Effect of Elevated CO₂ Levels and Leaf Area Removal on Sorbitol, Sucrose, and Phloridzin Content in ‘Gala’/Malling 9 Apple Leaves

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ABSTRACT. Apple seedlings (*Malus domestica* Borkh.) were grown under ambient (370), 700, and 1400 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO₂ regimes and artificially damaged by removal of leaf area (0%, 15%, and 30%). Increased CO₂ concentration had a highly significant effect on the concentrations of sucrose, sorbitol and phloridzin, however there were no significant interactions between CO₂ concentration and leaf damage. As CO₂ concentration increased there was an increase in levels of sucrose and phloridzin, whereas sorbitol concentration decreased. These findings are discussed in relation to the carbon nutrient balance hypothesis as well as other hypotheses regarding the production of plant primary and secondary compounds in response to elevated levels of CO₂ and mechanical damage and/or herbivory.

Carbon dioxide is a molecule that is necessary for photosynthesis and plant life, and is the carbon source for all primary and secondary metabolites. Atmospheric CO₂ concentrations are currently 370 $\mu\text{mol}\cdot\text{mol}^{-1}$ and may reach a concentration of 600 $\mu\text{mol}\cdot\text{mol}^{-1}$ by the middle of the 21st century (Wigley et al., 1996).

The expected rise in atmospheric CO₂ and its subsequent effect on plant leaf chemical composition is of great interest to researchers. Typically, plants exposed to elevated levels of carbon dioxide have demonstrated an increase in the partitioning of photosynthate to leaves and roots, which resulted in an increase in leaf and root area (Kozlowski et al., 1991). More specifically, an increase in photosynthate can mean an increase in carbohydrate, protein, fat, and secondary compound content of a leaf and/or other plant tissues. Apple trees exposed to elevated levels of CO₂ over the course of eight days had shown significant increases in leaf sorbitol and starch content, whereas sucrose content remained unchanged (Pan et al., 1998). In a review by Peñuelas et al. (1997), 17 different plant species exposed to high CO₂ concentrations had an overall increase in foliar carbon-based-secondary compounds (phenolics and terpenes) in the presence of both low and high nutrient availability. Based on literature data, Koricheva et al. (1998) developed a model of carbon secondary metabolism in woody plants that can be predicted by two source–sink hypotheses: 1) carbon-nutrient balance and 2) growth-differentiation balance. These models predict that under elevated levels of CO₂, woody plants will produce more carbon-based secondary compounds or starch from surplus carbon not used in growth. In a separate study, woody plants tended to produce more phenolics than nonwoody plants (Bezemer and Jones, 1998). However, the aforementioned

source–sink hypotheses do not predict what kinds of secondary compounds are produced but rather explain results in terms of specific evolutionary responses unique to a particular species.

A plant's chemical defenses and its ability to deter insect herbivory are related to the plant's secondary chemical composition. Therefore, in a situation where CO₂ levels are greater than ambient, one might expect an increase in defense chemical levels. Amounts of tannins, phenols, and terpenes, typically thought of as static insect defenses (Kozlowski et al., 1991), could increase under high CO₂. In support of this, Lavola et al. (2000) found that under elevated CO₂ (700 $\mu\text{mol}\cdot\text{mol}^{-1}$) there were significant increases in leaf concentrations of phenolic acids, some flavonoids, and condensed tannins in birch (*Betula pendula* Roth) seedlings. The concentration of plant phenolics could be influenced by mechanical damage to leaves, such as insect feeding. Yet recently summarized data (Walling, 2000) indicate that damage caused by chewing insects is substantially different than mechanical wounding with respect to changes in a plant's defensive chemistry. Leaf damage can also result from a number of other physical and biological factors as well. Pathogenic microorganisms, weather, and various cultural practices can also cause leaf damage. For example, leaf area removal during early stages of berry (*Vitis vinifera* Linn. cv. Cabernet Sauvignon) development strongly retarded berry growth (Ollat and Gaudilere, 1998). Berry juice composition relative to osmolarity, titratable acidity, malic acid, and soluble sugars were also adversely affected by leaf area removal.

