−SOD, APX, and GR in response to high irradiance than plants grown under medium or low N availability conditions, as well as having a higher carotenoid content. These results suggest that a higher N availability may provide increased protection from oxidative stress (*19*). On the other hand, in long-term studies using spinach, activities of SOD, APX, and GR were higher on a total protein basis in N-limited plants than N-replenished plants (*20*). In addition, different N fertilization regimes induce different responses in antioxidant enzyme activity (*21*). Supplementation of potassium either through soil or by foliar-spray increased lycopene levels and decreased fruit color disorders in ripe tomato (*22*). Furthermore, soil or foliar P fertilization during normal field growth conditions of tomato enhanced antioxidant enzyme activities (*23*). Soil or foliar P fertilization reduced superficial scald and core flush in apple (*24*, *25*) and enhanced lycopene level in tomato during some seasons (*26*). An increased level of antioxidant enzyme function may help preserve the cell structure longer by scavenging the activated oxygen species produced during the ripening phase of fruits. In general, this would tend to enhance the shelf life as well as preserve the nutritional ingredients in ripe fruits, thus improving the overall quality of fruits. In the present study, phosphorus was supplemented through the soil as superphosphate or by foliar spray as formulations (hydrophos, seniphos, Phosyn PLC, UK) and its effect on antioxidant enzyme activities was studied. The potential implications of phosphorus supplementation on fruit quality are discussed.

MATERIALS AND METHODS

Plant Materials and Growth Conditions. Tomatoes (*Lycopersicon esculentum* Mill. H9478 or H9997, Heinz processing varieties) were used. Tomato seeds were germinated in potting soil in the greenhouse, and four-week old plants were transplanted into the field in early June. H9478 was evaluated for two seasons, 2001 and 2002, and H9997, in 2002. The field trials were conducted at the Cambridge Research Station of the University of Guelph. Tomato plants were planted in plots of 1.8 m × 3.0 m, each plot containing 24 plants. Each plot was separated from the others by a minimum distance of 1.5 m. All soil P fertilizer applications were conducted for the plots as per the Ontario Ministry of Agriculture Food (OMAF) recommendations [250 kg of 5:20:20 (N:P:K) per hectare, 50 kg of P_2O_5 per hectare for soils with 30∼50 mg/L available P]. The following fertilization regimes were used. The regular phosphorus supplemented plots received 135 g of 5:20:20 (NPK) and 80 g of ammonium nitrate (46:0:0) at the time of planting. The phosphorus-supplemented plots received an additional 315 g of superphosphate (0:20:0, approximately 45 kg P_2O_5/acre, low phosphorus supplementation, LP) and the high phosphorus-supplemented plots received 630 g of superphosphate (approximately 90 kg P_2O_5/acre, HP). Phosphorus supplementation was also performed through foliar sprays of Hydrophos (a foliar formulation containing 440 g/L phosphorus, 74 g/L K_2O and 60 g/L magnesium, Phosyn UK; 300 mL in 64 liter water, 4 L per plot, each plot received 8 g of phosphorus, 1.48 g of K_2O and 1.2 g of magnesium per spray; equivalent to 35 L/7400 L water in a hectre, HYDRO) and Seniphos (a foliar formulation containing 310 g/L phosphorus and 40 g/L calcium, Phosyn UK; 600 mL in 64 L water, 4 L per plot, each plot received 11.6 g of phosphorus and 1.5 g of calcium per spray; equivalent to 70 L in 7400 L water/ hectre, SENI). The sprays were applied two times at 15-day intervals during the growing period. These plots received 135 g of 5:20:20 and 80 g of ammonium nitrate per plot at the time of planting. A no phosphorus control (NP; only nitrogen in the form of ammonium nitrate, 80 g/plot, and potassium, potash, 0:0:60, 60 g/plot) was also included. However, the soil contained 50 mg/L of available phosphorus, which is considered high. There were four randomly selected replicates for each treatment. In 2001, a combination treatment (COMB) including LP, HYDRO and SENI in applications was used instead HP in 2000.

Protein Extraction. The tomatoes were harvested at the mature green (MG), the orange (ORG) and the red (R) stages for each treatment. Protein extraction was carried out as described by Ahn et al. (*4*), pericarp (5 g) was homogenized (Polytron, Brinkmann) along with 5 mL of 100 mM sodium phosphate buffer (pH 7.5) containing 1 mM ethylenediamine tetraacetate (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF) dissolved in 1 mL dimethyl sulfoxide (DMSO) and 1% poly(vinylpyrrolidone) (pvp, mol. wt. 40 000, Sigma). The homogenate was filtered through four layers of cheesecloth and centrifuged (IEC 21 000R−Rotor 7851C, IEC) at 28350*g* (12 000 rpm) for 20 min. The supernatant was collected and 2.5 mL aliquots of the extract were passed through a Sephadex G-25 column (PD-10, Pharmacia) equilibrated with 100 mM sodium phosphate buffer (pH 7.5). The proteins were eluted with 100 mM sodium phosphate buffer (pH 7.5) and protein samples were stored at −80 °C for further analyses. Protein extraction for determining APX activity was performed essentially as described above, except that the homogenization buffer also contained 5 mM ascorbate.

All steps in preparation of the extract were carried out at 4 °C. Protein content was determined according to the method of Bradford (*27*) with bovine serum albumin as a standard.

Enzyme Activity Analysis. SOD activity was determined by measuring the ability of SOD to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm after illumination for 10 min using a 15 W fluorescent lamp (*28*). The assay mixture contained 50 mM sodium phosphate buffer (pH 7.8), 15 mM methionine, 80 μM NBT, 2 μM riboflavin and 0.1 mM EDTA. There was no measurable effect of diffuse room light on the assay. One unit of SOD activity is defined as the amount of enzyme that inhibits the NBT photoreduction by 50% under the assay conditions. SOD units/mL = $(V_o/V - 1) \times$ (dilution factor), where V_o is the slope of the change in absorbance in the absence of enzyme extract, and V is the slope of change in absorbance in the presence of enzyme extract, and was calculated as a linear correlation between the SOD activity and the amount of enzyme extract used. Enzyme activities are expressed as unit per microgram of protein and unit per gram per fresh weight, respectively.

