**Influence of Phostrade Ca on Color Development and Anthocyanin Content of ‘Braeburn’ Apple (Malus domestica Borkh.)**

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Abstract. The influence of two foliar applications of Phostrade Ca, which contains high concentrations of phosphorus and minor amounts of calcium and nitrogen, on color development and selected primary and secondary metabolites was investigated during advanced maturation of ‘Braeburn’ apple. Changes of hydroxycinnamic acids, flavanols, dihydrochalcones, flavonols, and anthocyanins were monitored six times during the advanced ripening until technological maturity of the fruits. Additionally, the changes in the chromatic values a*, h°, and the lightness coefficient L° were recorded weekly. The colorimetric parameters showed a significant difference in the intensity of red coloration between the treated and untreated apples. Spraying with Phostrade Ca also resulted in a significant increase in most individual sugars, total sugars, and concentration of anthocyanins and flavonols. Moreover, the amount of phosphorus (P) in the treated leaves was increased. However, the total phenolic content and accumulation of other classes of flavonoids such as hydroxycinnamic acids, flavonols, and dihydrochalcones were not influenced. Phostrade Ca treatment significantly increased dihydroflavonol 4-reductase (DFR) and slightly flavanone-3-hydroxylase (FIHT) activity, which were correlated with anthocyanin synthesis but had no effect on phenylalanine ammonia lyase (PAL) and chalcone synthase/chalcone isomerase (CHS/CHI) activity. The results indicate that two foliar applications of Phostrade Ca late in the growing season represent an effective way to improve the color of ‘Braeburn’ apples at commercial harvest.

An intense red skin color is an important quality parameter for consumers when purchasing apples and can contribute much to a higher market value of the fruit. In apple fruits, red color is mainly the consequence of the presence of anthocyanins, which accumulate as granules in the vacuoles (Bae and Kim, 2006), but flavonols (quercetin 3-O-glycosides) and proanthocyanidins also have some influence (Lister et al., 1994). Anthocyanin accumulation is usually restricted to the skin of apple. The pigments provide essential cultivar differentiation for consumers and are implicated in the health attributes of apple fruit (Espley et al., 2007). Fruit color and biosynthesis of anthocyanins can be regulated by light and ethylene (Saure, 1990), temperature (Arakawa, 1991), the use of a hail net and reflective foil (Blanke, 2008; Jakopic et al., 2010), nitrogen fertilization (Treuttet, 2001), wounding (Chalmers and Faragher, 1977), bagging (Hudina and Stampar, 2011), irrigation cooling (Iglesias et al., 2002), and different chemical applications (Whale et al., 2008). Moreover, color development also depends on a regular supply of sugars in the fruit (Lueangpraseart et al., 2010).

‘Braeburn’ Hillwell is classified as a striped red-colored apple cultivar. Striped fruits are less red on average than blushed fruits (Telias et al., 2008); therefore, it is useful for the growers to have a variety of techniques available that can help them to achieve better coloring, especially in regions and years with poor coloring conditions.

Phostrade Ca (Pho Ca) is a concentrated liquid P solution containing calcium and nitrogen recommended for foliar application at the beginning of fruit formation and during fruit enlargement and maturation. P-containing compounds have already been documented to increase anthocyanin concentration and improve fruit color (Gomez-Cordoves et al., 1996; Larrigaudiere et al., 1996; Li et al., 2002). The use of Seniphos seems to be promising because it stimulates anthocyanin accumulation without activation of ethylene production and advanced ripening (Larrigaudiere et al., 1996). Furthermore, it does not affect the storage life as reported for ethephon (Li et al., 2002). Li et al. (2002) suggested that increased PAL and CHI activity contributed to the observed improvement in anthocyanin formation and in red coloration of apple skin. The rapid increase of PAL activity directly related to the increase of anthocyanin biosynthesis was reported also by Larrigaudiere et al. (1996). However, the mode of action by which these compounds affect color development has been poorly investigated and remains unclear.