In the present study we examine the individual and combined effects of leaf area removal and elevated levels of CO₂ on soluble sugars and phloridzin content of apple leaf. The dihydrochalcone glucoside, phloridzin was selected because of its high abundance and allelochemical properties.

Materials and Methods

PLANT MATERIALS. A total of 60 1-year-old apple trees, ‘Gala’/Malling 9 (M9), were grown in 11.4-L plastic pots in a sandy loam type soil. One tree was planted per pot. Trees were initially cut back leaving ≈ 40 cm above the graft union. Trees were pruned to leave three shoots per tree prior to beginning the experiment.

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Trees were watered as needed and fertilized every 2 weeks with 1.5 cL of Peters 20N–8.8P–16.6K (The Scotts Co., Marysville, Ohio) per pot during the course of the experiment.

CO₂ TREATMENT AND LEAF DAMAGE. Forty healthy trees were selected with similar number of shoots, leaves, and branches. These trees were subsequently subjected to four different CO₂ environments and three different levels of leaf area removal (LAR). Six Mylar (Du Pont, Wilmington, Del.) balloon-like chambers, 2 m in diameter and 1.2 m in height, were constructed containing five trees each. Airflow (10,000 L·min⁻¹) for each chamber was generated by using two high-volume low-pressure furnace fans attached in parallel to 25-cm-diameter polyvinyl chloride (PVC) tubing. This airflow was sufficient to inflate and circulate air within chambers and maintain temperatures within 2 °C of outside balloon temperatures. Six chambers were used in this study, each consisting of the following CO₂ concentrations: 1400 μmol·mol⁻¹, 700 μmol·mol⁻¹, and ambient. There were two replicates per CO₂ treatment. Carbon dioxide concentrations were measured and with a CIRIS-1 infrared gas analyzer (PP Systems, Hitchin, U.K.) as previously described (Druta, 2001). Carbon dioxide was controlled by metering in pure CO₂ from a liquid source, with a needle valve for each concentration, into a constant flow of ambient air. Two sets of five trees were kept outside as ambient CO₂ controls. Ambient CO₂ concentrations were typically 370 μmol·mol⁻¹. Leaves were subjected to three levels of area removal; 0%, 15%, and 30%. There were four 0%, three 15%, and three 30% damaged trees in each of the CO₂ environments. Leaf area removal was carried out on all fully expanded leaves after trees had been exposed to the CO₂ enriched environments for a period of 3 weeks. Newly expanded leaves were damaged similarly. LAR or damage was achieved by the use of a hole punch (1 hole = 0.38 cm²). Average apple leaf areas were determined to be 21.18 ± 8.16 cm². Therefore, for example, an average sized leaf requiring 15% leaf removal had eight to nine holes punched. Whereas leaves requiring a larger or smaller leaf removal than 15% had 16 to 17 or five to six holes punched out, respectively. Leaf midribs were not damaged. Leaves were damaged according to previously published work (Layne and Flore, 1992).

EXTRACTION OF PLANT MATERIAL. Leaves were only collected after trees had been exposed to 3 months of the treatments. Leaves (20–30 g fresh weight) of various ages and positions were harvested from each of the separate treatment trees, freeze-dried, then milled in a coffee grinder to a 100-μm particle size. Freeze dried sub-samples (50 mg) were extracted in triplicate with 80% ethanol (1.75 mL) for 30 min at room temperature in test tubes. Following extraction, samples were centrifuged (5 min, 1966 g_n) and supernatants were decanted into separate tubes. Plant pellets were re-extracted two additional times as described above. Chlorophyll was removed by combining supernatants and partitioning, by first adding H₂O (2.5 mL) then CHCl₃ (2.5 mL). Tubes were tightly capped, gently mixed, and centrifuged (3 min, 874 g_n). The upper clear aqueous layers were collected in separate tubes and dried in a Speedvac SC200 (Savant Instruments, Holbrook, N.Y.) overnight to afford a crusty tan residue.

