Differences in Chlorophyll Loss at 13 °C for Two Broccoli (*Brassica oleracea* L.) Cultivars Associated with Antioxidant Enzyme Activities†

P. M. A. Toivonen*,† and Mark Sweeney‡

Agriculture and Agri-Food Canada, Pacific Agri-Food Research Centre, Summerland, British Columbia V0H 1Z0, Canada, and British Columbia Ministry of Agriculture, Fisheries and Food, Abbotsford, British Columbia V3G 2M3, Canada

Two broccoli cultivars which have been previously found to have large differences in yellowing susceptibility were studied to determine if there was a relationship between antioxidant capability and chlorophyll loss at a simulated retail display temperature. Greenbelt cultivar retained a stable chlorophyll content over 4 days at 13 °C, while Emperor cultivar showed a constant decline in chlorophyll content. These differences were not related to differences in water loss. Superoxide dismutase (SOD) and peroxidase (POD) activities were approximately 30% higher in Greenbelt than in Emperor. The ratio of superoxide dismutase to peroxidase activity was also lower in Greenbelt. The susceptibility of Emperor to oxygen radical damage and lipid peroxidation is expected to be higher since an excessive SOD activity, with respect to POD activity, can potentially lead to enhanced hydroxyl radical formation. Catalase (CAT) activity was higher in Emperor, suggesting that catalase is not important in providing resistance to chlorophyll loss in broccoli. Water soluble, nonenzymatic antioxidant activity was similar for both cultivars. These results support the hypothesis that antioxidant protection offered by superoxide dismutase and peroxidase is important to retention of green color in broccoli.

Keywords: *Senescence; superoxide dismutase; peroxidase; catalase; antioxidant activity*

INTRODUCTION

Significant differences in shelf-life potential with the two broccoli cultivars Emperor and Greenbelt have been consistently seen over several years of study (Toivonen, 1995). Greenbelt has had a superior shelf-life potential when compared to that of Emperor. Emperor is more susceptible to both yellowing and wilting. This difference is most dramatic at elevated temperatures such as those that might be experienced at the retail and consumer level. Differences in storage-life potential in low-temperature storage for certain broccoli cultivars has also be demonstrated (Morris, 1990). One of the superior cultivars in the storage study was also found to be Greenbelt.

The large difference in yellowing potential for Emperor and Greenbelt was considered a useful tool to investigate the physiological basis of senescence in broccoli. The importance of both endogenous ethylene emission and respiratory rate of cultivars has been studied (King and Morris, 1994). However, only basal ethylene emission was associated with the time to onset of yellowing (i.e. shelf-life). In another laboratory, yellowing in broccoli buds has been associated with lipid peroxidation (Zhuang et al., 1995). However, the increase in lipid peroxidation associated with yellowing is not linked to increased lipoxygenase activity (Zhuang et al., 1994). Senescence has been correlated with lipid peroxidation in other plants (Dhindsa et al., 1981; Lesham, 1988). Ethylene evolution has also been associated with the lipid oxidation reactions (Frenkel and Eskin, 1977; Lesham, 1988). The activity of antioxidant systems to quench various oxygen radicals is considered to be inversely proportional to lipid peroxidation levels, ethylene synthesis, and senescence (Dhindsa et al., 1981; Lesham, 1988).

Other workers have investigated the association of enzymes associated with oxidation and chlorophyll degradation (Baardseth and von Elbe, 1989; Barth et al., 1992, 1993). The mechanisms to prevent oxidation are associated with both antioxidant enzymes and endogenous antioxidants (Salin, 1987; Lesham, 1988). Both of these components are believed to be important in preventing oxidative injury through their abilities to scavenge free radicals before they can cause cellular damage. Superoxide dismutase, peroxidase, and catalase are considered to be important to the oxy-radical detoxification process in plant tissues (Salin, 1987). The focus of this study was to investigate activities of superoxide dismutase, peroxidase, catalase and endogenous nonenzymatic antioxidant levels in these two cultivars exhibiting large differences in yellowing potential.

MATERIALS AND METHODS

Plant Material. Broccoli cultivars were grown in plots located in commercial fields in the Abbotsford area of the Fraser Valley, British Columbia. Harvest maturity for the broccoli was defined as heads which were 3−6 in. in diameter, being firm and compact in texture and not showing any yellowing or flower opening (USDA, 1943). When the broccoli

* Author to whom correspondence should be addressed [telephone (250) 494-6386; fax (250) 494-0755; e-mail TOIVONENP@EM.AGR.CA].

