Reexamining Polyphenol Oxidase, Peroxidase, and Leaf-blackening Activity in *Protea*

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Abstract. Premature leaf blackening in *Protea* severely reduces vase life and market value. The current hypothesis suggests that leaf blackening is induced by a sequence of events related to metabolic reactions associated with senescence, beginning with total depletion of leaf carbohydrates. It is thought that this carbohydrate depletion may induce hydrolysis of intercellular membranes to supply respiratory substrate, and subsequently allow vacuole-sequestered phenols to be oxidized by polyphenol oxidase (PPO) and peroxidase (POD) (Whitehead and de Swardt, 1982). To more thoroughly examine this hypothesis, leaf carbohydrate depletion and the activities of PPO and POD in cut flower *Protea susannae* × *P. compacta* stems held under light and dark conditions were examined in relationship to postharvest leaf blackening. Leaf blackening proceeded rapidly on dark-held stems, approaching 100% by day 8, and was temporally coincident with a rapid decline in starch concentration. Blackening of leaves on light-held stems did not occur until after day 7, and a higher concentration of starch was maintained earlier in the postharvest period for stems held in light than those held in dark. A large concentration of the sugar alcohol, polygalatol, was maintained in dark- and light-held stems over the postharvest period, suggesting that it is not involved in growth or maintenance metabolism. Polyphenol oxidase activity in light- and dark-held stems was not related to appearance of blackening symptoms. Activity of PPO at pH 7.2 in light-held stems resulted in a 10-fold increase over the 8-day period. Activity in dark-held stems increased initially, but declined at the onset of leaf blackening. There was no significant difference in POD activity for dark- or light-held stems during the postharvest period. Total chlorophyll and protein concentrations did not decline over the 8-day period or differ between light- and dark-held stems. Total phenolics in the dark-held stems increased to concentrations ≈30% higher than light-held stems. Consequently, the lack of association between membrane collapse, leaf senescence, or activities of oxidative enzymes (PPO or POD) with leaf blackening does not support the hypothesis currently accepted by many *Protea* researchers. An alternative scenario may be that the rapid rate of leaf starch hydrolysis imposes an osmotic stress resulting in cleavage of glycosylated phenolic compounds to release glucose for carbohydrate metabolism and coincidentally increase the pool of free phenolics available for nonenzymatic oxidation. The physiology of such a carbohydrate-related cellular stress and its manifestation in cellular blackening remains to be elucidated.

Premature leaf blackening in many cut flower *Protea* species seriously reduces vase life and market value. When stems are held under dark postharvest conditions, blackening symptoms can appear within 3 days after harvest (Bielecki et al., 1992; McConchie et al., 1991). The current hypothesis suggests that postharvest leaf blackening in *Protea* is induced by a sequence of metabolic events associated closely with senescence, beginning with the total depletion of leaf carbohydrates and followed by subsequent hydrolysis of intercellular membranes to supply respiratory substrate for the developing inflorescence (Ferreria, 1986). Membrane hydrolysis, or other reactions associated with senescence, may allow vacuole-sequestered phenols to come in contact with oxidative enzymes (Jones and Clayton-Greene, 1992). Polyphenol oxidase (PPO) and peroxidase (POD) have been proposed as the enzymes responsible for leaf blackening symptoms (Whitehead and de Swardt, 1982). In fact, the general acceptance of PPO and POD involvement in

Protea leaf blackening has focused recent research efforts on modification of their postharvest activity, as by inhibition with anti-oxidant dips (Jones and Clayton-Greene, 1992).