ANALYTICAL HIGH-PRESSURE LIQUID CHROMATOGRAPHY (HPLC). *Soluble carbohydrates.* All samples were filtered through 0.2-μm syringe filters (PTFE) into HPLC vials prior to injection. All solvents used were HPLC grade. Samples were loaded into a Waters 717 autosampler (Waters Corp., Milford, Mass.) and 50 μL injections made onto an Alltech 1.6 × 300 mm 700 CH carbohydrate column (Alltech Associates, Deerfield, Ill.). The solvent system was 100% Millipore (Millipore Corp., Billerica,

Mass.) water at 0.6 mL·min⁻¹ with a Waters 515 pump and a Waters 410 refractometer as the detector. Column temperature was maintained at 85 °C with a Waters column heating unit and the detector temperature was set to 35 °C. Peaks obtained at ≈6.5 and 13.0 min (Fig. 1) were identified by co-injection with standards (100 μL of 1.0 mg·mL⁻¹) as sucrose and sorbitol. All standards were obtained from Sigma Aldrich Chemical Co. (St. Louis). Quantification of sucrose and sorbitol was achieved by injecting standard sucrose and sorbitol at 5.0, 2.5, 1.25, 0.625, and 0.313 mg·mL⁻¹ immediately prior to the extracted samples. The calibration curves were then calculated with Waters Millennium 32 software (sucrose $r^2 = 0.990$; sorbitol $r^2 = 0.999$) and quantities of sucrose and sorbitol in the crude apple leaf determined and expressed as mg·g⁻¹ dry weight.

Phloridzin (phloretin 2'-O-β-D-glucoside). Pure phloridzin standard was obtained from the Sigma Aldrich. The HPLC system consisted of a Waters 717 autosampler connected to a Waters 600 multi-solvent pump and a Waters 996 photodiode array detector. The column used was a Shiseido Capcell Pak reverse-phase C18 column (5 μm, 4.6 × 250 mm, AG 120; Shiseido Co., Tokyo) coupled to a guard column with the same packing material. The solvent system was isocratic acetonitrile:water (30:70, v/v) with a flow rate of 1.0 mL·min⁻¹. Ten microliters of 1 mg·mL⁻¹ of standard phloridzin and crude ethanolic extracts from one of the extracted leaf samples were then injected with a detection scan set for 210–600 nm. The peak at 6.0 min (Fig. 2) had the same retention time and UV maximum as the phloridzin standard. The leaf extract vial was then spiked with 100 μL of 1.0 mg·mL⁻¹ of standard phloridzin, and the peak area increased with no change in the UV spectra. The peak at 6.0 min in the leaf extracts was therefore identified as the dihydrochalcone glucoside, phloridzin. For quantification of phloridzin, a calibration curve was constructed by injecting standard phloridzin at 5.0, 2.5, 1.25, 0.625, and 0.313 mg·mL⁻¹ prior to the extracted samples. The curve was then calculated by Waters Millennium 32 software ($r^2 = 0.987$) and quantity of phloridzin in the crude apple leaf determined.

Statistical analyses. To determine the effects of CO₂, damage and the CO₂ per damage interaction, the SAS 7.0 (SAS Institute, Cary, N.C.) software package was used. A two-way analysis of variance (ANOVA) was conducted on the concentrations of the three compounds for the different treatments using the General Linear Model (GLM) procedure. Two one-way ANOVAs using the GLM procedure were performed to analyze differences between the means for CO₂ and damage treatments. Significant differences between means were determined using a Student–Newman–Keuls test.

Results

Sorbitol, sucrose, and phloridzin were detected in the ethanolic extracts of apple leaf by HPLC. Glucose was observed in some chromatograms, however good peak intensity and resolution were not attainable. According to a two-way ANOVA for the effects of CO₂ and leaf damage (Table 1), CO₂ was highly significant in affecting the concentrations of sucrose, sorbitol, and phloridzin in apple leaf. With the exception of sorbitol, the leaf concentrations of sucrose and phloridzin were not affected by leaf damage. However, when all concentrations of CO₂ were combined and only leaf damage analyzed in a one-way ANOVA there did not appear to be any significant relationship between leaf damage and concentration of sugars or phloridzin (Table 2). There were no significant interactions between CO₂ concentrations and leaf

Fig. 1. HPLC trace showing the sucrose peak at ≈ 6.5 min and the sorbitol peak at 13.0 min. The peak at ≈ 4.0 min is most likely phloridzin. Normal phase separation achieved with an Alltech 700 CH carbohydrate column.

