Plant Physiol. (1993) 103: 949–953

Oxidative Stress Affects α-Tocopherol Content in Soybean Embryonic Axes upon Imbibition and following Germination[1]

Marcela Simontacchi, Andres Caro, César G. Fraga, and Susana Puntarulo*

Physical Chemistry Division-Institute of Biochemistry and Biophysics, School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina

The content of α-tocopherol (αT) in isolated soybean (*Glycine max*, var Hood) embryonic axes was measured upon germination. Dry, high-vigor axes contained 1.2 ± 0.1, nmol/axis and after an increase during the initial 6 h of imbibition, there was a decline to 1.0 ± 0.1 nmol/axis at 24 h of incubation. Incubation in the presence of the redox-cycling agent paraquat (4 mM) for 24 h increased the αT content to 1.9 ± 0.2 nmol/axis. When the incubation medium was supplemented with 500 μM Fe-EDTA over 24 h, the content of αT increased to 1.8 ± 0.1 nmol/axis. Isolated axes from soybean seeds stored for 56 months contained 6.5 ± 0.3 nmol of αT/axis after 24 h of imbibition as compared to 1.0 ± 0.1 nmol of αT/axis in axes from soybean seeds stored for 8 months. In all of these experimental situations, oxidant production as assessed in vivo by a fluorometric assay was increased by 4 mM paraquat (8-fold), 500 μM iron (2-fold), and 56 months of storage (4-fold) after 24 h of imbibition. The data presented here suggest that the cellular content of αT is physiologically adjusted as a response to conditions of oxidative stress.

The set of intracellular or extracellular conditions that leads to an increase in the steady-state concentration of reactive oxygen species such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), singlet oxygen ($^1O_2$), lipid hydroperoxides, or related species is termed oxidative stress (Chance et al., 1979). Physiologically, metabolic activity in conjunction with the structural organization of the cell is able to minimize adverse effects of oxidative stress. Susceptibility to oxidative stress is a function of the overall balance between the factors that increase oxidant generation and those substances that exhibit antioxidant capability.

Tocopherols (with vitamin E activity) are critical antioxidants in eukaryotic organisms, ubiquitous in higher plants, and essential nutrients for animals (Tramontano et al., 1992). However, little is known about the endogenous content in plant tissues. αT (5,7,8-trimethyltocol) is the predominant tocopherol in vegetative tissue (Newton and Pennock, 1971; Hardy et al., 1991). Early studies in which two-dimensional chromatography was used showed that αT was predominant in young maize, wheat, barley, and peas and that other tocopherols appeared as the plant aged (Tramontano et al., 1992). It was reported that αT concentration increases with age in spruce seedlings (Franzen et al., 1991a, 1991b) and in leaves of soybean and pinto beans (Tramontano et al., 1992).

The content of flavonoids, which can also act as antioxidants (Fraga et al., 1987), increased in mesophyll cells of *Vicia faba* incubated in the presence of methyl viologen (paraquat) (Takahama, 1989) or H_2O_2 (Takahama et al., 1989) in the dark. It has been suggested that an increase in the steady-state concentrations of O_2^- and/or H_2O_2 in the cells is accompanied by increases in the level of flavonoids, presumably due to the induction of their synthesis (Takahama et al., 1989).

To study whether oxidative stress was able to affect antioxidant defenses, αT content was measured at the onset of germination of soybean embryonic axes. The effect of paraquat imbibition, increase in Fe concentration in the incubation medium, and aging were analyzed in terms of oxidative stress, assessed by a fluorescence technique (Bass et al., 1983; Szejda et al., 1984; Scott et al., 1988; LeBel et al., 1990) and αT content.

MATERIALS AND METHODS

Plant Material

Soybean seeds (*Glycine max* var Hood) were incubated for 2 to 48 h in the dark at 26°C over water-saturated filter paper. The composition of 1 L of the modified Steinberg nutrient solution (Tiffin, 1966) was: 51 mg of Ca, 6.6 mg of Mg, 59 mg of N, 3 mg of P, 44 mg of K, 5 mg of S, 0.13 mg of Mn, 0.07 mg of B, 0.04 mg of Zn, 0.01 mg of Cu, 0.01 mg of Mo. Germination (emergence of a radicle of 1 mm length) in intact seeds occurred at 20 to 24 h of incubation, and it was slowed to 30 h by aging. Fe was supplemented as an Fe-EDTA (1:1) complex, in a final concentration of 50 μM, unless otherwise indicated. Paraquat was added to the incubation medium to final concentrations of 1 to 8 mM. Aging was developed by the storage of the seeds in desiccators at room temperature. The buffers and water used to prepare all solutions were passed through columns containing Chelex 100 resin (Sigma Chemical Co.) to remove metal contaminants.