We believe this hypothesis requires further investigation because of limited supporting evidence. Although strong correlative data exist between the postharvest reductions in sucrose and starch concentrations and leaf blackening (Bielecki et al. 1992; McConchie and Lang, 1993a, 1993b; McConchie et al., 1991), carbohydrates are not totally depleted in leaves of dark-held stems. McConchie and Lang (1993b) found no evidence of a sequential loss of membrane integrity that would facilitate contact of phenols with oxidative enzymes. Low levels of lipid peroxidation, maintenance of the ascorbate-glutathione antioxidant system, and continued respiration in dark-held stems of *P. neriifolia* leaves after the occurrence of substantial leaf blackening suggest that membrane integrity is maintained. In addition, the potential association of leaf blackening with PPO and POD activity must be examined further since Whitehead and de Swardt (1982) did not demonstrate an actual relationship between the enzyme activity they reported and subsequent leaf blackening (which was not investigated experimentally). Their assumption that leaf senescence occurred during the postharvest interval was not based on measured parameters. In fact, their experimental stems were maintained under a 12-h photoperiod and exhibited no leaf blackening during the period in which PPO and POD activity was measured. Furthermore, PPO activity was measured without an appropriate control, such as catalase. Indeed, without the addition of catalase to inhibit POD indirectly (by decomposing H₂O₂), oxidation of phenolics by POD can often be mistaken for PPO activity (Mayer and Harel, 1979).

The present study was conducted to reexamine critically the

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involvement of oxidative enzymes in leaf blackening. Here we report on leaf carbohydrate depletion, leaf blackening, and the activity of PPO and POD in leaves of cut flower *P. Susannae* × *P. compacta* stems held under light and dark postharvest conditions. We also measured total phenolics to examine the possible involvement of substrate levels, as well as chlorophyll and protein concentrations to examine the possible role of senescence (Thompson, 1988) in the leaf blackening phenomenon.

Materials and Methods

Plant material. Eighteen uniform floral stems of *P. susannae* × *P. compacta* 'Pink Ice' (≈40 cm in length, at soft tip maturity) were harvested from a commercial plantation in Goleta, Calif., in Mar. 1992. All stems were packed under standard cut flower shipping conditions and air-freighted to Baton Rouge, La. Stems were re-cut on arrival, placed in 1-liter deionized distilled water containing 50 ppm hypochlorite, and were equally and randomly assigned to either a light or dark treatment. Stems assigned to the light treatment were placed in a growth chamber (Environmental Growth Chambers, Chagrin Falls, Ohio) and held at 25°C (± 1°C) with 12-h light each 24-h period (photosynthetically active radiation averaged 330 μmol·m⁻²·s⁻¹). Stems assigned to the dark treatment were placed in a similar growth chamber under 24 h darkness at 25°C (± 1°C).

In each treatment, three groups of three stems were divided randomly for determination of leaf blackening, carbohydrate concentration, enzyme activity, total phenolic, protein, and chlorophyll concentration. A visual assessment of the number of leaves per stem with 10% or more blackened leaf area was recorded daily for 8 days. Samples for all other analyses were taken at the same time every other day over the 8-day period and only green tissue was collected for analysis. Samples for carbohydrate analysis are described in the next section. To assay for PPO and POD activity, as well as to determine total phenolic, protein, and chlorophyll concentrations, three randomly selected leaves were removed, diced, and mixed to form a composite sample for each stem. Each composite sample was weighed, divided into four sub-samples (0.5 g each), and frozen (–80°C). Leaf samples for enzyme, carbohydrate, protein, phenolic, and chlorophyll analyses were also taken preharvest and shipped in liquid N₂ to Baton Rouge, La. On arrival the frozen samples were stored at –80°C until used for analysis.

Carbohydrate analysis. To determine nonstructural carbohydrate concentrations, four leaf discs (≈0.15 g) were removed from two randomly selected leaves on each of the three stems from each treatment and were frozen (–80°C). It was not possible to sample for carbohydrate analysis on day 8 due to the extent (nearly 100%) of leaf blackening in the dark-held stems. Samples were lyophilized and then homogenized (Brinkmann Polytron Homogenizer, Brinkmann Instruments, Westbury, N.Y.) for 1 min in 3 ml 80% (v/v) ethanol. After centrifugation, the residue was extracted two additional times with 2 ml 80% ethanol. The supernatants were combined and evaporated to dryness according to the method of Robbins and Pharr (1988). Samples were redissolved in 2 ml 80% ethanol, and soluble carbohydrates were determined using high-performance liquid chromatography (HPLC) as described by McConchie and Lang (1993b). Sugars were identified and quantified based on retention times and peak area integration using the following standards: 0.4% polygalatol (1,5-anhydro-D-glucitol), 0.1% fructose, 0.1% glucose, 0.2% sucrose, and 0.1% maltose.