damage observed (Table 1). Data on the effects of four CO_2 treatments is presented in Table 3. The overall trend was that as CO_2 increased, sucrose and phloridzin increased, whereas sorbitol decreased. Trees exposed to 700 and 1400 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO_2 demonstrated $\approx 10 \text{ mg}\cdot\text{g}^{-1}$ decrease in sorbitol, which coincided with a proportionate increase in phloridzin. Sucrose showed a modest increase when exposed to 1400 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO_2 . Interestingly, there was a significant increase in leaf phloridzin content of trees maintained within balloons at ambient CO_2 concentrations versus those outside the balloons.

Discussion

A number of recent studies have demonstrated that with increased concentrations of CO_2 , the level of secondary metabolites such as flavonoids and phenolics in plant leaves are affected. For example, in wheat (*Triticum aestivum* Linn. cv. Yecora Rojo) CO_2 enrichment led to increases in the concentrations of flavonoids and total nonstructural carbohydrates (TNC) (Estiarte et al., 1999). Flavonoid compounds were also shown to increase significantly in strawberry (*Fragaria xananassa* Duch. cv. Honeoye) under enhanced CO_2 concentrations (Wang et al., 2003).

Phloridzin is a well-known polyphenolic allelochemical present in apple leaves, which in artificial diets has been shown to reduce tolerance to azinphosmethyl for third instars of a susceptible strain of tufted apple bud moth (*Platynota idaeuslalis* Walker) (Hunter

et al., 1994). Phloridzin has also demonstrated feeding deterrent activity against adult Japanese beetle (*Popillia japonica* Newman) (Fulcher et al., 1998). In our study we demonstrated that there was a significant increase in the main defensive compound phloridzin and a reduction in sorbitol, the main soluble sugar compound in apple leaves, in response to elevated CO_2 levels (Tables 1 and 2). However, except for sorbitol, we found no significant effect of leaf damage or damage $\times \text{CO}_2$ on the levels of these compounds (Table 1). This indicates that there does not seem to be an induction (or reduction) in the production of phloridzin in response to damage and that our increase in phloridzin is caused by increased CO_2 concentration. Close and McArthur (2002) have proposed that phenolics evolved in plants primarily to protect them from photodamage and that in addition to this main function, they may also negatively affect herbivores. However, in our study, a significant increase in phloridzin is believed to be due to elevated CO_2 and not necessarily as a response to photodamage since light levels between the treatments and controls were the same. Hamilton et al. (2001), points out that few studies have measured the value of plant tissue as the difference in fitness between intact and similar plants in which a known amount of tissue was removed. We did not measure fitness in terms of seed produced but if an increase in phloridzin improves fitness by reducing herbivory and thus allocation of resources to seed, then we would expect increases in phloridzin to have adaptive value. However, the lack of significant increase in the phloridzin concentration related to simulated

Fig. 2. HPLC trace showing phloridzin peak at 6.0 min and structure of this dihydrochalcone glucoside. Reverse-phase separation achieved with a Shiseido C18 column.

Table 1. Two-way analysis of variance on the effect of CO₂ and damage levels on the concentration of three compounds in *Malus domestica* 'Gala'/M9.

Factor	df	Sucrose		Sorbitol		Phloridzin	
		F	P	F	P	F	P
CO ₂	3	13.12	0.0001	13.38	0.0001	30.19	0.0001
Damage	2	0.33	0.7213	3.78	0.0373	0.83	0.4492
Damage × CO ₂	6	1.52	0.2139	1.73	0.1564	0.83	0.5594

Table 2. Effect of four concentrations of CO₂ on the concentration of soluble carbohydrates and chalcone phloridzin in *Malus domestica* 'Gala'/M9 (all CO₂ levels combined).