† Agriculture and Agri-Food Canada, PARC.

‡ BCMAFF.

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was rated as mature, it was hand harvested, trimmed, and taken to the Agassiz site of the Pacific Agri-Food Research Centre (PARC). Within 2 h of harvest, the heads were hydrocooled in a pilot-scale shower-type hydrocooler. The core temperature after hydrocooling was 5 °C. The broccoli was then placed into styrofoam coolers, with ice packs, and transported to the Summerland site of PARC. Immediately on arrival the product was placed into a 1 °C cooler for 2 days. The broccoli was then removed from the 1 °C room and placed into a 13 °C at 95% relative humidity room for 4 days to simulate response at a retail display temperature.

Color and Weight Loss Evaluation. Broccoli color was evaluated on a daily basis at 13 °C using a previously described visual scale for color (Gillies and Toivonen, 1995). Samples were weighed daily and specific weight loss calculated as percent loss per day per unit vapor pressure deficit (Lownds et al., 1993). Vapor pressure deficit was estimated as reported previously (Gillies and Toivonen, 1995).

Sampling. An entire head per replicate was sampled by excising the flower bud tissue (Zhuang et al., 1995). Five replicates of each cultivar were taken on days 0, 2, and 4 at 13 °C. These samples were quick frozen at −40 °C and held for subsequent enzyme and antioxidant assays. Samples were analyzed within 4 months.

Chlorophyll Determination. Chlorophyll measurements were made on the tissue from days 0, 2, and 4. The chlorophyll was extracted in 85% acetone. Total chlorophyll was determined using the methods reported previously (AOAC, 1990). Absorbance (A) was read at 660 and 642.5 nm with a Beckman DU640-B UV/vis spectrophotometer (Beckman Instruments (Canada), Inc., Mississauga, ON). Total chlorophyll was estimated with the following equation (AOAC, 1990)

$$\text{total chlorophyll (mg/mL)} = 7.12A_{660} + 16.8A_{642.5}$$

Superoxide Dismutase Activity. Superoxide dismutase (EC 1.15.1.1) activity was measured on crude extract using the method of Dhindsa et al. (1981). Two grams of frozen tissue was homogenized, on ice, in 20 mL of 50 mM phosphate buffer (pH 7) with 1% (w/v) insoluble poly(vinylpyrolidone) (Sigma Chemical Co., St. Louis, MO) and 0.1 mM EDTA (Sigma). The homogenate was then placed into a refrigerated centrifuge set at 5 °C and centrifuged at 15000g for 10 min and the supernatant filtered through a Whatman no. 4 filter. The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine (Sigma), 75 μM nitroblue tetrazolium (Sigma), and 0.1 mM EDTA. To 2.7 mL of this reaction mixture was added an extract mixture of 250 μL. In this extract mixture, crude extract (described above) volumes ranged from 0 to 250 μL, with water being added to make up the total volume to 250 μL. Riboflavin (Sigma), 100 μL of a 12 μM aqueous solution, was added last, and the tubes were inverted to mix the contents. The tubes were then placed into a light box, illuminated with 2−20 W cool white fluorescent bulbs, for 7 min. Samples were read at 560 nM with a Beckman DU640-B spectrophotometer. The inhibition curves were linearized (Beyer and Fridovich, 1987), and the extract concentration corresponding to 50% inhibition of the oxidation was defined as 1 unit of SOD activity. The results were reported on a per milligram of protein basis.

Peroxidase and Catalase Activities. Samples for peroxidase and catalase were processed as follows. Two grams of frozen tissue was homogenized, on ice, in 50 mM phosphate buffer (pH 6.6), containing 1% insoluble poly(vinylpyrolidone). The homogenate was placed into a refrigerated centrifuge set at 5 °C and centrifuged at 15000g for 10 min. The supernatant was then filtered through no. 4 Whatman paper. This filtrate was the crude extract.

Peroxidase (EC 1.11.1.7) activity determination was done according to the method of Hemeda and Klein (1990). For peroxidase determination, 75 μL of the crude extract was added to a 3 mL substrate mixture. The substrate mixture contained 10 mL of 1% guaiacol (ICN), 10 mL of 0.3% H_2O_2 (J. T. Baker, Phillipsburg, NJ), and 100 mL of 50 mM phosphate buffer (pH 6.6). The formation of the oxidized tetraguaiacol polymer was monitored at 470 nm over 3 min. The linear portion of the curve was used for calculating activity rates. The peroxidase activity was calculated using the extinction coefficient of 2.66×10^4 M^{-1} cm^{-1} at 470 nm and a stoichiometry of 4 (Whitaker, 1994). One unit of activity was defined as the calculated consumption of 1 μmol of H_2O_2/min. The level of peroxidase activity is reported on a per milligram of protein basis.