Homogenate Preparation

Isolated soybean embryonic axes were homogenized with a Potter-Elvejhem homogenizer in 60 mM phosphate buffer-

[1] Supported by the International Foundation for Science (Sweden), Fundación Antorchas, and the University of Buenos Aires. S.P. and C.G.F. are Career Investigators from Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina.

* Corresponding author; fax 54–1–962–5341.

Abbreviations: αT, α-tocopherol; DCFDA, 2′,7′-dichlorofluorescein diacetate.

100 mM KCl (pH 7.0) and then centrifuged at 750*g* for 10 min.

αT Content

The content of αT in the homogenates was quantified by reverse-phase HPLC with electrochemical detection using a Bioanalytical Systems LC-4C amperometric detector with a glassy carbon working electrode at an applied oxidation potential of 0.6 V (Desai, 1984). Extraction from the samples was performed with 1 mL of methanol and 4 mL of hexane. After the samples were centrifuged at 1500*g* for 10 min, the hexane phase was removed and evaporated to dryness under N_2. Samples were dissolved in methanol:ethanol (1:1) and injected for HPLC analysis. *d,l*-αT from synthetic phytol (Sigma) was used as the standard.

Reactive Oxygen Species Generation

After soybean seeds were incubated for 2 to 24 h, three embryonic axes were isolated and placed in 2 mL of 40 mM Tris-HCl buffer (pH 7.0) in the presence of 0.1 mM DCFDA (Molecular Probes Inc., Eugene, OR) at 37°C (Bass et al., 1983; Szejda et al., 1984; Scott et al., 1988; LeBel et al., 1990). Supernatants were removed after 30 min, and fluorescence was monitored in a Hitachi spectrofluorometer with an excitation at 488 nm and emission at 525 nm. Corrections for autofluorescence (<10%) were made by the inclusion in each experiment of parallel blanks (assay mixture without axes).

Statistical Analyses

Data in the text, figures, and tables are expressed as means ± SE. Statistical tests were carried out using Statview SE+, version 1.03 (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Content of αT in Soybean Embryonic Axes

The content of αT of isolated embryonic axes from high-vigor soybean seeds was increased after 4 h of incubation and then declined after 24 h of incubation (Fig. 1A). Nonsignificant differences in the profile were measured in the presence of 0 to 50 μM Fe in the imbibition medium. Supplementation with 500 μM Fe-EDTA for 2 to 6 h did not affect αT content in the soybean embryonic axes. However, after 24 h, axes incubated with 0 to 500 μM Fe-EDTA showed increases in their αT content that were significant over 100 μM Fe-EDTA (Fig. 1B).

Imbibition in the presence of paraquat affects the αT content of soybean axes. After 2 h of imbibition, exposure to 8 mM paraquat decreased αT content by 50%, but no effect was observed with 1 or 4 mM paraquat. After 24 h of incubation with 1 mM paraquat, αT content increased by 40% over the control values (Table I). A different kinetic pattern was observed in the presence of paraquat, as compared with that determined in its absence. During 2 to 24 h of imbibition there was a decrease of 37% in the αT content. When paraquat was added as a supplement, decay was prevented (1 mM paraquat) or the αT content of soybean embryonic axes even increased (4 and 8 mM paraquat) (Table I). Incubation for 24 h with paraquat increased αT content of the axes.

Dry, isolated embryonic axes from aged soybean seeds showed a linear correlation between αT content and the storage period ($r = 0.91$; $P < 0.01$) (Table II). As compared to high-vigor axes, the pattern of αT content upon imbibition was altered by storage. Axes from soybean stored for 44 and 56 months showed an increase in αT content of 53 and 49%, respectively, when incubated for 24 h. At all incubation times tested, αT content was higher in axes from aged seeds (Table II).