Compound ^z	CO ₂ concn (μmol·mol ⁻¹)			
	Outside (370)	370	700	1400
	mean ± SE (mg·g ⁻¹ dry wt)			
Sucrose	5.88 b ± 0.29	5.64 b ± 0.33	6.03 b ± 0.31	8.0 a ± 0.34
Sorbitol	64.39 a ± 2.58	59.59 a ± 2.04	49.12 b ± 2.21	52.29 b ± 1.76
Phloridzin	43.70 c ± 1.21	51.28 b ± 1.46	63.16 a ± 2.37	64.53 a ± 1.83

^zMeans in a row followed by the same letter are not significantly different ($P > 0.05$). Student–Newman–Keuls comparison of means.

herbivory or LAR does not support this hypothesis. However, as reviewed by Walling (2000), the production of increased levels of defense compounds may require an elicitor only to be found in the oral secretions of feeding insects.

There is little information in the literature as to how trees as opposed to herbaceous plants and shrubs respond phytochemi-

cally to increases in carbon dioxide levels (Lindroth, 1993). In birch seedlings the levels of condensed tannins and flavonoids increased with CO₂ enrichment but decreased with nitrogen fertilization (Lavola and Julkunen-Tiitto, 1994). However, in a later study Lavola et al. (1998) found that CO₂ enhancement led to reduced levels of flavonoids and phenolic acids in birch. These authors suggested that secondary chemical responses of birch to various climatic factors are very compound dependent and relate more to ontogenic differences in carbon allotment of the individual seedlings. Lindroth et al. (1993) suggested that fast-growing species such as aspen (*Populus tremuloides* Mich.), which have greater chemical flexibility, should show greater increases in the accumulation of carbon-based secondary compounds (CBSC) than slow-growing species such as maple (*Acer saccharum* Marsh.). However, they found that increases in phenolic compounds under enhanced CO₂ were higher in maple than in aspen and suggested that the allocation of carbon to storage vs. defensive compounds may be more related to photosynthetic rate, which is higher in aspen.

The above studies, and our own, offer only partial support to the carbon nutrient balance hypothesis (CNB), which sug-

Table 3. Effect of four concentrations of CO₂ on the concentration of soluble carbohydrates and chalcone phloridzin in *Malus domestica* 'Gala'/M9 (injured and uninjured plants combined).

Compound ²	Leaf damage (%)		
	0	15	30
	mean ± SE (mg·g ⁻¹ dry wt)		
Sucrose	6.317 a ± 0.31	6.56 a ± 0.27	6.29 a ± 0.53
Sorbitol	58.77 a ± 3.09	57.54 a ± 2.26	52.73 b ± 1.87
Phloridzin	54.53 a ± 2.39	57.27 a ± 3.27	55.20 a ± 3.21

²Means in a row followed by the same letter are not significantly different ($P > 0.05$). Student–Newman–Keuls comparison of means.

gests that when plants have resources, such as CO₂, in excess of growth, they will invest these into the production of CBSC as a defense against herbivory (Bryant et al., 1983). While the CNB theory has stimulated research into the ecology of plant secondary metabolites for over 20 years, there have been indications, other than the above studies, that its application was limited. For example, Peñuelas et al. (1997) noted that, in general, while biomass and production of CBSC increased with elevated CO₂, there was considerable variation in the literature depending on the study, the species and the particular compounds examined. The authors suggest that phenolics, products of the shikimic acid pathway, may be more sensitive to increases in CO₂. The usefulness of CNB as an explanatory tool for the production of secondary metabolites was further limited when it was found that flavonoid concentrations increased in green, well-developed leaves but not in mature, or senescing leaves (Peñuelas et al., 1999). Recently, Hamilton et al. (2001), Nitao et al. (2002), and Koricheva (2002) have argued for abandoning CNB because, among other things, it has become an evolution-free hypothesis that even under various refinements (Lerdau, 2002) has lost its value as a predictive tool.

While our study did show a significant increase in phloridzin in response to CO₂ enhancement in accord with the CNB and its refinements (Lerdau, 2002) we also saw a very significant reduction in the main soluble sugar, sorbitol. In addition, sorbitol concentration was also affected by LAR while phloridzin was not. We therefore suggest, given our own, other studies, and in accordance with Koricheva (2002), that the CNB as an all-encompassing and simplified theory be replaced with more specific and perhaps more complex theories such as Hamilton et al. (2001). Finally, further studies on elevated CO₂, plant phytochemical response, insect feeding, and effects on insects are warranted to test various hypotheses.

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