The purpose of our work was to investigate if two foliar applications of P-containing compound Pho Ca during advanced maturation can enhance anthocyanin accumulation and consequently improve red coloration of ‘Braeburn’ apples. Moreover, we were also interested in evaluating the impact of the Pho Ca on the total phenolic content and some other phenolic compounds belonging to different phenolic groups. To get a more detailed insight into the action of Pho Ca in apple skin, changes in sugars and enzyme activity of several different enzymes were also recorded. The results should contribute to an improved understanding of the mechanism, by which P-containing compounds affect the red coloration of apples.

Materials and Methods

Plant materials. The experiment was carried out in 2011 on 10-year-old trees of striped ‘Braeburn’ (Malus domestica Borkh.) cultivar clone Hillwell grafted on M9 rootstock, grown according to the system of integrated production at the Ljubljana location (lat. 46°2′ N, long. 14°28′ E). On 9 Sept. (5 weeks before commercial harvest), Pho Ca (23.6% w/w P2O5, 4.3% w/w CaO, and 3% w/w nitrogen) was applied at a concentration (Larrigaudiere et al., 1996; Larrigaudiere et al., 1996; Li et al., 2002). Li et al. (2002) suggested that increased PAL and CHI activity contributed to the observed improvement in anthocyanin formation and in red coloration of ‘Braeburn’ apples. Moreover, we were also interested in evaluating the impact of the Pho Ca on the total phenolic content and some other phenolic compounds belonging to different phenolic groups. To get a more detailed insight into the action of Pho Ca in apple skin, changes in sugars and enzyme activity of several different enzymes were also recorded. The results should contribute to an improved understanding of the mechanism, by which P-containing compounds affect the red coloration of apples.

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in hue angle, calculated as (tan⁻¹ b/°a) in degrees from 0 to 360, where 0° = red, 90° = yellow, 180° = green, and 270° = blue (McGuire, 1992).

Fruit color was measured starting on 9 Sept. (sampling date 0) and continued weekly until commercial harvest of the fruit (sampling date 5). At each sampling date, color measurements were made on 20 fruit from the outside of the tree canopy (four fruit per tree) up to a height of 2 m from the ground. Fruit were randomly selected before the first Pho Ca application (sampling date 0).

**Fruit sampling.** Fruit sampling started on 9 Sept. (before the first spraying) and was performed weekly until commercial harvest, which was determined using the starch iodine test. At that time the value of firmness was 9.5 kg·cm⁻² and the starch index was 2.7. At each sampling date, 15 fruits were randomly harvested and combined in five samples with three fruit per sample (n = 5). Immediately after harvest, apples were transported to the laboratory, where the tissue of samples was frozen in liquid nitrogen to prevent oxidation until the preparation of the samples. For the determination of enzyme activity, one part of the apple skin was stored frozen at –20°C until enzymatic analysis.

**Analysis of individual sugars and organic acids.** Sucrose, glucose, fructose and sorbitol, and malic and citric acids were analyzed in the whole edible part of the fruit according to Mikulic-Petkovsek et al. (2007). For the extraction of individual sugars and organic acids, 10 g of the fresh weight of each sample was homogenized in 50 mL of bidistilled water using Ultra-Turrax T-25 (Ika-Labortechnik, Staufen, Germany). Samples were left for 30 min at room temperature and stirred frequently. After extraction, the homogenate was centrifuged (Eppendorf 5810 R Centrofuge, Hamburg, Germany) at 10,000 rpm for 5 min at 4°C. The supernatants were filtered through the 0.45-μm cellulose ester filter (Macherey-Nagel, Düren, Germany), transferred into a vial, and 20 μL of the sample was used for analysis. The analysis of sugars (fructose, glucose, and sucrose), sorbitol, malic, and citric acid content was carried out using high-performance liquid chromatography (HPLC) from the Thermo Separation Products equipment (Mikulic-Petkovsek et al., 2007).

