UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA

Jan BIZJAK

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DOKTORSKA DISERTACIJA

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DOKTORSKA DISERTACIJA

THE REGULATION OF THE PHENOLIC BIOSINTHETIC PATHWAY AND ITS INFLUENCE ON THE APPLE (*Malus domestica* Borkh.) COLOR DURING THE ADVANCED MATURATION

DOCTORAL DISSERTATION

Ljubljana, 2014

Doktorska disertacija je zaključek podiplomskega študija bioloških in biotehnoloških znanosti ter se nanaša na znanstveno področje agronomije. Praktični deli poskusov so bili opravljeni v sadjarskem centru Gačnik v Mariboru ter na Laboratorijskem polju Biotehniške fakultete v Ljubljani. Laboratorijski del je bil izveden na Katedri za sadjarstvo, vrtnarstvo in vinogradništvo, Oddelka za agronomijo, Biotehniške fakultete v Ljubljani in inštitutu; Institut für Verfahrenstechnik, Umwelttechnik und Technische Biowissenschaften, Technische Universität Wien.

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Tema in naslov doktorske disertacije sta bila sprejeta na podlagi Statuta Univerze v Ljubljani ter po sklepu Senata Biotehniške fakultete in sklepa 28. Seje Komisije za doktorski študij Univerze v Ljubljani (po pooblastilu Senata Univerze v Ljubljani z dne 20. 1. 2009) z dne 16. 5. 2012. Za mentorja je bil imenovan izr. prof. dr. Robert VEBERIČ.

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Jan BIZJAK

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AL V študiji smo želeli pridobiti informacije o spremembah, ki se dogajajo v jabolkih med njihovim dozorevanjem v biosintezi fenolnih spojin ter ovrednotiti njihov pomen za razvoj barve plodov. Z uporabo rastnega regulatorja proheksadion kalcija (Pro-Ca) in foliarnega gnojila Phostrade Ca smo želeli raziskati vlogo aktivnosti encimov za razvoj barve plodov ter ugotoviti ali lahko s pomočjo teh pripravkov v kožici jabolk uravnavamo biosintezno pot fenolnih spojin in s tem vplivamo na obarvanost plodov. Sladkorje, organske kisline in širok spekter fenolnih spojin smo analizirali s pomočjo sistema visoko-tlačne tekočinske kromatografije v kombinaciji z masno spektometrijo (HPLC-MS), encimsko aktivnost pa s pomočjo radiografskega skeniranja tankoplastne kromatografije. Jesenska uporaba Pro-Ca je v plodovih prehodno povečala sintezo hidroksicimetnih kislin in zmanjšala sintezo antocianidinov in flavonolov v jabolkih, kar se je odrazilo v manjši jakosti in manjšem deležu krovne rdeče barve plodov. Pro-Ca je zmanjšal aktivnost encimov flavanon-3-hidroksilaze (FHT) in flavonol sintaze (FLS) ter ekspresijo genov antocianidin sintaze (ANS), antocianidin reduktaze (ANR), flavonoid 3-O-glikoziltransferaze (FGT) in transkripcijskega faktorja MYB10. Aktivnost ostalih analiziranih encimov je bila po tretiranju s Pro-Ca le rahlo zmanjšana. Spremembe, ki jih je Pro-Ca v fenilpropanoidni poti povzročil, so bile v kožici jabolk zaznavne le od 7 do 15 dni in se niso obdržale do tehnološke zrelosti plodov. Jesenska foliarna uporaba Phostrade Ca, ki je bila opravljena pet oziroma tri tedne pred tehnološko zrelostjo plodov, je povečala sintezo antocianov in flavonolov ter izboljšala obarvanost jabolk sorte 'Braeburn' ob obiranju v njihovi tehnološki zrelosti. Ugotovili smo, da večja aktivnost encimov PAL, CHS/CHI, FHT in DFR še ni zagotovila tudi večje sinteze posameznih fenolov oziroma skupin fenolnih spojin. V zadnjih petih tednih zorenja plodov se vsebnost hidroksicimetnih kislin, dihidrohalkonov in flavanolov v kožici jabolk ni bistveno spreminjala, medtem ko se je v tem obdobju pojavila povečana sinteza kvercetin glikozidov in antocianov, ki se je pospešeno začela dogajati približno tri tedne pred tehnološko zrelostjo plodov.