Catalase (EC 1.11.1.6) activity determination was done according to the method of Aebi (1983). For catalase activity determination, 150 μL of crude extract was added to 3 mL of substrate mixture. The substrate mixture contained 85 μL of 30% H_2O_2 in 20 mL of 50 mM phosphate buffer (pH 7.0). The decomposition of H_2O_2 was followed as a decline in absorbance at 240 nm for 5 min with a Beckman DU640-B spectrophotometer. The activity of catalase in the broccoli extracts was calculated using the activity of catalase from *Aspergillus niger* (Sigma, $\sigma = 6600$ units/mg of protein) as a standard. One unit of activity was defined as the calculated consumption of 1 μmol of H_2O_2/min. The level of catalase activity is reported on a per milligram of protein basis.

Protein Determination. Protein content of samples was determined after Bradford (1976) using 100 μL aliquots of crude extracts per 5 mL of reagent.

Water Soluble Antioxidant Content. The method of Al-Saikhan et al. (1995) was used for extraction. Frozen flower bud tissue (2.5 g) was homogenized in 10 mL of distilled water and the resultant homogenate centrifuged at 5000g for 20 min. The supernatant was then filtered through Whatman no. 4 filter paper. The filtrate was put into a capped tube and placed into a boiling water bath for 5 min to completely inactivate enzymes. The boiled extract was then centrifuged at 5000g for 15 min and filtered through Whatman no. 4 filter paper. The resultant clear extract was assayed for antioxidant activity using the method of McKersie et al. (1982). The extract (100 μL) was put into a tube containing a mixture of 0.2 mL of 0.2 M linoleic acid (Sigma) in 95% ethanol, 15% Tween 20 (Sigma) and 3.0 mL of 0.02 M phosphate buffer (pH 5.6). To initiate the oxidation, 1.5 mL of freshly prepared 0.5 mM ferrous-EDTA (Rhee, 1978) was added, and the tube was inverted several times. A 0.5 mL sample was immediately taken, and 2.0 mL of 0.1 N NaOH (in 10% ethanol) was added to clear the solution. The remainder of the reaction solution was incubated for 60 min at 37 °C in a temperature-controlled water bath. An aliquot was taken at 60 min and cleared with the 0.1 N NaOH solution, as above. The cleared sample was read at 232 nm with a Beckman DU649-B spectrophotometer. A control consisted of the above reaction mixture, using 100 μL of water in place of the sample extract. Antioxidant activity was calculated as percent inhibition relative to oxidation levels in the control (McKersie et al., 1982)

$$[(\Delta OD_{control} - \Delta OD_{extract})/\Delta OD_{control}] \times 100$$

Statistical Analysis. The entire experiment was conducted two times within a period of 3 months, and there were five replicates per experiment. Results were similar in both experiments; however only the data from the first experiment are reported here. Analysis of variance was performed over time at 13 °C, using the General Linear Model (GLM) Procedure on the SAS (Cary, NC) statistical software package. Significance was determined at the 0.05 level by least-squares difference (LSD). Data presented in graphs show calculated means and standard errors of the means.

RESULTS

Color, Weight Loss Evaluation, and Chlorophyll. The initial color of Emperor broccoli was visually rated darker as compared to Greenbelt (Table 1). Decline in green color proceeded twice as fast in Emperor as in Greenbelt. The rate of weight loss was similar for both cultivars. The chlorophyll content at day 0 was higher

Table 1. Water Loss, Initial Color, and Color Changes in Two Broccoli Cultivars Held at 13 °C and 95% Relative Humidity for 4 Days

cultivar	specific water loss (%/day per kPa vpd)	initial color rating (1−5 scale)[a]	color rating at 4 days (1−5 scale)[a]	change in color rating (units/day)
Greenbelt	11.3	4.3	3.8	−0.15
Emperor	10.8	4.8	3.3	−0.37
LSD[b]	1.3	0.4	0.5	0.08

[a] Visual color rating scale, where 1 = yellow, and 5 = dark green (Gillies and Toivonen, 1995). [b] Least significant difference at the $P \leq 0.05$ level, $n = 5$.