Separation of sugars and sorbitol was carried out using a Rezex RCM-monosaccharide column (300 × 7.8 mm; Phenomenex, Torrance, CA) with a flow of 0.6 mL·min⁻¹ and column temperature maintained at 65°C. The mobile phase was bidistilled water; the total run time was 30 min, and a refractive index detector Shodex RI-71 was used to monitor the eluted carbohydrates as described by Mikulic-Petkovsek et al. (2007). Organic acids were analyzed using a Rezex ROA-organic acid column (300 × 7.8 mm; Phenomenex) and the ultraviolet detector set at 210 nm with a flow of 0.6 mL·min⁻¹ maintaining the column temperature at 65°C. The duration of the analysis was 30 min.

The concentrations of carbohydrates and organic acids were calculated with the help of corresponding external standards. The concentrations were expressed in g·kg⁻¹ fresh weight (FW).

**Chemicals.** For the quantification of phenolic compounds, the following standards were used: chlorogenic acid (5-cafeoylquinic acid), phloretin, and rutin (quercetin 3-O-rutinoside) from Sigma Aldrich Chemie (Steinheim, Germany); cyanidin 3-O-galactoside chloride, quercetin 3-O-rhamnose, quercetin 3-O-galactoside, quercetin 3-O-glucoside, (–)-epicatechin, p-coumaric acid, procyanidin B2, and phlorizin dihydrate from Fluka Chemie (Buchs, Switzerland); quercetin 3-O-arabinofuranoside and quercetin 3-O-xylloside from Apin Chemics (Abingdon, U.K.); and (+)-catechin from Roth (Karlsruhe, Germany). Methanol for the extraction of phenolics was acquired from Sigma Aldrich Chemie. Chemicals for the mobile phases were the HPLC-mass spectrometry (MS)-grade acetoneitrile and formic acid from Fluka Chemie. Water for the mobile phase was bidistilled and purified with the Milli-Q system (Millipore, Bedford, MA). For determination of the total phenolic content, Folin–Ciocalteu phenol reagent (Fluka Chemie), sodium carbonate (Merck, Darmstadt, Germany), gallic acid, and ethanol (Sigma Aldrich Chemie) were used. Substrates for enzyme assays were synthesized as described (Gosch et al., 2003).

**Extraction and determination of phenolic compounds.** The extraction of fruit samples for phenolic compounds was done as described by Mikulic-Petkovsek et al. (2010) with some modification. Apple samples were ground to a fine powder in a mortar chilled with liquid nitrogen. Five grams of apple skin were extracted with 10 mL of methanol containing 3% (v/v) formic acid and 1% (w/v) 2,6-di-tert-butyl-4-methylphenol (BHT) in a cooled ultrasonic bath for 1 h. BHT was added to the samples to prevent oxidation. After extraction, the treated samples were centrifuged for 5 min at 12,000 × g. The supernatant was filtered through a Chromafil AO-45/25 polyamide filter (Macherey-Nagel, Düren, Germany) and transferred to a vial before the injection into the HPLC system.

The individual phenolic compounds were analyzed using a Thermo Finnigan Surveyor HPLC system (Thermo Scientific, San Jose, CA) with a diode array detector at 280 nm, 350 nm, and 530 nm. The hydroxycinnamic acids, dihydrochalcones, and flavonols were detected at 280 nm, flavonols at 350 nm, and anthocyanins at 530 nm. For the separation of phenolic compounds, a Phenomenex HPLC column C18 (150 × 4.6 mm, Gemini 3 μ) protected with a Phenomenex security guard column operated at 25°C was used. The injection volume for the fruit extract was 20 μL and the flow rate maintained at 1 mL·min⁻¹. The elution solvents were aqueous 1% formic acid and 5% acetonitrile (A) and 100% acetonitrile (B). Samples were eluted according to the linear gradient described by Marks et al. (2007): 0 to 5 min, 3% to 9% B; 5 to 15 min, 9% to 16% B; 15 to 45 min, 16% to 50% B; 45 to 50 min, 50% isocratic; this step was followed by the washing and reconditioning of the column. The compound identification was achieved by comparing the retention times and their ultraviolet-VIS spectra from 200 to 600 nm as well as by the addition of an external standard. The identity of the phenolic compounds was also confirmed and quantified using a mass spectrometer (Thermo Scientific, LCQ Deca XP MAX) with an electrospray ionization operating in the negative and positive ion modes. For the analyses, full-scan data-dependent MS scanning from m/z 115 to 2000 was performed. Quantification was achieved according to the concentrations of a corresponding external standard. For the compounds lacking reference compounds, related compounds were used as standards for the quantification. Therefore, 4-O-p-coumaroylquinic acid was quantified in equivalents of p-coumaric acid, phloretin-2-O-xyllosylglucoside in equivalents of phloridzin, quercetin 3-O-arabinofuranoside and anthocyanins (cyanidin 3-arabinoside, cyanidin 7-arabinoside, cyanidin 3-glucoside, and cyanidin 3-xylloside) were quantified in equivalents of cyanidin 3-galactoside. Concentrations of the phenolic compounds were expressed in mg·kg⁻¹ FW.