The pellet remaining from the soluble carbohydrate ethanolic extraction was used for starch analysis. Starch concentrations were determined enzymatically by detecting released glucose (Robbins and Pharr, 1988).

Polyphenol oxidase. To successfully assay PPO activity it is necessary to remove the high concentrations of phenolic compounds found in *Protea* species (Rheede van Oudtshoorn, 1963) that may interact with the enzyme (Smith and Montgomery, 1985) as well as release it from the chloroplast membrane (Mayer and Harel, 1979; Vaughn and Duke, 1984). Removal of phenolic compounds can be accomplished by washing isolated chloroplast membranes with buffer containing ascorbic acid (Lax and Vaughn, 1991). Solubilization of chloroplast membranes by detergents (Sato and Hasegawa, 1976), fatty acids (Mayer and Harel, 1981), and trypsin (Tolbert, 1973) have been reported to have the dual effect of releasing and activating the latent form of the enzyme. To establish an extraction and assay protocol for removal of phenolic substrates and release of PPO, chloroplast membranes were isolated from leaf tissue of vegetative stems of glasshouse-grown *P. Susannae* × *P. compacta*. Leaf extracts were prepared by homogenizing 0.5 g (fresh wt) of diced leaves for 20 sec in 5 ml ice-cold grinding medium containing 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)/KOH buffer (pH 7.6), 2 mM ethylenediaminetetraacetate (EDTA), 1 mM MgCl₂, 1 mM MnCl₂, 330 mM sorbitol, and 5 mM sodium ascorbate. The polytron generator and grinding tube were rinsed with 10 ml grinding medium, and the brei was filtered through two layers of Miracloth. The extract was then centrifuged up to 5000× *g* and stopped immediately. The supernatant was discarded and the pellet was rinsed twice to remove phenols. Grinding medium minus sodium ascorbate was used for the second rinse so as not to interfere with the subsequent assay procedure. The pellet was resuspended in 200 μl solubilization buffer (Webber et al., 1988) containing Triton X-100 or sodium dodecyl sulfate (SDS) at 0%, 0.5%, 1%, 2%, or 5% each and incubated on ice in the dark for 1 h with periodic mixing, followed by microfuge centrifugation (Microfuge E, Beckman Instruments, San Ramon, Calif.) for 1 min. Polyphenol oxidase activity was assayed as described above at pH 7.2 (Vaughn and Duke, 1981) and at pH 4.5, for which PPO activity has been reported in *P. neriifolia* (Whitehead and de Swardt, 1982). Optimum activity was obtained using SDS at 2% at pH 7.2 and pH 4.5 (data not shown); higher concentrations of SDS released not only PPO, but also chlorophylls, which interfered with the assay procedure. Chlorophyll interference has also been noted by Sanchez-Ferrer et al. (1989). Triton X-100 extracts yielded no enzyme activity at pH 7.2 or pH 4.5. Based on these results, PPO was extracted as described for all treatment samples using 2% SDS.

To distinguish between measurement of PPO and POD activity in a *Protea* chloroplast extract, PPO activity was determined in the presence and absence of catalase by oxidation of DL-3,4-dihydroxyphenyl-alanine (DL-DOPA) to DOPA quinone (Vaughn and Duke, 1981). A total assay volume of 1000 μl contained 25 mM DL-DOPA plus/minus catalase (420 units/100 μl) in phosphate buffer (pH 7.2), and 50-μl sample extract. Each assay was repeated at pH 4.5 using phosphate buffer. Control samples were boiled to determine if the reaction was enzymatic. Change in absorbance at 490 nm (30°C) was recorded over 5 min, and one unit of activity represents Δ*Abs*⁴⁹⁰·min × 10³. Activity at pH 7.2 was reduced by ≈27% following addition of catalase; however, catalase addition at pH 4.5 did not lower activity (data not shown). The reported pH range for catalase reactions in vitro is between 6.5 to 7.0 (Maehly and Chance, 1954); thus, decomposition of H₂O₂ by catalase may not have been effective at pH 4.5. Although addition of higher catalase concentrations at pH 4.5 failed to reduce activity (data not shown), PPO activity for all treatment samples was assayed in the presence of catalase.