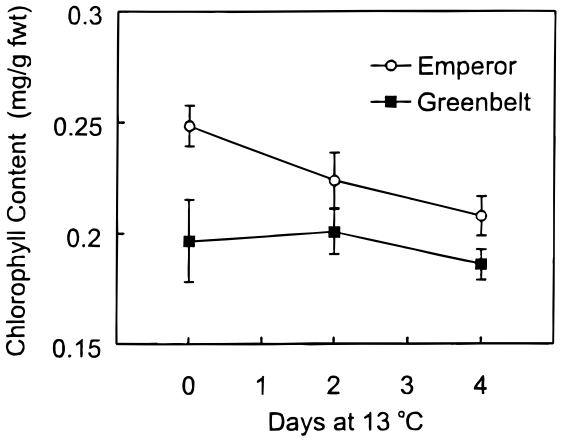

Figure 1. Chlorophyll loss in flower bud tissue of two broccoli cultivars during 4 days at 13 °C, 95% RH (means ± SEM, n = 5).

for Emperor (Figure 1), which parallels the visual color ratings (Table 1). However, the visual color rating on day 4 was lower for Emperor than for Greenbelt. The decline in total chlorophyll was much greater for Emperor than Greenbelt (Figure 1), parallelling the decline in color ratings for these two cultivars (Table 1). The chlorophyll content of Greenbelt was relatively stable over the 4 day period of the experiment. Barth et al. (1992) showed that color loss in broccoli was related to water loss and if water loss was controlled by misting, yellowing could be reduced. Despite the fact that water loss accompanied chlorophyll loss in the current study, there were no differences in the rates of water loss for the two cultivars, indicating that other endogenous factors can determine the magnitude of impact which water loss has on chlorophyll loss.

Superoxide Dismutase Activity. SOD activity was found to be initially ~30% higher in Greenbelt broccoli (Figure 2). The SOD activity increased for both cultivars, and the differences in activity between the two cultivars decreased somewhat with time. The increase in SOD activity is not unexpected, since Dhindsa et al. (1981) found that in the initial stages of senescence in leaves, SOD activity increased on a per milligram of protein basis. Spychalla and Desborough (1990) have shown activity to increase in storage for potatoes.

Peroxidase and Catalase Activities. The POD activity in Greenbelt was 30% greater than in Emperor (Figure 3). As with SOD, the POD activity increased with time at 13 °C. The increase in POD for Greenbelt was linear with time, while changes POD activity for Emperor appeared to level off at 2 days. Catalase activity was found to be higher in Emperor (Figure 4). This pattern is the reverse of that seen for SOD and POD. CAT activity also increased over time as did SOD and POD activities. This increase in enzyme activities

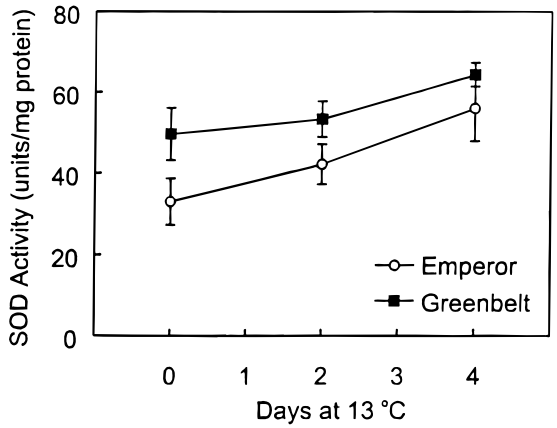

Figure 2. Superoxide dismutase activity in crude extracts from flower bud tissue of two broccoli cultivars during 4 days at 13 °C, 95% RH (means ± SEM, n = 5).

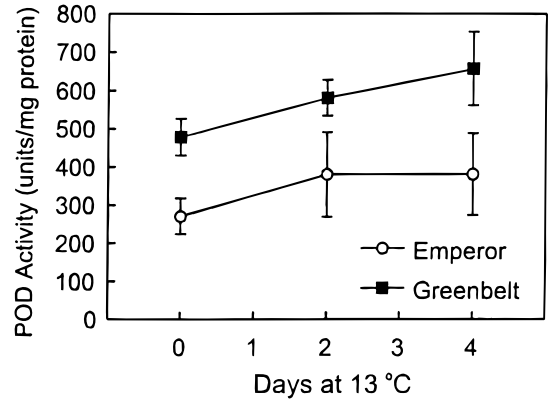

Figure 3. Peroxidase activity in crude extracts from flower bud tissue of two broccoli cultivars during 4 days at 13 °C, 95% RH (means ± SEM, n = 5).