**Determination of the total phenolic content.** Extracts of the samples were obtained in the same way as for the individual phenolic compounds with the difference that no BHT was added. The total phenolic content of extracts was assessed using the Folin–Ciocalteu phenol reagent method, as described by Singleton and Rossi (1965). To 100 μL of sample extracts, 6 mL of bidistilled water and 500 μL of Folin–Ciocalteu reagent were added; after resting between 8 and 8 min at room temperature, 1.5 mL of sodium carbonate (20% w/v) and 1.9 mL of bidistilled water were added. The extracts were mixed and allowed to stand for 30 min at 40°C. After that the absorbance was measured in a spectrophotometer (Perkin Elmer, ultraviolet/VIS Lambda Bio 20) at 765 nm. A mixture of water and reagents was used as a blank. The total phenolic content was expressed as gallic acid equivalents in mg·kg⁻¹ FW. Absorption was measured in three replications.

**Analysis and determination of phosphorus and calcium concentration in apple leaves.** Samples for leaf P and calcium (Ca) concentrations were taken three times during the advanced ripening: on 9 Sept. (before the first application of Pho Ca), on 30 Sept. (1 week after the second application), and on 14 Oct. (commercial harvest). A combined sample of 10 fully developed, healthy leaves per tree was analyzed in three repetitions for each treatment. Plant samples had been washed first in water to remove surface residues and then dried at 40°C until constant weight was achieved. The sample was reduced to ashes in a muffle furnace at 550 ± 15°C. The ash was then dissolved in hydrochloric acid and the silica compounds present removed by precipitation and filtration. The filtrate was diluted...
to the desired volume (100 mL) with demineralized water (Milli Q; Millipore).

For the determination of P, an aliquot portion of the filtrate was mixed with molybdenum reagent and the absorbance of the yellow complex was measured at a wavelength of 430 nm on the ultraviolet/VIS spectrometer (Cary 100; Varian). The absorbance of the sample was compared with the absorbance of standards with a known concentration to determine leaf P concentration. Calcium was determined by flame atomic absorption spectrometry ( Analyst 800; Perkin Elmer). The absorbance of Ca in the sample solution was determined by comparison with the absorbance of calibration solutions. The ionization and chemical interferences were controlled by the addition of caesium and lanthanum buffer solutions to standards and samples.

**Extraction and assay of enzyme.** Buffers used were Buffer A (phenylalanine ammonia lyase—PAL assays; electrical conductivity (EC) 4.3.1.5): 0.1 M H₃BO₃ + 0.4% Na-ascorbate, pH 8.5; Buffer B (CHS/CHI assays; CHS; EC 3.2.1.74; CHI; EC 3.2.1.14): 0.1 M KPi (KH₂PO₄/K₂HPO₄) + 0.4% Na-ascorbate, pH 7.0; Buffer C (FHT assays; FHT; EC 1.14.11.9): 0.1 M Tris/HCl + 0.4% Na-ascorbate, pH 7.25; and Buffer D (DFR assays; DFR; EC 1.1.1.219): 0.1 M KPi + 0.4% Na-ascorbate, pH 7.25.