POX activity was determined by the rate of guaiacol oxidation in the presence of H_2O_2 (extinction coefficient, 26.6 mM^{-1} cm^{-1}) at 470 nm for 4 min as described by Rao et al. (*2*). The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.5), 16 mM guaiacol and 20 μg protein in 2 mL assay volume, and the reaction was initiated by adding 10 μL of 15% H_2O_2.

APX activity was determined by the decomposition of H_2O_2, essentially using the same method used for catalase assay as described by Du and Bramlage (*29*). Fifty μL of enzyme extract was added to 4 mL of assay mixture containing 50 mM Tris-HCl buffer (pH 6.8) and 5 mM H_2O_2. After incubation for 10 min at room temperature, the reaction was terminated by adding 0.5 mL of Titanium reagent (titanium(IV) chloride, 0.09 M solution in 20% hydrochloric acid, v/v). A time zero samples was obtained by adding 0.5 mL of Titanium reagent immediately after adding the enzyme extract to the assay mixture. The difference in absorbance measured at 415 nm after 10 min of incubation and the initial time was calculated. The consumption rate of H_2O_2 was calculated using a calibration curve of H_2O_2 in a concentration range from 1.00 to 2.00 mM. One unit of APX activity is defined as the amount of enzyme that decomposes 10 nmol of H_2O_2 per minute under the assay conditions.

Enzyme activities for SOD, POX, and APX determined by spectrophotometry, total activities were also expressed on the basis of fresh weight at each developmental stage.

Native Polyacrylamide Gel Electrophoresis (PAGE) and Activity Staining. Equal amounts of concentrated protein (10 μg) from tomato fruits from plants subjected to different phosphorus application regimes and harvested at the mature green, orange, and red stages were subjected

to PAGE under nondenaturing, nonreducing conditions, as described by Rao et al. (*2*). Electrophoretic separation was performed on 8% polyacrylamide gel for 4.0 h at a constant current of 10 mA per gel for SOD and POX. For separating APX isozymes, electrophoresis was performed on 10% polyacrylamide gel for 3.0 h under the same condition except that the carrier buffer contained 2 mM ascorbate and the gel was pre-run for 30 min to allow ascorbate to enter the gel. All native PAGE runs were carried out at 4 °C. After completion of electrophoresis, the gels were stained for the activities of SOD, POX, and APX as described by Rao et al. (*2*). SOD isoenzymes were stained by incubating in a solution containing 2.5 mM NBT for 25 min, followed by incubation in 50 mM potassium phosphate buffer (pH 7.8) containing 28 μM riboflavin and 28 mM tetramethylethylenediamine for 20 min in the dark. The gel was placed in distilled water and exposed on a fluorescent light box for 15 min at room temperature. POX isoenzymes were stained by incubating the gel in 100 mM sodium acetate buffer (pH 4.5) containing 2 mM benzidine dihydrochloride dissolved in 1 mL DMSO. The reaction was initiated by adding 3 mM H_2O_2 and continued for 20 min. For localization of APX isoenzymes, the gel was incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate, 3 times for 10 min each, for a total of 30 min. The gel was further incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 4 mM ascorbate and 2 mM H_2O_2 for 20 min. The gel was washed with buffer for 1 min and submerged in a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 30 mM tetramethylethylenediamine and 3.0 mM NBT with gentle agitation. The reaction was continued for 15 min and stopped by a brief wash in distilled water and the gel was stored in 10% acetic acid solution.

Sampling and Statistical Analysis. Various phosphorus supplementation protocols were applied through a randomized block design. Each treatment was replicated four times. Tomatoes (1 kg) at the mature green, orange and red stages were harvested from each of the four blocks independently. These four sets of tomatoes were pooled, randomized and divided into three equal sets. Samples were taken from these three sets for analysis. Enzyme activities were measured from the three preparations independently, and all data are reported as mean value ± SD. The data obtained were analyzed between various treatments and between the three developmental stages studied. The data were subjected to ANOVA and significant differences are reported at a confidence level of $p < 0.05$.

RESULTS

The shelf life and quality of field-grown fruits can vary tremendously from season to season. The parameters that determine the shelf life and quality include firmness, color, texture, flavor, pathogen resistance etc. In general, there is a decline in firmness due to the breakdown of cell wall components, a decline in membrane lipid components, an increase in sugars, lycopene and flavor components, all contributing to the enhancement in quality. The levels of antioxidants and antioxidant enzyme activity can influence the shelf life and quality by removing activated oxygen species produced during ripening. At present, we do not have any information on the effects of phosphorus supplementation on the levels of antioxidant enzyme activities. In the previous study (*30*), no significant differences were observed in the levels of vitamin C as a result of phosphorus supplementation. Therefore, the activities of several antioxidant enzymes were analyzed in tomato fruits harvested from plants subjected to different regimes of phosphorus fertilization. The present study was conducted during two seasons with contrasting features. The 2001 season was extremely hot and dry which resulted in poor yield. The 2002 season was ideal for growth and development with abundant rainfall during the early stages and dry, sunny periods toward the mid growth period when the fruit set had been initiated. Despite these differences, there are several similarities in antioxidant enzyme activities resulting from phosphorus supplementation.