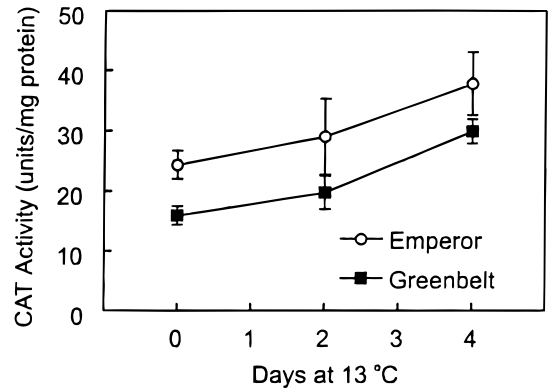

Figure 4. Catalase activity in crude extracts from flower bud tissue of two broccoli cultivars during 4 days at 13 °C, 95% RH (means ± SEM, n = 5).

involved with oxidation reactions seems to be universal, and Spychalla and Desborough (1990) have reported this phenomena for SOD and CAT.

Water Soluble Antioxidant Content. The antioxidant content in both cultivars were similar (Figure 5), and while there is some rise in activity, there was no significant increase over the 4 days at 13 °C. The antioxidant activity was relatively high (>80%) as measured here. High values for antioxidant activity in broccoli have been reported previously, and possibly this may be due to soluble phenolic compounds (Oszmianski and Lee, 1990; Al-Saikhan et al., 1995).

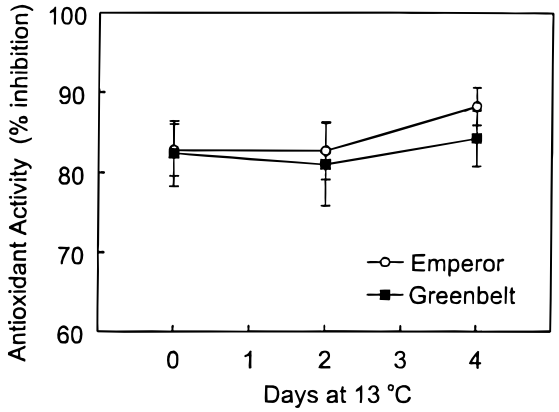

Figure 5. Water soluble antioxidant activity in crude extracts from flower bud tissue of two broccoli cultivars during 4 days at 13 °C, 95% RH (means ± SEM, $n = 5$).

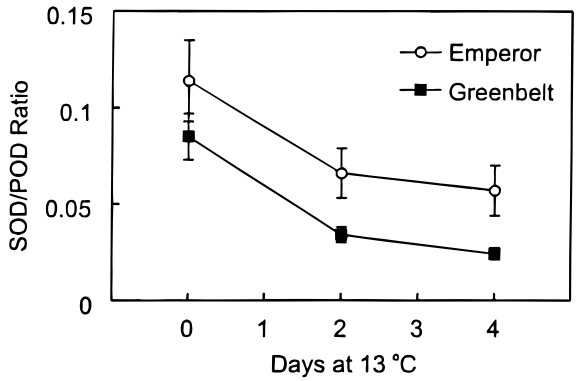

Figure 6. Superoxide dismutase/peroxidase ratios in crude extracts from flower bud tissue of two broccoli cultivars during 4 days at 13 °C, 95% RH (means ± SEM, $n = 5$).

DISCUSSION

Increases in SOD, POD, and CAT occurred during the 4 days at 13 °C. This increase has been found in previous work (Dhindsa et al., 1981; Spychalla and Desborough, 1990). Spychalla and Desborough (1990) interpreted such increases to be associated with the rate of oxygen radical production in tissues as they aged in storage. Zhuang et al. (1995) found lipid peroxidations to increase over time. Thompson et al. (1987) have shown that lipid peroxidation is mediated via oxygen radicals, particularly hydroxyl radicals. The increases in SOD, POD, and CAT are likely response to increases in oxygen radical production in the broccoli, which could subsequently lead to yellowing.