Enzyme preparations. Shock-frozen apple skin was ground to powder with liquid nitrogen. A total of 1 g fine apple skin powder was homogenized with 0.5 g quartz sand, 0.5 g Polyclar AT, and 6 mL 0.1 M Tris/HCl (containing 0.4% Na-ascorbate, pH 7.25) in a mortar. The homogenate was centrifuged for 10 min at 4 °C and 10,000 g. To remove low-molecular compounds, 400 μL of supernatant was passed through a gel chromatography column (Sephadex G25 medium).

**Analyticals of enzyme activity.** Enzyme assays were performed as previously described (Slatnar et al., 2010) using the assay conditions optimized for apple skin. Values on each sampling date represent an average of four independent biological repetitions for each treatment. Enzyme activity for PAL, CHS/CHI, FHT, and DFR was calculated and expressed in nkat·g⁻¹ FW.

**Statistics.** Statistical analysis was conducted using the Statgraphics Plus 4.0 program (Manugistics, Inc., Rockville, MD). All data were subjected to two-way analysis of variance including the treatment and maturation time as factors. Significant differences among treatments were determined using the Duncan test. In both cases, the significance level was 0.05. The relationship between sampling dates were monitored.

**Results**

To evaluate the potential of Pho Ca applications of enhancing red coloration during advanced ripening, the changes in the colorimetric parameters a*, L*, and hue angle (h°) were monitored.

The hue angle (h°), the best indicator of color changes during fruit development (Greer, 2005), decreased during the advanced ripening, indicating a greater intensity of red color (Fig. 1A). Treatment with Pho Ca significantly influenced the hue angle. During the 5 weeks of ripening, the average values for the treated apples were 41.5 and for the control 46.1 (P = 0.0002). Similar effect was also detected for the parameter a*, which increased during the fruit ripening (Fig. 1B). In this parameter, the average values were 26.9 for treated apples and 23.5 for the control (P = 0.00001). Furthermore, the Pho Ca treatment also influenced the parameter L*, which decreased during the advanced maturity, indicating darker fruit skin (Fig. 1C). During 5 weeks of ripening, the average L* value of the treated apples was 44.68, whereas the average value of the control was 46.1. However, the effect of the treatment was slightly less significant (P = 0.008) in this parameter.

The anthocyanin concentration increased during the advanced ripening (Fig. 1D). The highest concentration of total anthocyanins was measured at commercial harvest, when
Total phenolic content 1197.08 1213.38 0.70
Dihydrochalcones 72.71 68.35 0.27
Flavonols 362.89 336.84 0.35
Hydroxycinnamic acids 33.27 31.75 0.57
Quercetin 3-rhamnoside 68.28 67.59 0.90
Quercetin 3-xyloside 75.17 59.23 0.0154
Quercetin 3-arabinopyranoside 4.85 3.07 0.0007
Quercetin 3-rutinoside 14.66 8.90 0.0004
Quercetin 3-glucoside 39.77 28.48 0.0031
Quercetin 3-galactoside 166.83 124.25 0.0133
Cyanidin 3-xyloside 2.14 1.13 0.000001
Cyanidin 3-glucoside 0.91 0.45 0.000001
Cyanidin 3-galactoside 49.59 28.03 0.000002

In addition, cyanidin 3-

The presented data are the average values of five replicates at five sampling dates (n = 25). Corresponding P values of the treatment are also listed.

FW = fresh weight; GAE = gallic acid equivalents.

Pho Ca also significantly enhanced the formation of flavonols, which increased during the advanced ripening when comparing the zero and the fifth sampling dates (Fig. 2B). However, the difference between treated and untreated apples was less significant than in the case of anthocyanins. During the 5 weeks of ripening, the average values of total flavonols (mg kg⁻¹ FW) were 410.98 for the treated apples and 337.14 for the control (P = 0.04). The influence of Pho Ca was noticed in all individual quercetin–glycosides, except quercetin 3-O-rhamnoside. The most pronounced was in the case of quercetin 3-O-arabinopyranoside and quercetin 3-O-rutinoside (Table 1).