Table 1. Protein Concentrations of H9478 and H9997 Tomato Fruits Harvested in 2001 and 2002 Seasons

	protein concentration (μg/g fresh weight)		
phosphorus application	mature green stage	orange stage	red stage
	H9478 Harvested in 2001		
NP[a]	$^{A}187 \pm 15^{a}$	$^{A}157 \pm 25^{a}$	$^{A}153 \pm 66^{ab}$
RP	$^{A}174 \pm 52^{ab}$	$^{A}144 \pm 69^{a}$	$^{A}226 \pm 11^{a}$
HYDRO	$^{A}135 \pm 70^{bc}$	$^{A}149 \pm 73^{a}$	$^{A}147 \pm 36^{b}$
SENI	$^{C}120 \pm 23^{c}$	$^{B}157 \pm 43^{a}$	$^{A}202 \pm 35^{ab}$
LP	$^{A}124 \pm 35^{c}$	$^{A}122 \pm 50^{a}$	$^{A}161 \pm 15^{ab}$
COMB	$^{A}144 \pm 17^{abc}$	$^{A}121 \pm 40^{a}$	$^{A}161 \pm 10^{ab}$
	H9478 Harvested in 2002		
NP	$^{A}300 \pm 41^{b}$	$^{A}377 \pm 32^{ab}$	$^{A}318 \pm 61^{ab}$
RP	$^{A}423 \pm 30^{a}$	$^{B}268 \pm 58^{c}$	$^{B}244 \pm 11^{b}$
HYDRO	$^{A}417 \pm 60^{a}$	$^{A}428 \pm 28^{a}$	$^{A}360 \pm 27^{a}$
SENI	$^{A}462 \pm 52^{a}$	$^{B}322 \pm 60^{bc}$	$^{B}355 \pm 81^{a}$
LP	$^{A}475 \pm 97^{a}$	$^{AB}317 \pm 18^{bc}$	$^{B}291 \pm 35^{ab}$
HP	$^{A}489 \pm 42^{a}$	$^{B}292 \pm 29^{c}$	$^{B}335 \pm 26^{a}$
	H9997 Harvested in 2002		
NP	$^{A}75 \pm 5^{ab}$	$^{A}81 \pm 30^{a}$	$^{A}58 \pm 10^{a}$
RP	$^{A}53 \pm 9^{c}$	$^{A}58 \pm 8^{a}$	$^{A}46 \pm 8^{a}$
HYDRO	$^{A}77 \pm 5^{ab}$	$^{A}82 \pm 7^{a}$	$^{A}58 \pm 23^{a}$
SENI	$^{A}60 \pm 9^{bc}$	$^{A}76 \pm 26^{a}$	$^{A}52 \pm 7^{a}$
LP	$^{A}81 \pm 13^{a}$	$^{A}62 \pm 12^{a}$	$^{A}67 \pm 31^{a}$
HP	$^{A}73 \pm 13^{ab}$	$^{A}58 \pm 33^{a}$	$^{A}64 \pm 10^{a}$

[a] NP; no phosphorus, RP; regular phosphorus, LP; low phosphorus supplementation, HP; high phosphorus supplementation, HYDRO; hydrophos, SENI; Seniphos, COMB; combination phosphorus treatments (LP+Hydro+Seni). The values are mean ± standard deviation and different superscripts in the same column vertically (within treatments, a–c) or horizontally (within different stages, A–C) are significantly different at $p < 0.05$.

Protein Levels in Tomato Fruits. Protein levels were variable during the two seasons and the varieties. H 9478 fruits possessed more protein on a fresh weight basis than the H9997 fruits at all the developmental stages analyzed (**Table 1**). During 2001 season, protein levels of H 9478 fruits were considerably lower than the levels observed during the 2002 season. During the 2001 season, protein levels differed between different treatments in the mature green fruits of H 9478. The fruits from phosphorus-supplemented plots did not show a statistically significant difference in protein levels at the orange and red stages. Fruit protein levels from Seni and LP plots were significantly lower than fruits from RP plots at the mature green stage (**Table 1**). Fruits from seniphos treated plots showed a significant increase in protein levels during development (**Table 1**). During 2002 season, fruits from all the phosphorus-supplemented plots possessed similar levels of protein at the mature green stage when compared to the fruits from RP plots. In general, protein levels declined during the ripening process. During 2002 season, protein levels declined during the transition from mature green to orange and red in all phosphorus treatments except hydrophos treatment. Fruits from hydrophos, seniphos and HP treated plots maintained slightly elevated levels of protein at the red stage when compared to fruits from RP plots. Protein levels in H 9997 were much lower compared to the protein levels in 9478, and did not show appreciable changes in response to phosphorus supplementation or during development (**Table 1**).

Activity Staining of Antioxidant Enzymes. Activity staining for SOD activity revealed two major isozymes, the intensities of which appeared to undergo minor changes during development and in response to phosphorus fertilization (**Figure 1A**). The less intense, fast-moving isoform was distinguishable at

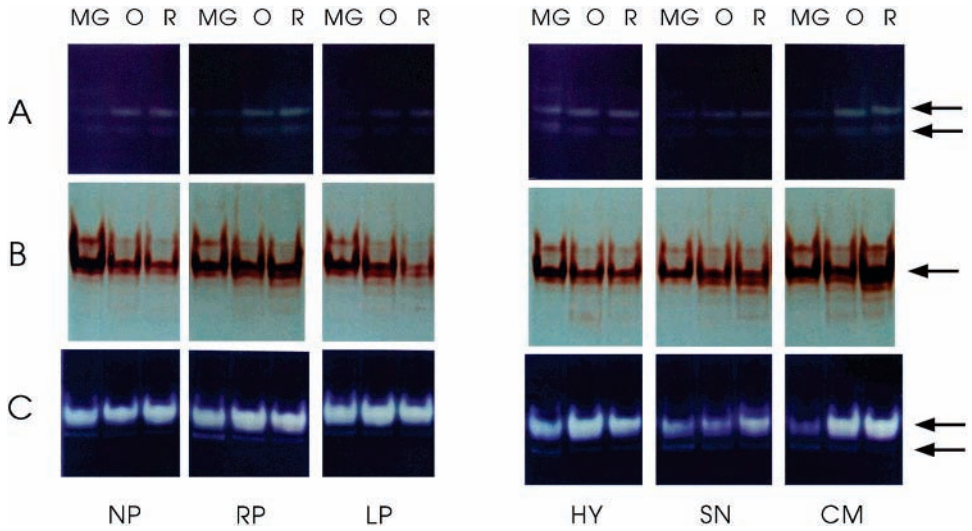

Figure 1. Native gels stained for the activity of SOD (A), POX (B), and APX (C). Proteins were isolated at the mature green (MG), orange (O), and red (R) stages from H 9478 tomato fruits collected from plots subjected to various phosphorus supplementation regimes during the 2001 season. The treatments shown include no phosphorus (NP), regular phosphorus (RP), low phosphorus supplementation (LP), hydrophos (HY), seniphos (SN) and combination treatments (CM). The arrows to the right indicate the isozymes.