Peroxidase. POD activity was determined by homogenizing

0.5 g (fresh wt) frozen leaf tissue in 6 ml ice-cold medium containing 50 mM sodium phosphate buffer (pH 7.0), 5 mM 2-mercaptoethanol, 2 mM dithiothreitol (DTT), 2 mM Na₂-EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1% (w/v) bovine serum albumin (BSA), and 1% insoluble polyvinylpyrrolidone (PVP), as described by Badiani et al. (1990). The brei was filtered through two layers of Miracloth and centrifuged for 30 min at 37,000×g. The supernatant was assayed for POD activity at pH 6.8 by measuring formation of tetraguaiacol (Maehly and Chance, 1954) using guaiacol as the hydrogen donor. To test whether the reaction was due to peroxidase, control assays contained catalase (420 units/100 µl). To determine if the reaction was enzymatic, sample extract was boiled and assayed.

Chlorophyll, total protein, and total phenolic determination. Total chlorophyll was extracted in 80% (v/v) acetone and absorbance was measured spectrophotometrically at 645 and 663 nm using the method of Arnon (1949). Total soluble protein concentrations were determined using the Bio-Rad (Bio-Rad Laboratories) microassay, which is based on the method of Bradford (1976), using bovine serum albumin as the standard. Total phenolics were determined as described by Swain and Hillis (1959), with minor modification, using Folin Ciocalteu Phenol Reagent (Sigma Chemical, St. Louis). Phenols were extracted in 80% ethanol (v/v) and diluted (1:400) before reaction with the Folin Ciocalteu Phenol Reagent, to obtain an absorbance reading in the range of standards (0 to 100 µg caffeic acid/ml). Total phenolic concentration was determined from a standard curve generated using caffeic acid.

Statistical analysis. Samples for carbohydrate, protein, chlorophyll, phenol, and enzyme analyses were taken on day 0, 1, 2, 4, 6, and 8. Leaf blackening was recorded daily for 8 days. Treatments (light and dark) were not applied until day 1. The data sets were analyzed using a one-way analysis of variance (ANOVA) to test the effect of day. The rate of change for each variable was compared between light and dark treatments using regression ANOVA (from day 2 to day 8). Differences between independent regression lines were tested for homogeneity by comparing the slopes obtained from regression analysis using two-tailed *t* tests (Steel and Torrie, 1980). All analyses were performed using the SAS statistical package (SAS Institute, 1987).

Results

Leaf blackening under dark postharvest conditions proceeded rapidly after day 3, approached 100% by day 8 (Fig. 1), and was significantly higher on day 7 to day 8 than all other days ($P < 0.05$).

Fig. 1. Percent leaves with 10% or more area blackened on cut floral stems of *Protea susannae* × *P. compacta* held at 25C (±1C) under 12 h light each 24-h period (□), or 24 h darkness (■). Bars indicate the SE of means (n = 3).

In contrast, blackening symptoms in the light did not occur until after day 7. The rate of blackening in the dark was greater than in the light ($b_{\text{dark}} = 17.76$, $b_{\text{light}} = 3.43$; $t_{38} = 20.17$; $P < 0.001$), which is consistent with previous results for other *Protea* species (McConchie and Lang, 1993a and 1993b; McConchie et al., 1991).

Leaf starch concentrations declined dramatically (≈58%) during the 24-h dark shipping period immediately after harvest (Table 1). In the dark, leaf starch had declined by 88% by day 4. Leaf starch concentrations in the light declined similarly, but the rate of decrease was significantly less than in the dark ($b_{\text{dark}} = -27.73$, $b_{\text{light}} = -4.90$; $t_{17} = 25.34$; $P < 0.001$). By day 6, leaves in both treatments had low starch concentrations.