No significant influence of treatment was observed either in the case of the total phenolic content or in the levels of hydroxycinnamic acids, flavonols, and dihydrochalcones (Table 1), although in the treated apples, an increase in flavonols after the second application of the Pho Ca was detected (data not shown).

Among sugars, the highest sugar concentration was fructose, representing up to 55% of the total sugars followed by sucrose, glucose, and few sorbitol. Our results are in accordance with those obtained by Veberic et al. (2007).

Before the first application of Pho Ca (sampling date 0), there were no differences in individual and total sugar concentration in apples that were later subjected to different treatments. Analyzing the sampling dates 0 and 1, a significant interaction was observed in the case of sucrose (P = 0.02), whereas in the case of glucose (P = 0.08), fructose (P = 0.09), and total sugar concentration (P = 0.06), an interaction was marginally significant. The results obtained indicate that the first application of the Pho Ca had a significant effect on the synthesis of sugars in the treated apples. In the next 4 weeks of ripening (sampling dates 1 to 5), the sugars showed a similar accumulation pattern in both treatments (Fig. 2A). However, a significantly higher concentration of all the individual sugars, except fructose in the treated apples, was detected (Table 2). During ripening, the level of sucrose and glucose was significantly and positively (P ≤ 0.0002) correlated with the total anthocyanin concentration. The correlation between sucrose and anthocyanins was closer (r = 0.53) than between glucose and anthocyanins (r = 0.50).

The effect of the Pho Ca was also pronounced in the amount of total sugars. Over the 5 weeks of ripening, an average total sugar concentration (mg kg⁻¹ FW) in the treated apples was 101.1 and 94.97 in control apples.

Table 1. Concentration of analyzed phenolics (mg kg⁻¹ FW) and total phenolic content (mg GAE kg⁻¹ FW) in the skin of apples treated with the Phostrade Ca and control.

| Sugar/Glycoside | Phostrade Ca | Control | P Value |
|-----------------|-------------|---------|---------|
| Fructose        | 64.72       | 68.35   | 0.0001  |
| Glucose         | 34.66       | 31.75   | 0.0001  |
| Sucrose         | 410.98      | 337.14  | 0.04    |

Fig. 2. The concentration of total sugars (A) and flavonols (B) in apples treated with Phostrade Ca (○) and control (●) at different sampling dates (n = 5). Least significant differences (LSD) (5%) of the treatment are presented. Arrows indicate treatment application dates but samples have been taken before the respective application. FW = fresh weight.
(P = 0.0054). Regarding the amount of malic and citric acid, no influence of the Pho Ca treatment was detected (Table 2).

During the 5 weeks of ripening, no significant changes in PAL (P = 0.76) and CHS/CHI (P = 0.77) activities were detected (Fig. 3A–B). However, an increase in FHT and DFR activities (P < 0.001) was observed (Fig. 3C–D). At commercial harvest, the DFR activity was almost 2-fold and FHT activity almost 4-fold higher than at the beginning of advanced ripening (sampling date 0). FHT and DFR were significantly correlated with anthocyanin accumulation (P < 0.0001). The correlation between the anthocyanins and FHT (r = 0.70) was closer than between the anthocyanins and DFR (r = 0.61).

The significant influence of Pho Ca was seen only in the case of DFR (P = 0.01), in which the average activity (nkat·g⁻¹ FW) during ripening in the treated apples (0.001173) was 25.6% higher than the control ones (0.000934). Slightly higher activity was noticed also by FHT; however, the difference was not significant (P = 0.35).

Before the first application (sampling date 0), the leaf P concentration in the control (1.67 g·kg⁻¹) as well as in the treated leaves (1.41 g·kg⁻¹) was under the critical value of 1.8 g·kg⁻¹ proposed by Bergmann (1992) (Fig. 4). The measured values were similar to those in ‘Jonagold’ apple leaves reported by Wojcik and Wojcik (2007). The foliar application of the Pho Ca significantly increased the amount of P in the leaves of the treated apples. At commercial harvest, 28% higher P concentration in the treated leaves was detected when compared with the amount measured before the first application reaching the value of the critical concentration of 1.8 g·kg⁻¹. However, in the control apples, almost a 10% lower amount compared with the first sampling date was observed. Regarding the amount of Ca in the leaves, no significant influence of the Pho Ca treatment was detected (data not shown).