Generation of Reactive Oxygen Species by Soybean Embryonic Axes

The assay to assess in vivo generation of reactive oxygen species in isolated axes was based on DCFDA oxidation to a

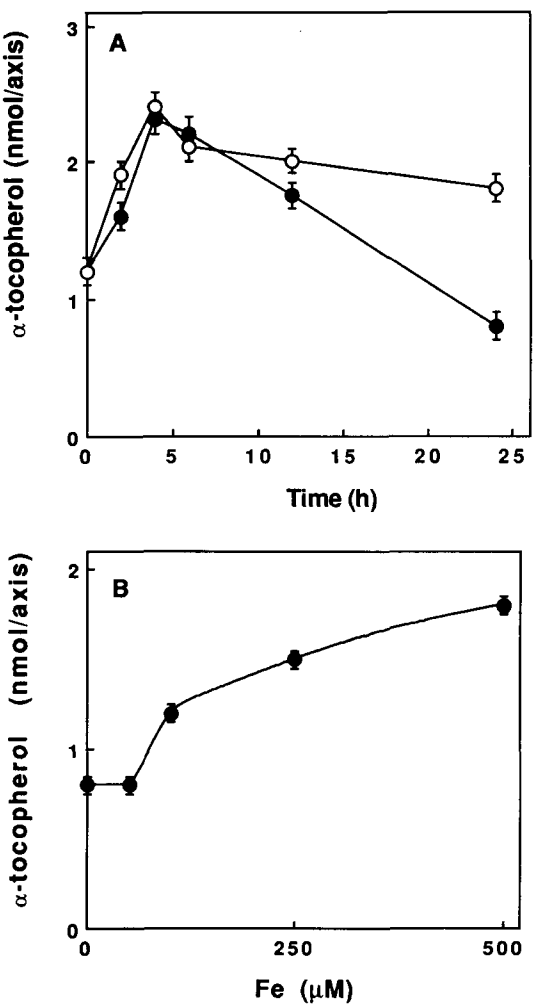

Figure 1. A, Effect of Fe supplemetation on αT content of soybean embryonic axes upon imbibition. Axes were excised after the indicated incubation periods. High-vigor soybean seeds were incubated as indicated in "Materials and Methods," in the presence of either 50 μM (●) or 500 μM (○) Fe-EDTA. B, αT content in embryonic axes isolated from high-vigor soybean seeds allowed to imbibe for 24 h in the presence of variable Fe-EDTA concentrations. Data are means ± SE.

Table I. *Paraquat effect on αT content of soybean embryonic axes during imbibition*

Paraquat studies were performed with axes from soybean seeds stored for 8 months. Significantly different ($P < 0.05$, one way analysis of variance) compared to: [a] values at 2 h of incubation for each paraquat concentration; [b] values in absence of paraquat for each time. Data are expressed as means ± SE.

Paraquat	αT	
	2 h	24 h
mM	nmol axis^{-1}	
0	1.6 ± 0.1	1.0 ± 0.1[a]
1	1.5 ± 0.1	1.4 ± 0.1
4	1.6 ± 0.1	1.9 ± 0.3[b]
8	0.8 ± 0.2[b]	1.6 ± 0.1[a]

fluorescent compound (Bass et al., 1983; Szejda et al., 1984; Scott et al., 1988; LeBel et al., 1990). The absence of fluorescence observed when the axes were incubated in the presence of DCFDA and then separated from the medium and homogenized indicates that DCFDA was not incorporated into the axes. Soybean embryonic axes generate oxidants capable of diffusing and/or being released into the medium and reacting with DCFDA. To differentiate reactive oxygen species from other substances able to react with DCFDA, at each imbibition time fluorescence was determined by adding DCFDA to the medium obtained after incubating the axes for 30 min. This fluorescence value was defined as the contribution of nonspecific substances to the total fluorescence and was subtracted from all readings to assess reactive oxygen speciesdependent fluorescence. Essentially the same profile of sensitivity to oxygen radical scavengers was observed with axes allowed to imbibe for 2 or 24 h (Table III). Fluorescence was inhibited by catalase, superoxide dismutase, and the ·OH scavenger DMSO, suggesting that H_2oxygen diffused from the axes and oxygen$^-$ and ·OH generated in the outer membranes could be responsible for DCFDA fluorescence. Fe addition to the reaction medium (Fe-EDTA or Fe-ADP) was more effective in promoting DCFDA oxidation with axes incubated for 2 h, as compared with axes incubated for 24 h. From 1 to 24 h of imbibition, there was a significant decrease in fluorescence that was probably due to an increase in the function of cellular membranes. At each imbibition time incubation with paraquat or

Table II. *Effect of aging on αT content in soybean embryonic axes during incubation*

Significantly different ($P < 0.05$, one way analysis of variance) compared to: [a] values in axes from seeds stored for 8 months for each incubation time; [b] values at 0 h incubation for each time of storage. Data are expressed as means ± SE.