The rate of chlorophyll loss in Emperor was much greater than in Greenbelt, and this was associated with lower SOD and POD activities in the flower bud tissue. The presence of significant activities of both SOD and POD are important in protection against oxygen radical damage in tissue (Beyer et al., 1991; Sen Gupta et al., 1993). The balance in their activities is also critical to effective antioxidant activity and peroxide detoxification (Beyer et al., 1991; Sen Gupta et al., 1993). The ratio of SOD/POD activity was calculated for the two cultivars (Figure 6). Greenbelt had significantly lower ratios throughout the experiment. According to current thought, Greenbelt has a better balance in SOD/POD activity and so would have less potential for hydroxyl radical formation (Beyer et al., 1991; Sen Gupta et al., 1993). If SOD activity far exceeds the capacity for POD to detoxify the H_2O_2 formed through SOD action on superoxide ions (eq 1), then the H_2O_2 can react with superoxide anions directly to produce singlet oxygen and hydroxyl radicals (eq 2). Hydroxyl radicals are very active in lipid peroxidation (Thompson et al., 1987; Beyer et al., 1991). The relationship of lipid peroxidation and yellowing reported by Zhuang et al. (1995) could be attributed to the reaction in eq 2, since they could not find a correlation with lipoxygenase and yellowing.

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H \rightarrow H_2O_2 + O_2 \quad (1)$$

$$O_2^{\bullet -} + H_2O_2 \rightarrow {}^1O_2 + OH + OH^{\bullet} \quad (2)$$

The role of CAT as a component of an antioxidant system in the prevention of yellowing in broccoli is questionable. CAT activity in Emperor was consistently higher than in the yellowing resistant Greenbelt. Therefore there appears to be a negative correlation between chlorophyll loss and endogenous CAT activity. Two factors are likely to minimize the importance of CAT in this situation: (1) catalase activity is largely restricted to the peroxisomes and no significant activity is found in the chloroplasts (Thompson et al., 1987) and (2) the H_2O_2 detoxification capacity of CAT was found to be 10 times lower than for POD (Figures 3 and 4).

The onset of yellowing in broccoli has often been associated with ethylene production (Tian et al., 1994; King and Morris, 1994). King and Morris (1994) studied three cultivars of broccoli with differences in susceptibility to yellowing and were able to associate basal ethylene production only with the time to onset of yellowing. Lipid peroxidation is also strongly linked with yellowing (Zhuang et al., 1995). Ethylene synthesis is intimately linked to lipid metabolism, and the rate of lipid oxidation in tissue is associated with ethylene evolution and presence of free radicals (Frenkel and Eskin, 1977; Leshem, 1988). SOD in concert with POD is a very effective scavenging system for free radicals, preventing the formation of hydroxyl radicals (Salin, 1987; Thompson et al., 1987; Leshem, 1988). Therefore it is expected that a cultivar of broccoli with higher SOD and POD activity would have lower basal rates of ethylene production and lower lipid peroxidation rates and therefore it would take a longer time for onset of yellowing.

It is interesting to note that despite high endogenous antioxidant levels in broccoli, it is very susceptible to yellowing. Pokorny (1971) demonstrated that antioxidants of moderate strength (e.g. tocopherols) when they exceeded a certain concentration would actually decline in antioxidant efficiency. It may be that, with high concentrations in the tissue, the nonenzymatic antioxidants are very inefficient in free radical scavenging ability. That may also explain why there was no relationship of antioxidant content in the broccoli cultivar and susceptibility to yellowing.

The broccoli in this work did not show visible signs of yellowing by the end of the experiments. At some point in time, during senescence, it would be expected that both SOD and POD activities would decline as shown in the work of Dhindsa et al. (1981). We did not carry the experiments to that point in this study. However, a decline in antioxidant enzyme activities would be expected at a point when the tissue loses most of its self-regulatory capability, and it is likely to occur when yellowing becomes obvious.

In summary, this work shows that susceptibility to chlorophyll loss in two cultivars of broccoli is associated with both SOD and POD activities as well as the relative proportions of their activities. The role of this antioxidant system may be critical in controlling yellowing in broccoli, likely as a consequence of the systems ability to modulate other phenomena associated with senescence, namely lipid peroxidation and ethylene production.

ABBREVIATIONS

SOD, superoxide dismutase; POD, peroxidase; CAT, catalase; $O^{\bullet -}$, superoxide anion; $OH^{\bullet}$, hydroxyl radical; $^1O_2$, singlet oxygen; vpd, vapor pressure deficit; fwt, fresh weight; OD, optical density.

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