### Discussion

Application of Pho Ca significantly increased parameter a* and lowered the hue angle and lightness (Fig. 1). The improvement in the apple skin color as a result of foliar sprays of P-containing compounds has also been documented in several apple cultivars, including ‘Starking Delicious’ (Gómez-Cordovés et al., 1996; Larrigaudiere et al., 1996), ‘Fuji’ (Li et al., 2002), ‘Elstar’ (Funke and Blanke, 2006), and ‘Jonagold’ (Wojcik and Wojcik, 2007). In the first three cited studies, the positive effect of foliar P applications on the fruit color was related to increased skin anthocyanin concentration, whereas in the studies of Funke and Blanke (2006) and Wojcik and Wojcik (2007), the apple color improvement was evaluated with the percentage of red blush.

In our study, the onset of rapid anthocyanin formation occurred between the second and the third sampling dates, ≈3 weeks before the commercial harvest (Fig. 1D). Similar findings were also reported by Awad and de Jager (2002) who detected rapid color formation in ‘Jonagold’ apples ≈20 d before the harvest.

Two applications of Pho Ca also increased synthesis of anthocyanins and flavonols (Figs. 1D and 2B). Our results are similar to those of Li et al. (2002) who reported that accumulation of anthocyanins and flavonols in Seniphos-treated ‘Fuji’ apples was much higher than the untreated fruit. Seniphos, a P–Ca mixture (23.6% w/w P2O5, 3% w/w Ca, and 3% w/w nitrogen), has almost the same chemical composition as Pho Ca, which was used in our experiment. Higher accumulation of anthocyanins in Seniphos-treated apples was reported also in the study of Gómez-Cordovés et al. (1996) and Larrigaudiere et al. (1996) in ‘Starking Delicious’ apples. However, Awad and de Jager (2002) found no influence of Seniphos on the formation of anthocyanins and quercetin 3-glycosides in ‘Jonagold’ apples.

Generally, no significant effect of Pho Ca on the accumulation of other classes of flavonoids and total phenolics was observed (Table 1); however, an increase of the flavonoids in the treated apples after the second application (data not shown) corresponded well to the increase of DFR activity at the late stage of ripening (Fig. 3D).

So far the mechanism by which P-containing compounds affect the coloration of apples has been studied poorly and remains unclear. Li et al. (2002) reported that foliar spraying with the P-containing compound Seniphos greatly increased the PAL and CHI activities, suggesting that these enzymes are closely related to anthocyanin accumulation. The rapid increase of PAL activity directly related to the increase of anthocyanin biosynthesis was reported also by Larrigaudiere et al. (1996) in ‘Starking Delicious’ apples. However, PAL

### Table 2. Concentration of individual sugars (mg·kg⁻¹ FW), malic and citric acid (mg·kg⁻¹ FW) in the fruit of apples treated with Phostrade Ca and the control.¹

| Sugar          | Phostrade Ca | Control | P Value  |
|----------------|--------------|---------|---------|
| Fructose       | 50.86        | 50.17   | 0.52    |
| Sucrose        | 36.22        | 33.22   | 0.0048  |
| Glucose        | 9.90         | 8.06    | 0.0017  |
| Sorbitol       | 4.12         | 3.44    | 0.0093  |
| Malic acid     | 5.03         | 5.12    | 0.60    |
| Citric acid    | 0.91         | 0.96    | 0.20    |

¹The presented data are the average values of five replicates at five sampling dates (n = 25). Corresponding P values of the treatment are also listed.

FW = fresh weight.

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Fig. 3. Changes in enzyme activity (nkat·g⁻¹ FW) of PAL (A), CHS (B), FHT (C), and DFR (D) in ‘Braeburn’ apples treated with Pho Ca (□) and control (●) at different sampling dates. FW = fresh weight; PAL = phenylalanine ammonia lyase; CHS = chalcone synthase; FHT = flavanone-3-hydroxylase; DFR = dihydroflavonol 4-reductase.