Storage	αT		
	0 h	2 h	24 h
months	nmol axis^{-1}		
8	1.2 ± 0.1	1.6 ± 0.1	1.0 ± 0.1
32	2.7 ± 0.1[a]	2.5 ± 0.2[a]	2.2 ± 0.1[a]
44	3.6 ± 0.2[a]	5.1 ± 0.3[a,b]	5.5 ± 0.3[a,b]
56	4.4 ± 0.5[a]	5.7 ± 0.2[a]	6.5 ± 0.3[a,b]

Table III. *Effect of antioxidants and added Fe on the generation of reactive oxygen species in soybean embryonic axes*

Significantly different ($P < 0.05$, one way analysis of variance) compared to: [a] values at 2 h incubation; [b] values with no additions. Data are expressed as means ± SE.

	Relative Fluorescence	
	2 h	24 h
	units min^{-1} axis^{-1}	
No additions	3.0 ± 0.1	1.1 ± 0.2[a]
+50 units/mL of catalase	1.5 ± 0.1[b]	0.1 ± 0.1[a,b]
+300 units/mL of superoxide dismutase	2.2 ± 0.1[b]	1.2 ± 0.1[a]
+50 mM DMSO	1.4 ± 0.2[b]	0.3 ± 0.1[a,b]
+50 μM Fe-EDTA	5.4 ± 0.4[b]	0.8 ± 0.1[a,b]
+50 μM Fe-ADP	6.2 ± 0.2[b]	0.8 ± 0.1[a,b]

an excess of Fe increased fluorescence significantly (Table IV). Embryonic axes from aged seeds also showed increased fluorescence, depending on the storage period. Dry axes isolated from seeds stored for 8, 32, 44, and 56 months showed fluorescence values of 2.1 ± 0.3, 6.0 ± 0.7, 10 ± 1, and 5.0 ± 0.7 units min^{-1} axis^{-1}, respectively. After 24 h of imbibition, significant differences between the high- and low-vigor axes were observed (Table IV).

DISCUSSION

αT has been identified as a nonenzymic protector against peroxidation of lipids (Tappel, 1980; Kunert and Boger, 1984).

Table IV. *Generation of reactive oxygen species in soybean embryonic axes. Effect of Fe, aging, and paraquat*

Paraquat and Fe studies were performed with axes from soybean seeds stored for 8 months. Aging studies were on axes allowed to imbibe as described in "Materials and Methods," in the presence of 50 μM Fe-EDTA. Significantly different ($P < 0.05$, one way analysis of variance) compared to: [a] values in absence of paraquat; [b] values in absence of iron; [c] values in axes from soybean seeds stored for 8 months. Data are presented as means ± SE.

Treatment	Relative Fluorescence			
	1 h	2 h	4 h	24 h
	units min^{-1} axis^{-1}			
Paraquat (mM)				
0	2.8 ± 0.3	3.0 ± 0.1	2.7 ± 0.2	1.1 ± 0.1
1	3.5 ± 0.4	3.5 ± 0.3	17.1 ± 0.8[a]	6.6 ± 0.8[a]
4	8.6 ± 0.3[a]	9.0 ± 0.9[a]	20.2 ± 1.0[a]	10.2 ± 0.9[a]
8	6.2 ± 0.3[a]	18.6 ± 1.0[a]	28.6 ± 1.1[a]	10.8 ± 0.7[a]
Fe (μM)				
0	1.6 ± 0.1	0.9 ± 0.2	3.2 ± 0.1	1.3 ± 0.1
50	2.8 ± 0.3[b]	3.0 ± 0.1[b]	2.7 ± 0.2	1.1 ± 0.1
100	7.1 ± 0.3[b]	6.0 ± 0.7[b]	3.9 ± 0.3	2.9 ± 0.2[b]
500	7.0 ± 0.4[b]	4.6 ± 0.5[b]	4.4 ± 0.3	2.4 ± 0.4[b]
Storage (months)				
8	2.8 ± 0.3	3.0 ± 0.1	2.7 ± 0.2	1.1 ± 0.1
32	9.8 ± 0.9[c]	8.3 ± 0.4[c]	10.0 ± 0.9[c]	2.8 ± 0.4[c]
44	4.4 ± 0.8[c]	7.6 ± 0.5[c]	8.3 ± 0.9[c]	4.1 ± 0.1[c]
56	4.5 ± 0.5[c]	4.8 ± 0.6[c]	5.3 ± 0.5[c]	5.0 ± 0.4[c]