all stages in all treatments. This isoform was slightly more intense in hydrophos treatment. The intensity of the slow-moving isoform increased during ripening. Hydrophos treatment appeared to increase the intensity of this isoform at all stages.

There were several peroxidase isozymes noticeable in tomato fruits, of which one isozyme showed much higher intensity (**Figure 1B**, arrow). In general, except for the combination treatment, the intensities of the peroxidase isozymes declined during a transition from mature green to orange to red. Phosphorus supplementation did not appear to alter the isozyme profiles and the intensities markedly, except in low phosphorus supplementation, hydrophos and seniphos treatments where the intensity of the major isozyme was considerably lower than the others.

There were two ascorbate peroxidase isozymes noticeable in the gel, with the fast moving isozyme showing considerably low intensity. The staining intensities remained more or less similar in response to phosphorus supplementation except in seniphos treatment, where the levels were noticeably low at all stages of development (**Figure 1C**).

Changes in SOD Activity. SOD activity was highly variable depending on the season and the treatment. During 2001 season, SOD activity estimated on a fresh weight basis (units/gfw) was more or less similar in all the treatments and at all three stages analyzed (**Table 2**). However, when analyzed on a specific activity basis, in all treatments except the RP treatment, SOD activity was maintained during development at mature green, orange and red stages. SOD activity was maintained at a higher level in fruits from hydrophos plots at the red stage. During 2002, SOD activity was nearly half of that observed during the 2001 season (**Table 3**). On a fresh weight basis, phosphorus supplementation did not enhance SOD activity at the mature green stage when compared to that of RP fruits (**Table 3**). No significant differences were observed at other stages in all treatments, and when expressed on a specific activity basis. SOD activities in H 9997 showed nearly similar patterns of change during development (**Table 4**). On a fresh weight basis, fruits obtained from hydrophos plots possessed significantly higher activity (100%) at the red stage as compared to its level at the mature green stage. There were no major differences in SOD activity with phosphorus supplementation or with stage of development in other treatments. When expressed on a specific activity basis, SOD activity was the highest at the red stage in fruits from plots subjected to regular phosphorus, hydrophos and low phosphorus supplementation (**Table 4**). Among all the

Table 2. Activities of SOD in H9478 Tomato Fruits Harvested in 2001 Season

	phosphorus application	developmental stage		
		mature green	orange	red
specific activity of SOD (unit/μg protein)	NP[a]	$^{A}0.35 \pm 0.17^{b}$	$^{A}0.25 \pm 0.04^{b}$	$^{A}0.37 \pm 0.14^{ab}$
	RP	$^{A}0.49 \pm 0.18^{ab}$	$^{AB}0.31 \pm 0.14^{ab}$	$^{B}0.23 \pm 0.01^{b}$
	HYDRO	$^{A}0.57 \pm 0.22^{ab}$	$^{A}0.44 \pm 0.22^{a}$	$^{A}0.47 \pm 0.14^{a}$
	SENI	$^{A}0.73 \pm 0.38^{a}$	$^{A}0.35 \pm 0.08^{ab}$	$^{A}0.27 \pm 0.03^{ab}$
	LP	$^{A}0.56 \pm 0.22^{ab}$	$^{A}0.41 \pm 0.07^{ab}$	$^{A}0.34 \pm 0.13^{ab}$
	COMB	$^{A}0.34 \pm 0.11^{b}$	$^{A}0.32 \pm 0.20^{ab}$	$^{A}0.31 \pm 0.23^{ab}$
total activity of SOD (unit/gfw)	NP	$^{A}63 \pm 25^{a}$	$^{A}40 \pm 8^{ab}$	$^{A}61 \pm 37^{a}$
	RP	$^{A}79 \pm 11^{a}$	$^{B}40 \pm 11^{ab}$	$^{B}53 \pm 4^{a}$
	HYDRO	$^{A}67 \pm 13^{a}$	$^{A}55 \pm 8^{a}$	$^{A}68 \pm 21^{a}$
	SENI	$^{A}87 \pm 43^{a}$	$^{A}52 \pm 3^{a}$	$^{A}54 \pm 6^{a}$
	LP	$^{A}67 \pm 25^{a}$	$^{A}47 \pm 10^{ab}$	$^{A}56 \pm 21^{a}$
	COMB	$^{A}47 \pm 15^{a}$	$^{A}35 \pm 19^{b}$	$^{A}47 \pm 32^{a}$

[a] The values are mean ± standard deviation and different superscripts in the same column vertically (within treatments, a–c) or horizontally (within different stages, A–C) are significantly different at $p < 0.05$.