The major leaf soluble carbohydrates were the sugar alcohol polygalatol (1,5-anhydro-D-glucitol) and sucrose (Table 1). Polygalatol concentrations were ≈4 times higher than sucrose concentrations throughout the postharvest period, and did not change significantly for either dark- or light-held stems ($P = 0.87$ and $P = 0.51$, respectively). In the dark, sucrose was undetectable on day 4 (Table 1), which coincided with the rapid appearance of leaf-blackening symptoms (Fig. 1). In the light, sucrose concentrations were maintained until day 8 (Table 1), which also coincided with the appearance of leaf blackening (Fig. 1). Although the variability in these data precludes establishment of a statistically

Table 1. Changes² in leaf nonstructural carbohydrates (mg·g⁻¹ fresh wt) from *Protea susannae* × *P. compacta* cut flower stems at harvest (day 0), after 24-h shipping period (day 1) and held at 25C (±1C) in 12 h light each 24-h period or 24 h darkness (days 2 to 8).

Day	Starch		Polygalatol (mg·g ⁻¹ fresh wt)		Sucrose	
			Pretreatment			
0	14.29 ± 3.76		38.6 ± 2.6		6.15 ± 3.52	
1	5.95 ± 1.19		40.6 ± 4.8		5.17 ± 1.17	
Postharvest treatments						
	Light	Dark	Light	Dark	Light	Dark
2	6.37 ± 0.17	5.17 ± 2.35	38.8 ± 2.5	33.21 ± 0.2	6.98 ± 1.80	3.33 ± 1.78
4	2.98 ± 0.93	1.76 ± 0.17	36.1 ± 7.6	39.70 ± 5.7	7.85 ± 4.02	0.00 ± 0.00
6	1.56 ± 0.06	1.67 ± 0.14	35.5 ± 8.8	44.00 ± 6.1	5.35 ± 1.91	1.92 ± 0.13
8	1.41 ± 0.33	---	46.0 ± 2.2	---	3.97 ± 1.11	---

²Each datum represents the mean ± SE of three replications.

significant relationship of the apparent temporal relationship of sucrose depletion with leaf blackening, this trend is consistent with previous studies (McConchie and Lang, 1993a and 1993b).

No activity was detected for PPO (pH 4.5 = PPO_{4.5} and pH 7.2 = PPO_{7.2}) in control assays using boiled extract (data not shown). In the presence of catalase, PPO_{4.5} activity ranged from ≈5 to 20 times the PPO_{7.2} activity for both treatments (Fig. 2 A and B). Over the postharvest period there was no significant difference in PPO_{4.5} activity in light or dark conditions (*P* = 0.38 and 0.30, respectively). In contrast, PPO_{7.2} activity increased linearly with time in the light (*P* = 0.005) (Fig. 2B). By the end of the postharvest period, PPO_{7.2} activity in the light was about 10 times the activity at harvest (day 0). In the dark, PPO_{7.2} activity did not change significantly (*P* = 0.16). Although PPO_{7.2} activity increased sharply in the dark through day 2, it declined thereafter until, by the end of the postharvest period, it was lower than in the light (Fig. 3B). There was a significant difference in the rate of change in PPO_{7.2} activity between dark- and light-held stems (*b*_{dark} = -2.87, *b*_{light} = 5.75; *t*₁₉ = 3.79; *P* < 0.01), although changes in activities were not related to leaf blackening in either treatments.

No POD activity was detected using control assays that included catalase in the reaction mix or used boiled extract (data not shown). Peroxidase activity declined ≈30% during shipping (Fig. 3); however, there was no significant difference thereafter for either light- or dark-held stems (*P* = 0.47 and *P* = 0.22, respectively).

Total leaf phenolic content was measured to determine possible changes in PPO and POD substrate concentration over the

Fig. 2. Changes in leaf polyphenol oxidase activity assayed at pH 4.5 (A) and pH 7.2 (B) in *Protea Susannae* x *P. compacta* cut flower stems at harvest (day 0), after a 24-h shipping period (day 1), and held at 25C (± 1C) in 12 h light each 24-h period (□), or 24 h darkness (■) (days 2 to 8). Vertical lines equal 1 SE (n = 3). Bars smaller than symbols are not shown.