In plants, vitamin E is able to protect Chl damage in vivo (Kunert and Boger, 1984) and even slow senescence (Leshem et al., 1979; Dhindsa et al., 1982), although this latter effect could not be confirmed (Kar and Feierabend, 1984). In the present study we observed different effects on the αT content of soybean embryonic axes at the onset of germination, depending on the oxidative stress. The decrease observed in the content of αT in isolated axes at the early stages of germination could reflect the consumption of the antioxidant due to an increase in active oxygen species production as imbibition progressed. It was previously reported that, during the initial steps of germination, isolated soybean embryonic axes showed a significant increase in chemiluminescence consistent with increased lipoxygenase activity and $^1O_2$ generation (Boveris et al., 1984) and enhanced oxygen$^-$ production (Puntarulo et al., 1991). Vitamin E is one of the best quenchers for $^1O_2$, with a quenching rate constant of approximately 6×10^8 (in methanol) (Foote et al., 1974), and it also appears to react with oxygen$^-$ to give a phenoxyl radical (Larson, 1988). Because in dicotyledonous plants the tocol precursors of αT appear during germination (Takahama et al., 1989), it seems that at the onset of germination the stored αT is consumed by the oxidants generated as soon as imbibition starts.

The connection between oxidative damage and senescence, on one hand, and antioxidant defense mechanisms, on the other, has been postulated for both animals and plants. In this context, αT content could be considered a potential indicator of oxidative stress in plants. Increases in αT content with aging have been reported in potato tubers (Spychalla and Desborough, 1990), spruce needles (Franzen et al., 1991b), and leaves and needles of beech and fir (Kunert and Ederer, 1985), among others. The data presented here indicate that the αT content of isolated soybean embryonic axes increased in dried axes not only as storage progressed (3-fold increase after 48 months) but upon imbibition as well. During the initial 24 h of incubation the decline in αT content in high-vigor axes was not detected in axes from aged soybeans. Moreover, a significant increase in αT was measured compared to its content in dry, aged axes.

This pattern of response was reproduced upon germination when other conditions of oxidative stress were given. The bipyridyl herbicide paraquat is considered to be responsible for increasing the rate of generation of oxygen$^-$ and H_2O_2 in plant tissues (Halliwell, 1982), and Fe is a catalyst for many reactions involving free radicals. The presence of both agents during the initial steps of imbibition led to an increase in the αT content of the axes.

The use of DCFDA provides experimental evidence for the presence of oxidative stress conditions in the axes upon imbibition with paraquat, Fe, or aging. At all imbibition times and conditions tested, oxidative stress development paralleled the increase in αT content. Relative fluorescence of embryonic axes was similar after paraquat and Fe treatments at 1 h of incubation; however, it was vastly different at 2 to 24 h. This profile of response could reflect the different characteristics of each oxidative situation.

Our data suggest that enhanced oxidant production is limited by the content of αT. In spite of the fact that the membrane composition determines the maximal amount of αT that can be incorporated, the content of antioxidants appears to be also regulated by oxidative stress. The efficiency of the natural response is limited by the extent of the stress; thus, under conditions of severe oxidation, antioxidant defenses could be overwhelmed and the plant fails to become established. Consequently, supplementation with appropriate antioxidants could afford a novel strategy for improving seed germination and plant growth.

Received May 3, 1993; accepted July 10, 1993.