Table 3. Activities of SOD in H9478 Tomato Fruits Harvested in 2002 Season

	phosphorus application	developmental stage		
		mature green	orange	red
specific activity of SOD (unit/μg protein)	NP[a]	$^{A}0.08 \pm 0.01^{a}$	$^{A}0.09 \pm 0.01^{a}$	$^{A}0.08 \pm 0.03^{a}$
	RP	$^{A}0.08 \pm 0.01^{a}$	$^{A}0.15 \pm 0.06^{a}$	$^{A}0.11 \pm 0.01^{a}$
	HYDRO	$^{B}0.07 \pm 0.03^{a}$	$^{A}0.10 \pm 0.02^{a}$	$^{AB}0.08 \pm 0.02^{a}$
	SENI	$^{B}0.08 \pm 0.01^{a}$	$^{A}0.13 \pm 0.02^{a}$	$^{AB}0.11 \pm 0.02^{a}$
	LP	$^{B}0.06 \pm 0.01^{a}$	$^{A}0.12 \pm 0.01^{a}$	$^{A}0.11 \pm 0.02^{a}$
	HP	$^{A}0.07 \pm 0.01^{a}$	$^{A}0.13 \pm 0.05^{a}$	$^{A}0.09 \pm 0.02^{a}$
total activity of SOD (unit/gfw)	NP	$^{A}24 \pm 2^{b}$	$^{A}35 \pm 7^{a}$	$^{A}26 \pm 5^{b}$
	RP	$^{AB}34 \pm 1^{ab}$	$^{A}37 \pm 7^{a}$	$^{B}26 \pm 2^{b}$
	HYDRO	$^{AB}31 \pm 12^{ab}$	$^{A}42 \pm 9^{a}$	$^{B}29 \pm 7^{ab}$
	SENI	$^{A}38 \pm 3^{a}$	$^{A}42 \pm 9^{a}$	$^{A}37 \pm 5^{a}$
	LP	$^{B}30 \pm 7^{ab}$	$^{A}37 \pm 3^{a}$	$^{B}31 \pm 3^{ab}$
	HP	$^{A}35 \pm 5^{a}$	$^{A}38 \pm 9^{a}$	$^{A}30 \pm 7^{ab}$

[a] The values are mean ± standard deviation and different superscripts in the same column vertically (within treatments, a–c) or horizontally (within different stages, A–C) are significantly different at $p < 0.05$.

Table 4. Activities of SOD in H9997 Tomato Fruits Harvested in 2002

	phosphorus application	developmental stage		
		mature green	orange	red
specific activity of SOD (unit/μg protein)	NP[a]	$^{A}0.42 \pm 0.11^{b}$	$^{A}0.36 \pm 0.14^{a}$	$^{A}0.45 \pm 0.02^{bc}$
	RP	$^{AB}0.60 \pm 0.24^{ab}$	$^{B}0.34 \pm 0.11^{a}$	$^{A}0.95 \pm 0.42^{ab}$
	HYDRO	$^{B}0.35 \pm 0.06^{b}$	$^{B}0.38 \pm 0.11^{a}$	$^{A}0.98 \pm 0.34^{a}$
	SENI	$^{A}0.88 \pm 0.58^{a}$	$^{A}0.46 \pm 0.25^{a}$	$^{A}0.62 \pm 0.30^{abc}$
	LP	$^{B}0.40 \pm 0.23^{b}$	$^{B}0.48 \pm 0.30^{a}$	$^{A}0.67 \pm 0.34^{abc}$
	HP	$^{A}0.37 \pm 0.28^{b}$	$^{A}0.32 \pm 0.08^{a}$	$^{A}0.37 \pm 0.04^{c}$
total activity of SOD (unit/gfw)	NP	$^{A}31 \pm 7^{a}$	$^{A}27 \pm 8^{a}$	$^{A}26 \pm 5^{bc}$
	RP	$^{A}33 \pm 17^{a}$	$^{A}19 \pm 4^{a}$	$^{A}43 \pm 14^{ab}$
	HYDRO	$^{B}27 \pm 6^{a}$	$^{AB}30 \pm 7^{a}$	$^{A}53 \pm 14^{a}$
	SENI	$^{A}52 \pm 33^{a}$	$^{A}31 \pm 7^{b}$	$^{A}31 \pm 10^{bc}$
	LP	$^{A}32 \pm 19^{a}$	$^{A}30 \pm 20^{a}$	$^{A}38 \pm 4^{abc}$
	HP	$^{A}26 \pm 16^{a}$	$^{A}17 \pm 7^{a}$	$^{A}23 \pm 6^{bc}$

[a] The values are mean ± standard deviation and different superscripts in the same column vertically (within treatments, a–c) or horizontally (within different stages, A–C) are significantly different at $p < 0.05$.

Table 5. Activities of POX in H9478 Tomato Fruits Harvested in 2001

	phosphorus application	developmental stage		
		mature green	orange	red
specific activity of POX (nmol guaiacol/min/μg protein)	NP[a]	$^{A}1.4 \pm 0.16^{b}$	$^{A}1.2 \pm 0.21^{b}$	$^{A}1.0 \pm 0.79^{a}$
	RP	$^{A}1.6 \pm 0.38^{b}$	$^{A}0.8 \pm 0.46^{b}$	$^{A}1.4 \pm 0.34^{a}$
	HYDRO	$^{A}2.2 \pm 0.34^{a}$	$^{B}1.1 \pm 0.73^{b}$	$^{AB}1.4 \pm 0.44^{a}$
	SENI	$^{A}2.2 \pm 0.24^{a}$	$^{B}1.4 \pm 0.50^{ab}$	$^{AB}1.8 \pm 0.28^{a}$
	LP	$^{A}2.3 \pm 0.44^{a}$	$^{A}2.2 \pm 0.57^{a}$	$^{A}1.9 \pm 0.63^{a}$
	COMB	$^{A}2.2 \pm 0.17^{a}$	$^{A}1.4 \pm 0.67^{ab}$	$^{A}1.4 \pm 0.44^{a}$
total activity of POX (nmol guaiacol/min/gfw)	NP	$^{A}256 \pm 33^{a}$	$^{AB}182 \pm 23^{ab}$	$^{B}122 \pm 49^{c}$
	RP	$^{A}265 \pm 11^{a}$	$^{B}93 \pm 20^{b}$	$^{A}314 \pm 89^{ab}$
	HYDRO	$^{A}302 \pm 15^{a}$	$^{A}136 \pm 41^{b}$	$^{A}196 \pm 17^{bc}$
	SENI	$^{AB}269 \pm 62^{a}$	$^{B}202 \pm 27^{ab}$	$^{A}354 \pm 51^{a}$
	LP	$^{A}286 \pm 108^{a}$	$^{A}267 \pm 100^{a}$	$^{A}308 \pm 129^{ab}$
	COMB	$^{A}313 \pm 21^{a}$	$^{B}169 \pm 96^{ab}$	$^{B}200 \pm 75^{bc}$

[a] The values are mean ± standard deviation and different superscripts in the same column vertically (within treatments, a–c) or horizontally (within different stages, A–C) are significantly different at $p < 0.05$.

treatments, hydrophos treatment provided a consistent increase in SOD activity during development.