Fig. 3. Changes in leaf peroxidase activity (units/g fresh weight) from *Protea Susannae* x *P. compacta* cut flower stems at harvest (day 0), after a 24-h shipping period (day 1), and held at 25C (± 1C) in 12 h light each 24-h period (□), or 24 h darkness (■) (days 2 to 8). Vertical lines equal 1 SE (n = 3). Bars smaller than symbols are not shown.

Fig. 4. Changes in total leaf phenolic concentration in *Protea Susannae* x *P. compacta* cut flower stems at harvest (day 0), after a 24-h shipping period (day 1), and held at 25C (± 1C) in 12 h light each 24-h period (□), or 24 h darkness (■) (days 2 to 8). Vertical lines equal 1 SE (n = 3). Bars smaller than symbols are not shown.

Table 2. Changes² in total leaf chlorophyll and total leaf protein concentration (mg·g⁻¹ fresh wt) from *Protea Susannae* x *P. compacta* cut flower stems at harvest (day 0), after a 24-h shipping period (day 1) and held at 25C (± 1C) in 12 h light each 24-h period or 24 h darkness (days 2 to 8).

Day	Chlorophyll	Protein		
	(mg·g ⁻¹ fresh wt)			
	<i>Pretreatment</i>			
	13.9 ± 0.10	13.9 ± 0.1		
	1.43 ± 0.09	14.8 ± 1.0		
	<i>Postharvest treatments</i>			
	Light	Dark	Light	Dark
	1.41 ± 0.10	1.41 ± 0.02	12.4 ± 1.1	13.2 ± 1.0
	1.48 ± 0.10	1.57 ± 0.03	13.4 ± 0.4	12.6 ± 0.2
	1.62 ± 0.06	1.63 ± 0.13	12.7 ± 0.2	13.3 ± 0.7
	1.49 ± 0.08	1.57 ± 0.13	13.2 ± 0.6	12.8 ± 0.5

²Each datum represents the mean ± SE of three replications.

postharvest interval (Fig. 4). Concentrations of total phenolics were similar in both the dark and light through day 2, after which phenolic concentrations increased linearly in the dark, while remaining essentially unchanged in the light. By day 6, phenolic concentrations in the dark were $\approx 30\%$ higher than in the light, a trend which continued through day 8. The rate of phenolic content increase during the postharvest interval was significantly greater in the dark than in the light ($b_{\text{dark}} = 5725$, $b_{\text{light}} = 1963$; $t_{20} = 8409$; $P < 0.001$).

Due to the assertion that both leaf senescence and increased PPO activity are responsible for *Protea* leaf blackening (Whitehead and de Swardt, 1982), total protein and chlorophyll concentrations were measured as two biochemical indicators of the onset of senescence (Thompson, 1988). Total chlorophyll in leaves of both dark- and light-held stems did not change significantly over the 8-day postharvest period ($P = 0.19$ and 0.66 , respectively) (Table 2). Similarly, over the postharvest interval total protein did not change significantly ($P = 0.30$ and 0.39 , respectively) (Table 1).

Discussion

Whitehead and de Swardt (1982) first reported PPO activity in *Protea* and their study has been widely accepted as evidence that PPO activity and leaf blackening are closely related. Although their work is consistently cited, it does not demonstrate an association between PPO and leaf blackening. Their enzymatic assays were based on leaf samples from *Protea* stems that were maintained under a 12-h photoperiod, for which no leaf blackening was reported; no dark-held (and therefore, blackening-induced) stems were sampled. They assumed that leaf senescence occurred during the postharvest interval in their study without testing that assumption. Peroxidative oxidation of phenolics is often mistaken for PPO activity (Mayer and Harel, 1979); thus, it is important to use catalase to indirectly inhibit POD by decomposing H_2O_2 . In their assays Whitehead and de Swardt used no boiled enzyme extracts for controls or catalase as a POD inhibitor, rendering tenuous the assumption that activity measured for PPO was due to PPO activity alone. Polyacrylamide gel electrophoresis of PPO isoenzymes revealed activity at pH 4.5 (not a common pH optimum for PPO activity), yet no activity at pH 7 (within the optimum for PPO activity), which is further evidence that the activity reported may not be attributed entirely to PPO.