Fig. 4. Phosphorus (P) levels in the leaves of apples treated with the Phostrade Ca (□) and control (●) at different sampling dates. Horizontal line presents the critical content for phosphor (according to Bergmann, 1992).
may not be a key enzyme during fruit maturation in apple (Lister and Lancaster, 1996; Saure, 1990) and CHS is not a regulatory enzyme in anthocyanin biosynthesis (Ju et al., 1995). In our study no increase in activities of these enzymes was detected (Fig. 3A–B), suggesting that PAL and CHS/CHI were not decisive for the increased anthocyanin accumulation. However, we found an increase in FHT and DFR, which was significantly correlated with the observed anthocyanin accumulation. The increase in DFR activity in treated apples corresponded well to the increase in flavonol concentration after the second application of Pho Ca (data not shown). Higher FHT and DFR activity in the last stages of ripening was observed also in the study of Slatnar et al. (2012) in ‘Florina’ apples.

The accumulation of anthocyanins and flavonols is also dependent on temperature (Arakawa, 1991) and on a regular supply of sugars in the plant (Lueangprasert et al., 2010). It has been suggested that low temperatures may reduce the loss of sugars in the skin by reducing respiration, which allows more sugar substrate for anthocyanin production (Lancaster, 1992). In our experiment, the amount of total sugars decreased at the beginning of the advanced ripening and at commercial harvest somehow reached the value measured at the beginning of the sampling (Fig. 2A). The decrease could be the result of the weather conditions, specifically high day and night temperatures, which stimulated the respiration processes in fruits. Approximately 3 weeks before the harvest, the night temperatures dropped, whereas day temperatures still remained quite high (Fig. 5). Just the drop in night temperatures may have reduced the loss of sugars in the skin of apples and thus stimulated anthocyanin biosynthesis. Rapid anthocyanin formation observed in our study at that time (Fig. 1D) confirms the mentioned hypothesis. Cold night temperatures followed by warm day temperatures have already been reported to stimulate anthocyanin synthesis in some apple cultivars (Revelle, 1990). It could be speculated that a higher accumulation of anthocyanins in the Pho Ca-treated apples could be the result of higher sugar concentration, especially those of sucrose and glucose (Table 2). The positive correlation between the sucrose and the anthocyanins and glucose and anthocyanins confirms that assumption. The stimulatory effects of sugars, especially sucrose, on anthocyanin biosynthesis have been already reported in several plant species, including *Vitis vinifera* cells (Vitrac et al., 2000), radish (*Raphanus sativus*) hypocotyls (Hara et al., 2003), mango (*Magnifera indica* Linn. cv. Mahajana) fruit exocarp (Lueangprasert et al., 2010), and *Arabidopsis* (*Arabidopsis thaliana*) seedlings (Teng et al., 2005).

In our study, the reason for higher synthesis of sugars in the treated apples could be the higher amount of P in the leaves of the treated apples (Fig. 4), because starch synthesis in the chloroplasts and transport of sugars across the chloroplast envelope into the cytoplasm are directly controlled by the concentration of inorganic phosphate (Heldt et al., 1977). However, so far the importance of the P and its influence on sugar concentration in apple fruit has been poorly investigated.

**Conclusions**

In the present study, we were able to demonstrate that two applications of the Pho Ca ≥5 weeks before harvest can markedly enhance the synthesis of anthocyanins and improve the red coloration of ‘Braeburn’ apple skin. In addition, the synthesis of most of the flavonols was also increased. Through the analysis of sugars and enzymatic activities, we were capable to evaluate some of the possible mechanisms by which the P-containing compounds affect the red coloration of apples. However, the color development is a complicated process; therefore, in the future, further research is needed to determine the function of P, sugar levels, and enzyme activities on red coloration of apple skin and thus get better insight in the mode of action of the Pho Ca or similar P-containing compounds.

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