Changes in POX Activity. As observed in the case of SOD activity, peroxidase activity was highly variable during development and did not show any consistent pattern of changes in response to phosphorus supplementation. When expressed on a fresh weight basis, peroxidase activities were significantly higher in red fruits from plants (H9478) subjected to regular phosphorus fertilization, low phosphorus supplementation and seniphos treatment when compared to the NP control fruits (**Table 5**). The activities were similar in all treatments at the mature green stage, which declined to varying degrees at the orange stage, before increasing to their highest levels at the red stage. On a specific activity basis, peroxidase activities did not show any significant increase in any treatments. During the 2002 season, mature green tomatoes (H9478) showed high levels of peroxidase activity as compared to that in 2001 season (**Table 6**). On a fresh weight basis, fruits from seniphos treatment showed significantly enhanced peroxidase activity at the mature green stage as compared to fruits of RP plots (**Table 6**). Peroxidase activities declined during ripening in all treatments except the NP treatment. When expressed on a specific activity basis, similar trends were observed. Specific activities of peroxidase were nearly similar in all treatments at the orange and red stages (**Table 6**), and significantly lower in fruits from hydrophos, seniphos and LP plots. No significant differences

Table 6. Activities of POX in H9478 Tomato Fruits Harvested in 2002

	phosphorus application	developmental stage		
		mature green	orange	red
specific activity of POX (nmol guaiacol/min/μg protein)	NP[a]	$^{A}0.6 \pm 0.09^{c}$	$^{A}0.6 \pm 0.13^{a}$	$^{A}0.5 \pm 0.02^{bc}$
	RP	$^{A}1.0 \pm 0.26^{b}$	$^{AB}0.6 \pm 0.04^{a}$	$^{B}0.6 \pm 0.03^{b}$
	HYDRO	$^{A}1.2 \pm 0.05^{a}$	$^{B}0.8 \pm 0.07^{a}$	$^{C}0.4 \pm 0.06^{cd}$
	SENI	$^{A}1.5 \pm 0.08^{a}$	$^{B}0.7 \pm 0.01^{a}$	$^{C}0.3 \pm 0.06^{d}$
	LP	$^{A}0.9 \pm 0.08^{b}$	$^{B}0.7 \pm 0.18^{a}$	$^{C}0.4 \pm 0.11^{d}$
	HP	$^{A}0.9 \pm 0.19^{b}$	$^{A}0.6 \pm 0.40^{a}$	$^{A}0.8 \pm 0.01^{a}$
total activity of POX (nmol guaiacol/min/gfw)	NP	$^{A}180 \pm 22^{c}$	$^{A}215 \pm 41^{b}$	$^{A}155 \pm 23^{b}$
	RP	$^{A}411 \pm 133^{b}$	$^{B}69 \pm 43^{b}$	$^{B}145 \pm 9^{bc}$
	HYDRO	$^{A}493 \pm 90^{ab}$	$^{B}337 \pm 10^{a}$	$^{C}159 \pm 18^{b}$
	SENI	$^{A}634 \pm 76^{a}$	$^{B}229 \pm 43^{b}$	$^{C}117 \pm 11^{cd}$
	LP	$^{A}436 \pm 108^{b}$	$^{B}222 \pm 44^{b}$	$^{C}103 \pm 22^{d}$
	HP	$^{A}450 \pm 106^{b}$	$^{B}180 \pm 127^{b}$	$^{AB}261 \pm 22^{a}$

[a] The values are mean ± standard deviation and different superscripts in the same column vertically (within treatments, a–c) or horizontally (within different stages, A–C) are significantly different at $p < 0.05$.

Table 7. Activities of POX in H9997 Tomato Fruits Harvested in 2002

	phosphorus application	developmental stage		
		mature green	orange	red
specific activity of POX (nmol guaiacol/min/μg protein)	NP[a]	$^{A}1.7 \pm 0.11^{a}$	$^{A}1.4 \pm 0.15^{a}$	$^{A}1.1 \pm 0.21^{a}$
	RP	$^{A}2.0 \pm 0.34^{a}$	$^{AB}1.5 \pm 0.64^{a}$	$^{B}0.8 \pm 0.18^{a}$
	HYDRO	$^{A}1.5 \pm 0.44^{a}$	$^{A}1.1 \pm 0.24^{a}$	$^{A}1.1 \pm 0.19^{a}$
	SENI	$^{A}1.7 \pm 0.06^{a}$	$^{A}0.9 \pm 0.26^{a}$	$^{A}1.3 \pm 1.13^{a}$
	LP	$^{A}1.6 \pm 0.43^{a}$	$^{A}1.0 \pm 0.37^{a}$	$^{A}1.3 \pm 0.15^{a}$
	HP	$^{A}1.8 \pm 0.76^{a}$	$^{A}1.2 \pm 0.52^{a}$	$^{A}0.8 \pm 0.13^{a}$
total activity of POX (nmol guaiacol/min/gfw)	NP	$^{A}124 \pm 6^{a}$	$^{AB}117 \pm 54^{a}$	$^{B}63 \pm 22^{a}$
	RP	$^{A}109 \pm 38^{a}$	$^{AB}84 \pm 32^{ab}$	$^{B}35 \pm 6^{a}$
	HYDRO	$^{A}112 \pm 26^{a}$	$^{A}88 \pm 26^{ab}$	$^{A}66 \pm 33^{a}$
	SENI	$^{A}100 \pm 14^{a}$	$^{A}65 \pm 15^{ab}$	$^{A}70 \pm 62^{a}$
	LP	$^{A}127 \pm 40^{a}$	$^{A}64 \pm 29^{ab}$	$^{A}81 \pm 31^{a}$
	HP	$^{A}123 \pm 39^{a}$	$^{B}56 \pm 6^{b}$	$^{B}52 \pm 10^{a}$

[a] The values are mean ± standard deviation and different superscripts in the same column vertically (within treatments, a–c) or horizontally (within different stages, A–C) are significantly different at $p < 0.05$.