Given the lack of direct correlation between PPO and POD activity with *Protea* leaf blackening and the trend for prevention of postharvest *Protea* leaf blackening to focus predominantly on inhibition of oxidative enzymes, we reexamined the prevailing hypothesis regarding PPO, POD, and leaf blackening. In contrast to the Whitehead and de Swardt experiment (1982), our study sampled blackening-induced and non-induced leaves, utilized controls of boiled extract and catalase in the enzyme assays, and tested the relationship between senescence processes and leaf blackening.

Previous studies (McConchie and Lang, 1993b; McConchie et al., 1991) have demonstrated that *P. neriifolia* leaf blackening can be delayed by lighted postharvest conditions, probably due to the contribution of photosynthetic activity to the carbohydrate pool. These photoassimilates, as well as storage reserves are partitioned into transport carbohydrates in response to sink demand; however, as the flower senesces, assimilate export rate decreases from leaves and photoassimilates are partitioned into starch (McConchie et al., 1991). In the current study using *P. Susannae* $\times$ *P. compacta*, our results confirm that leaf starch concentrations decline quickly in the dark and that leaf blackening symptoms develop soon

afterward (Fig. 1 and Table 1). Even though the starch loss by day 6 was similar in the dark and light, the light-held stems maintained higher starch concentrations early in the postharvest interval (when sink demand is greatest) due to the contribution of photoassimilates (Table 1). The complete depletion of sucrose in the dark between day 2 and 4 (Table 1) reflects the decline of starch reserves to a critical threshold, becoming unavailable for hydrolysis to sucrose and subsequent export to the inflorescence sink. Thus, decline of storage carbohydrates to a critical threshold appears to precede leaf-blackening symptoms.

While it is possible that this critical storage carbohydrate threshold may lead to membrane hydrolysis, previous research (McConchie and Lang, 1993b) found no such loss of membrane integrity. Furthermore, the manifestation of leaf blackening symptoms does not appear to be related to increased PPO activity in *P. Susannae* $\times$ *P. compacta*. In our study, PPO_{4.5} activity was similar in the light or dark (Fig. 2A). Although PPO_{7.2} activity differed between treatments, it did not correlate with leaf blackening (Fig. 2B). The rapid appearance of leaf blackening symptoms on day 4 in the dark (Fig. 1) coincided with a decrease in PPO_{7.2} activity (Fig. 2B), despite the low incidence of leaf blackening in the light (Fig. 1), and PPO_{7.2}, there was a 10-fold linear increase during the postharvest period. In fact, the data of Whitehead and de Swardt (1982) are similar to our data for light-held stems, which is expected, since their report of increased PPO activity occurred in light-held stems that did not blacken. Consequently, neither the Whitehead and de Swardt (1982) experiment nor the present study supports the hypothesis that an increase in PPO activity is associated with leaf blackening.

The reason for increased PPO activity in the light-held stems is unclear. Whitehead and de Swardt (1982) attributed their increased PPO activity to senescence processes. Indeed, PPO activity has been reported to increase during senescence (Meyer and Biehl, 1981; Spencer and Titus, 1972), albeit in a species-specific manner (Patra and Mishra, 1979). Having measured some common parameters associated with senescence, our data suggest that senescence did not occur during the 8-day postharvest period. Lack of chlorophyll degradation in both dark- and light-held stems (Table 2) indicates that chloroplasts probably remained intact before the appearance of leaf-blackening symptoms. This would suggest that PPO remained in the chloroplast, separate from phenolic substrates, and was not responsible for initial leaf-blackening symptoms, which is supported by the data that showed PPO_{7.2} activity increased in nonsenescent leaves under lighted postharvest conditions and not in leaves that turned black. Furthermore, the maintenance of chlorophyll and protein levels (Table 2) and continued leaf respiration in addition to a lack of significant lipid peroxidation in dark-held stems (McConchie and Lang, 1993b) suggest that membrane degradation does not precede the appearance of leaf blackening symptoms in *Protea* leaves.