LITERATURE CITED

Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M (1983) Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J Immunol **130:** 1910–1917

Boveris A, Puntarulo S, Roy A, Sanchez R (1984) Spontaneous chemiluminescence of soybean embryonic axes during imbibition. Plant Physiol **76:** 447–451

Chance B, Sies H, Boveris A (1979) Hydroperoxide metabolism in mammalian organs. Physiol Rev **59:** 527–605

Desai I (1984) Vitamin E analysis methods for animal tissues. Methods Enzymol **105:** 138–146

Dhindsa RS, Plumb-Dhindsa PL, Reid DM (1982) Leaf senescence and lipid peroxidation: effects of some phytohormones, and scavengers of free radicals and singlet oxygen. Physiol Plant **56:** 453–457

Foote CS, Ching TY, Geller GG (1974) Chemistry of singlet oxygen-18. Rate of reactions and quenching of α-tocopherol and singlet oxygen. Photochem Photobiol **20:** 511–513

Fraga CG, Martino VS, Ferraro GE, Coussio JD, Boveris A (1987) Flavonoids as antioxidants evaluated by *in vitro* and *in situ* liver chemiluminescence. Biochem Pharmacol **36:** 717–720

Franzen J, Bausch J, Glatzle D, Wagner E (1991a) Distribution of vitamin E in spruce seedlings and mature tree organs, and within the genus. Phytochemistry **30:** 147–151

Franzen J, Glatzle D, Bausch J, Wagner E (1991b) Age-dependent accumulation of vitamin E in spruce needles. Phytochemistry **30:** 2147–2150

Halliwell B (1982) The toxic effect of oxygen on plant tissues. *In* LW Oberley, ed, Superoxide Dismutase, Vol 1. CRC Press, Boca Raton, FL, pp 89–123

Hardy DJ, Violana Gallegos MA, Gaunt JK (1991) Metabolism of tocopherol by *Pisum sativum*. Phytochemistry **30:** 1099–1105

Kar M, Feierabend J (1984) Metabolism of activated oxygen in detached wheat and rye leaves and its relevance to the initiation of senescence. Planta **160:** 385–391

Kunert KJ, Boger P (1984) The diphenyl ether herbicide oxyfluorfen: action of antioxidants. J Agric Food Chem **32:** 725–728

Kunert KJ, Ederer M (1985) Leaf aging and lipid peroxidation: the role of the antioxidants vitamin C and E. Physiol Plant **65:** 85–88

Larson RA (1988) The antioxidants of higher plants. Phytochemistry **27:** 969–978

LeBel CP, Ali SF, McKee M, Bondy SC (1990) Organometal-induced increases in oxygen reactive species: the potential of 2′,7′-dichlorofluorescein diacetate as an index of neurotoxic damage. Toxicol Appl Pharmacol **104:** 17–24

Leshem YY, Grossman S, Frimer A, Ziv Y (1979) Endogenous lipoxygenase control and associated radical scavenging as mode of cytokinin action in plant senescence. *In* LA Appelquist, C Liljenberg, eds, Advances in Biochemistry and Physiology of Plant Lipids, Vol 3. Elsevier/North Holland Press, Amsterdam, pp 193–198

Newton RP, Pennock JF (1971) The intracellular distribution of tocopherols in plants. Phytochemistry **10:** 2323–2328

Puntarulo S, Galleano M, Sanchez RA, Boveris A (1991) Superoxide anion and hydrogen peroxide metabolism in soybean embryonic axes during germination. Biochim Biophys Acta **1074:** 277–283

Scott JA, Homcy CJ, Khaw B, Rabito CA (1988) Quantitation of intracellular oxidation in a renal epithelial cell line. Free Radical Biol Med **4:** 79–83

Spychalla JP, Desborough SL (1990) Superoxide dismutase, catalase, and α-tocopherol content of stored potato tubers. Plant Physiol **94:** 1214–1218

Szejda P, Parce JW, Seeds MS, Bass DA (1984) Flow cytometric quantitation of oxidative product formation by polymorphonuclear leukocytes during phagocytosis. J Immunol **133:** 3303–3307

Takahama U (1989) A role of hydrogen peroxide in the metabolism of phenolics in mesophyll cells of *Vicia faba* L. Plant Cell Physiol **30:** 295–301

Takahama U, Egashira T, Wakamatsu K (1989) Hydrogen peroxide-dependent synthesis of flavonols in mesophyll cells of *Vicia faba* L. Plant Cell Physiol **30:** 951–955

Tappel A (1980) Measurement of and protection from *in vivo* lipid peroxidation. *In* WA Pryor, ed, Free Radicals in Biology, Vol 4. Academic Press, New York, pp 1–47

Tiffin LO (1966) Iron translocation I. Plant culture, exudate sampling, iron uptake analysis. Plant Physiol **41:** 510–514

Tramontano WA, Ganci D, Pennino M, Dierenfeld ES (1992) Age dependent α-tocopherol concentrations in leaves of soybean and pinto beans. Phytochemistry **31:** 3349–3351