Table 8. Activities of APX in H9478 Tomato Fruits Harvested in 2001

	phosphorus application	developmental stage		
		mature green	orange	red
specific activity of APX (nmol H_2O_2/10 min/mg protein)	NP[a]	$^{A}2.7 \pm 1.6^{ab}$	$^{A}6.4 \pm 0.31^{a}$	$^{A}5.2 \pm 2.8^{a}$
	RP	$^{A}1.2 \pm 0.2^{b}$	$^{A}4.9 \pm 6.0^{ab}$	$^{A}0.44 \pm 0.48^{b}$
	HYDRO	$^{A}5.2 \pm 4.7^{ab}$	$^{A}5.4 \pm 4.9^{ab}$	$^{A}8.2 \pm 4.8^{a}$
	SENI	$^{A}2.5 \pm 1.1^{b}$	$^{A}1.3 \pm 1.4^{ab}$	$^{A}0.5 \pm 0.14^{b}$
	LP	$^{A}8.1 \pm 2.7^{a}$	$^{A}4.9 \pm 4.2^{ab}$	$^{A}3.7 \pm 0.62^{a}$
	COMB	$^{A}2.8 \pm 1.2^{ab}$	$^{A}1.6 \pm 1.3^{b}$	$^{A}1.9 \pm 2.0^{b}$
total activity of APX (nmol H_2O_2/10 min/gfw)	NP	$^{A}0.5 \pm 0.30^{ab}$	$^{A}1.0 \pm 0.05^{a}$	$^{A}0.8 \pm 0.43^{ab}$
	RP	$^{A}0.2 \pm 0.03^{b}$	$^{A}0.7 \pm 0.87^{ab}$	$^{A}0.1 \pm 0.11^{b}$
	HYDRO	$^{A}0.7 \pm 0.64^{ab}$	$^{A}0.8 \pm 0.73^{ab}$	$^{A}1.2 \pm 0.70^{a}$
	SENI	$^{A}0.3 \pm 0.13^{b}$	$^{A}0.2 \pm 0.22^{b}$	$^{A}0.1 \pm 0.03^{b}$
	LP	$^{A}1.0 \pm 0.33^{a}$	$^{A}0.6 \pm 0.51^{ab}$	$^{A}0.6 \pm 0.10^{ab}$
	COMB	$^{A}0.4 \pm 0.18^{b}$	$^{A}0.2 \pm 0.16^{b}$	$^{A}0.3 \pm 0.33^{b}$

[a] The values are mean ± standard deviation and different superscripts in the same column vertically (within treatments, a–c) or horizontally (within different stages, A–C) are significantly different at $p < 0.05$.

were observed in peroxidase activities of H 9997 fruits from all treatments and at all stages except in RP treatment (**Table 7**).

Changes in APX Activity. Ascorbate peroxidase activity was relatively low during the 2001 season in all treatments (**Table 8**). Much higher levels of activity were observed during the 2002 season (**Table 9**). On a fresh weight basis, hydrophos and LP treatment showed enhanced APX activity at the mature green stage as compared to fruits from RP plots during 2001 season (**Table 8**). The activities remained similar during the transition

Table 9. Activities of APX in H9478 Tomato Fruits Harvested in 2002

	phosphorus application	developmental stage		
		mature green	orange	red
specific activity of APX (nmol H_2O_2/10 min/mg protein)	NP[a]	$^{A}40 \pm 9^{c}$	$^{A}45 \pm 4^{a}$	$^{A}31 \pm 8^{b}$
	RP	$^{A}42 \pm 8^{bc}$	$^{B}71 \pm 6^{ab}$	$^{B}74 \pm 14^{a}$
	HYDRO	$^{A}53 \pm 9^{a}$	$^{A}44 \pm 9^{ab}$	$^{A}33 \pm 7^{b}$
	SENI	$^{A}54 \pm 4^{ab}$	$^{A}68 \pm 12^{a}$	$^{A}65 \pm 19^{a}$
	LP	$^{A}42 \pm 4^{abc}$	$^{AB}50 \pm 5^{ab}$	$^{B}48 \pm 8^{b}$
	HP	$^{A}38 \pm 6^{abc}$	$^{A}44 \pm 11^{b}$	$^{A}39 \pm 14^{b}$
total activity of APX (μmol H_2O_2/10 min/gfw)	NP	$^{AB}12 \pm 2.8^{c}$	$^{A}17 \pm 1.8^{abc}$	$^{B}10 \pm 2.8^{b}$
	RP	$^{A}18 \pm 3.5^{b}$	$^{A}19 \pm 1.7^{abc}$	$^{A}18 \pm 3.4^{ab}$
	HYDRO	$^{A}22 \pm 4.0^{ab}$	$^{A}19 \pm 4.0^{ab}$	$^{B}12 \pm 2.7^{b}$
	SENI	$^{A}25 \pm 2.0^{a}$	$^{A}22 \pm 4.1^{a}$	$^{A}23 \pm 6.6^{a}$
	LP	$^{A}20 \pm 1.9^{ab}$	$^{AB}16 \pm 1.6^{bc}$	$^{B}14 \pm 2.5^{b}$
	HP	$^{A}19 \pm 3.1^{ab}$	$^{A}13 \pm 3.4^{c}$	$^{A}13 \pm 4.5^{b}$

[a] The values are mean ± standard deviation and different superscripts in the same column vertically (within treatments, a–c) or horizontally (within different stages, A–C) are significantly different at $p < 0.05$.

to orange and red stage. Similar trends were observed when the APX activities were expressed on a specific activity basis. Hydrophos and LP treatments provided the highest levels of APX activity in fruits. APX activities were much higher in all treatments and during development in 2002 season (**Table 9**). Fruits from RP and seniphos plots showed the highest level of activity, which was also maintained at the orange and red stages. The APX activity of H 9997 fruits did not show any changes with phosphorus treatments and the data are not shown.