Peroxidases have been associated with mechanical injury and environmental stress responses involving cell wall metabolism and intracellular transport (Gaspar et al., 1985; Lagrimini, 1992). Whitehead and de Swardt (1982) reported a 5-fold increase in POD activity, but did not report use of a control or specific inhibitor of POD; therefore, it is difficult to verify whether PPO or POD activity was being measured. Using such controls, we found no significant change in peroxidase activity for dark- or light-held stems. POD activity was actually highest in leaves before harvest (day 0) (Figs. 1 and 3).

Stress responses may also include release of polyphenols from the vacuole (Lagrimini, 1992), as well as *de novo* phenol synthesis (Hahlbrock and Scheel, 1989). Indeed, under dark postharvest

conditions that induced leaf blackening, total phenolics increased in *P. Susannae* x *P. compacta* (Fig. 4). Thus, *Protea* leaf-blackening symptoms may be enhanced by polyphenol synthesis and/or transport from the vacuole. Furthermore, many phenolics in leaves of *Protea* species are glycosylated compounds (Rheede van Oudtshoorn, 1963) that may be cleaved under periods of carbohydrate stress (Dey and Dixon, 1985), releasing glucose for carbohydrate metabolism and increasing the concentration of free phenolics available for oxidation.

It is clear that the *Protea* leaf-blackening phenomenon is complex, involving a cascade of events that may begin with carbohydrate depletion and eventually lead to oxidation of phenolic substrates. The immature *Protea* inflorescence represents a substantial sink that can deplete the majority of leaf starch reserves within 24 h of harvest (Table 1) (McConchie and Lang, 1993b; McConchie et al., 1991). Although it has been hypothesized that decreased leaf carbohydrate status can result in hydrolysis of membrane-bound macromolecules (Ferreira, 1986), there is no evidence of membrane disruption before the onset of leaf blackening (McConchie and Lang, 1993b). Alternatively, considering that starch has minimal osmotic activity (Setter, 1990), the rapid rate of starch hydrolysis in response to sink demand may impose osmotic stress on leaf cells. It has been suggested that the sugar alcohol, polygalatol, may be involved in cellular osmotic adjustment during stress, since it does not decline under depleted carbohydrate conditions (Bialeski et al., 1992; McConchie and Lang, 1993b), as would be expected if it were involved in growth and maintenance metabolism (Cheeseman, 1988). Instead, concentrations either increase slightly or remain stable (Table 1) (Bialeski et al., 1992; McConchie and Lang, 1993b; McConchie et al., 1991). Thus, maintenance of high polygalatol concentrations, which are several times greater than sucrose concentrations both preharvest (McConchie and Lang, 1993a) and postharvest (Table 1), appears to be important under depleted carbohydrate conditions. In theory, during rapid starch hydrolysis the subsequently altered osmotic status of the cell may induce polygalatol synthesis and thus become a secondary competitive sink to stabilize osmotic status. The lack of evidence of membrane collapse, lack of relationship between PPO and blackening, and lack of significant differences in POD activity in dark- and light-held stems suggest that blackening symptoms may involve a nonenzymatic oxidation of increased phenolic concentrations. Highly reactive phenolic O-glycoside esters such as trisphenol 1,2,4-trihydroxybenzene have been isolated from *Protea* species (Perold, 1993) and may be oxidized by free O₂ in the cell. Additionally, free phenols may rapidly modify enzymes and proteins, resulting in metabolic dysfunction (Chalker-Scott and Fuchigami, 1989). Under such conditions of osmotic stress and carbohydrate depletion, and lacking evidence of sequential cellular deterioration or senescence, such a catastrophic cellular response might be hypothesized to be responsible for leaf blackening. The physiology of such a cellular chain of events remains to be elucidated.

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