DISCUSSION

Although several agronomic studies have evaluated the effects of phosphorus nutrition on yield parameters in crop plants, the effects of such enriched nutritional regimes on the quality of the produce are less well understood. In general, soil P is found in different pools, such as organic P (20−80% of total soil P), mineral P (available to plants by desorption and solubilization) and phosphate (Pi, available to plants <10 μM in soil solution) (*31*). The absorption and metabolism of phosphorus could be influenced by several environmental and physiological factors. Maintenance of an active antioxidant system is critical to the preservation of cellular structure because of its role in the detoxification of active oxygen species produced under stressful conditions (*1*−*3*). In rapidly senescing systems such as ripening fruits, maintenance of antioxidant enzyme activity can in turn provide enhanced shelf life and quality. In this study, the effect of P fertilizer supplementation on antioxidant enzyme activities during tomato fruit development was investigated in the field during two years. P was supplemented through the soil as superphosphate (0:20:0) or spray as a foliar formulation (hydrophos and seniphos). Protein levels were much higher in H 9478 than in H 9997. As well, phosphorus supplementation enhanced protein levels in red tomatoes under ideal conditions as in 2002 season when compared to the fruits from RP plots. Protein concentrations in tomato fruits may show considerable variations depending on the stage of development and the variety (*4*, *32*).

Maintenance of stable cytoplasmic Pi concentrations is essential for many enzyme reactions. During detoxification of AOS by antioxidant enzymes, $O_2^{\bullet -}$ is initially dismutated to H_2O_2 by SOD. Subsequently, H_2O_2 is decomposed to water by POX and APX. Glutathione (GSH, reduced form) is an efficient scavenger of AOS and GSH is regenerated by the action of glutathione reductase in a reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$)-dependent reaction (*2*, *3*). Consequently, soil or foliar P supplementation could affect antioxidant enzyme activities during development and under conditions of stress in fruits and vegetables. Thus, limited phosphorus availability may reduce antioxidant enzyme function in turn affecting their stress tolerance. For example, under limited supply of P, tomato plants are more susceptible to chilling (*33*). Increased activity of SOD and APX can enhance chilling-tolerance in alfalfa and maize (*3*, *17*). Furthermore, P fertilization during the normal growth conditions of cherry tomato increased the activities of SOD, POX, and APX (*23*). In apples, foliar P spray increased fruit P concentration and reduced superficial scald and breakdown (*24*, *25*). Antioxidant enzyme activities were affected to various degrees by phosphorus supplementation. SOD activity was nearly similar at all stages and in response to phosphorus supplementation in H 9478. An increase in activity was observed at the red stage in H9997 fruits from RP, hydro and LP plots. Peroxidase activities were quite variable depending on the season. In 2002 season, peroxidase activity was higher at the mature green stage of H 9478. There was a general decline in peroxidase activity during ripening. In cherry tomato, activities of SOD and POX increased at the orange stage and decreased at the red stage, whereas the APX activity increased at the red stage (*4*). As well, the activities of SOD, APX, CAT, and GR in salad tomato increased at turning orange stage until over ripening stage (*32*). In this study, most of the activities of SOD, POX, and APX appears to be influenced by the environmental factors that may affect the expression of the enzymes under field conditions. Thus, seasons can influence the relative composition of antioxidant enzymes in fruits that may in turn affect their quality (*34*). The results from the present study suggest that certain forms of P fertilizer supplementation may have a positive influence on the quality of tomato fruits by affecting antioxidant enzyme systems. During early stages of growth, this may be translated into increased stress protection. The changes in enzyme activities observed in this study did not appear to have any direct influence on the processing qualities of tomatoes (*30*). However, the activation of antioxidant enzymes may enhance the shelf life of tomatoes by better preserving the cellular organelles, membrane integrity and compartmentalization.

ABBREVIATIONS USED

AOS, active oxygen species; APX, ascorbate peroxidase; ASA, reduced ascorbate; SOD, superoxide dismutase; POX, guaiacol peroxidase; CAT, catalase; COMB, combination; DMSO, dimethyl sulfoxide; EDTA, ethylenediamine tetraacetate; GR, glutathione reductase; HP, high phosphorus; HYDRO, Hydrophos; LP, low phosphorus; M, mature green; NBT, nitro blue tetrazolium; NP, no phosphorus supplemented; ORG, orange; P, phosphorus; PMSF, phenylmethylsulfonyl fluoride; PVP, poly(vinylpyrrolidone); R, red; RP, regular phosphorus; SENI, Seniphos.

ACKNOWLEDGMENT

We are extremely grateful to Tom Bruulsema, Director, Potash and Phosphate Institute of Canada, Eastern Region, and Kevin Moran, Technical Director, Phosyn PLC, UK, for their active interest and continued support for this project. We would like to thank Valsala Kallidumbil and Ramany Paliyath for helping with the experiments.

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Received for review May 21, 2004. Revised manuscript received November 19, 2004. Accepted November 22, 2004. We gratefully acknowledge the financial assistance from The Potash and Phosphate Institute of Canada, Phosyn PLC, UK, H.J. Heinz Company of Canada Ltd., Agriculture and Agri-Food Canada and the Agricultural Adaptation Council.

JF040248Y