KEY WORD DOCUMENTATION

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AB In the study we wanted to obtain information on changes occurring in the phenolic biosynthetic pathway during the advanced maturation of apples and evaluate their importance for the peel color development of the fruit. Through the application of the plant growth regulator prohexadione calcium (Pro-Ca) and foliar fertilizer Phostrade Ca we wanted to investigate the role of enzymatic activity on the development of fruit color and determine whether we can regulate the biosynthetic pathway in the peel of apples and thereby influence the apple color development. Sugars, organic acids and a wide range of phenolic compounds were analyzed by the use of high performance liquid chromatography coupled with mass spectrometer (HPLC-MS), whereas enzymatic activity was measured by thin layer radiography. The application of Pro-Ca transient increased the synthesis of hydroxycinnamic acids and decreased the synthesis of anthocyanins and flavonols in apple peel, which resulted in a lower intensity and share of red coloration of apple peel. The application of Pro-Ca led to a decrease in the enzyme activities of the flavanone-3-hydroxylase (FHT) and flavonol synthase (FLS) while other enzymes analyzed were slightly inhibited by the Pro-Ca treatment. At the same time the expression of anthocyanidin synthase *(ANS),* anthocyanidin reductase *(ANR),* flavonoid 3-O-glycosyltransferase *(FGT)* and the transcription factor *MYB10* was downregulated after the Pro-Ca treatment. The changes in the flavonoid pathway in the Pro-Ca treated apple peel were detectable only from 7 up to 15 days and were not kept up till the technological maturity of the fruit. Autumn foliar application of Phostrade Ca, which took place five or three weeks before the technological maturity of the fruit increased the synthesis of anthocyanins and flavonols and improved the color of 'Braeburn' apples at their commercial harvest. We found out that the higher activity of the enzymes PAL, CHS/CHI, FHT and DFR did not guarantee a higher synthesis of individual phenols and phenolic groups. In the last five weeks of ripening, the content of hydroxycinnamic acids, flavanols and dihydrochalcones in apple peel did not change significantly, while an intensive accumulation of quercetin glycosides and anthocyanins took place during this period, starting with the onset of rapid formation approximately 3 weeks before the technological maturity of apples.

KAZALO VSEBINE

KAZALO SLIK

KAZALO PRILOG

OKRAJŠAVE IN SIMBOLI

Okrajšava/simbol	**Pomen**
ANR	antocianidin reduktaza
ANS	antocianidin sintaza
BF	Biotehniška fakulteta
CHI	halkon izomeraza
CHS	halkon sintaza
DFR	dihidroflavonol 4-reduktaza
FGT	flavonoid 3-O-glikoziltransferaza
FHT	flavanon 3-hidroksilaza
FLS	flavonol sintaza
GalT	galaktozil transferaza
GT	glikozil transferaza
HPLC-MS	visoka tekočinska kromatografija masnega spektra
PAL	fenilalanin amonijak liaza
Pho Ca	Phostrade Ca
Pro-Ca	proheksadion kalcij
SM	sveža masa
SUM	suha masa

1 UVOD

Obarvanost kožice jabolk predstavlja enega od ključnih parametrov zunanje kakovosti jabolk, ki lahko pomembno vpliva na odločitev potrošnikov za njihov nakup. Velik pomen ima tudi za pridelovalca, saj dobra oziroma zadostna obarvanost plodov omogoča prodajo jabolk po višji tržni ceni. Barva kožice jabolk je določena z odtenki zelene in rumene osnovne barve na eni ter odtenki rdeče krovne barve na drugi strani (Janick in sod., 1996). Zelena oziroma rumena barva kožice je posledica prisotnosti klorofilov in karotenoidov, medtem ko se rdeča krovna barva tvori na račun akumulacije antocianov in flavonolov (Lancaster, 1992). Poleg antocianov in flavonolov lahko na obarvanost jabolk v manjši meri vpliva tudi prisotnost procianidinov (Lister in sod., 1994). Rdeča barva se lahko izrazi v obliki črt ali pa je prelita in pokriva večino ploda.

Intenzivnost krovne rdeče barve je pri jabolkih večinoma pogojena z razmerjem, količino in vrsto antocianov, ki se v obliki granul kopičijo v vakuolah celic (Bae in Kim., 2006). Antociani se uvrščajo v eno od najpomembnejših in najraznolikejših podskupin fenolnih spojin, imenovano flavonoidi, ki obsega več kot 4000 različnih spojin (Hyson, 2011). Na podlagi molekulske strukture fenolne spojine v jabolkih razvrščamo v pet glavnih skupin, in sicer poleg antocianov med najpomembnejše spadajo še hidroksicimetne kisline, flavanoli, dihidrohalkoni in flavonoli (Tsao in sod., 2005). Z izjemo hidroksicimetnih kislin kopičenje ostalih flavonoidov in drugih fenolnih spojin poteka predvsem v kožici plodov, ki v primerjavi z mesom jabolka vsebuje od dva do šest krat več fenolnih spojin (Lata, 2009; Mikulic-Petkovsek in sod., 2007) ter dva do trikrat več flavonoidov (Wolfe, 2003). Tudi sinteza antocianov večinoma poteka le v kožici jabolk, čeprav so obstajale in se še vedno vzgajajo sorte, pri katerih se antociani akumulirajo tudi v mesu plodov (Espley, 2009).

Flavonoidi so eden od razredov fenolnih spojin, katere uvrščamo med sekundarne metabolite, ki v nasprotju s primarnimi niso bistveni za preživetje rastlin. Vendar pa s svojo prisotnostjo v jabolkih pomembno vplivajo tako na notranjo, kakor tudi izboljšujejo zunanjo kakovost plodov kot je na primer obarvanost kožice jabolk (Treutter, 2001). Antociani so glavni pigmenti v cvetovih jablane, kjer s privabljanjem čebel in ostalih opraševalcev igrajo pomembno vlogo pri opraševanju. V večjih količinah pa so prisotni tudi v kožici jabolk, kjer s privabljanjem ptic in ostalih frugivorov pripomorejo k hitrejšemu širjenju in razmnoževanju jablan. Flavonoli in flavoni ščitijo celice pred premočnim UV-B sevanjem, med drugim pa flavonoidi sodelujejo tudi pri fiksaciji dušika, transportu avksinov in obrambi rastline (Ronald in sod., 1994; Taiz in Zeiger, 2002).

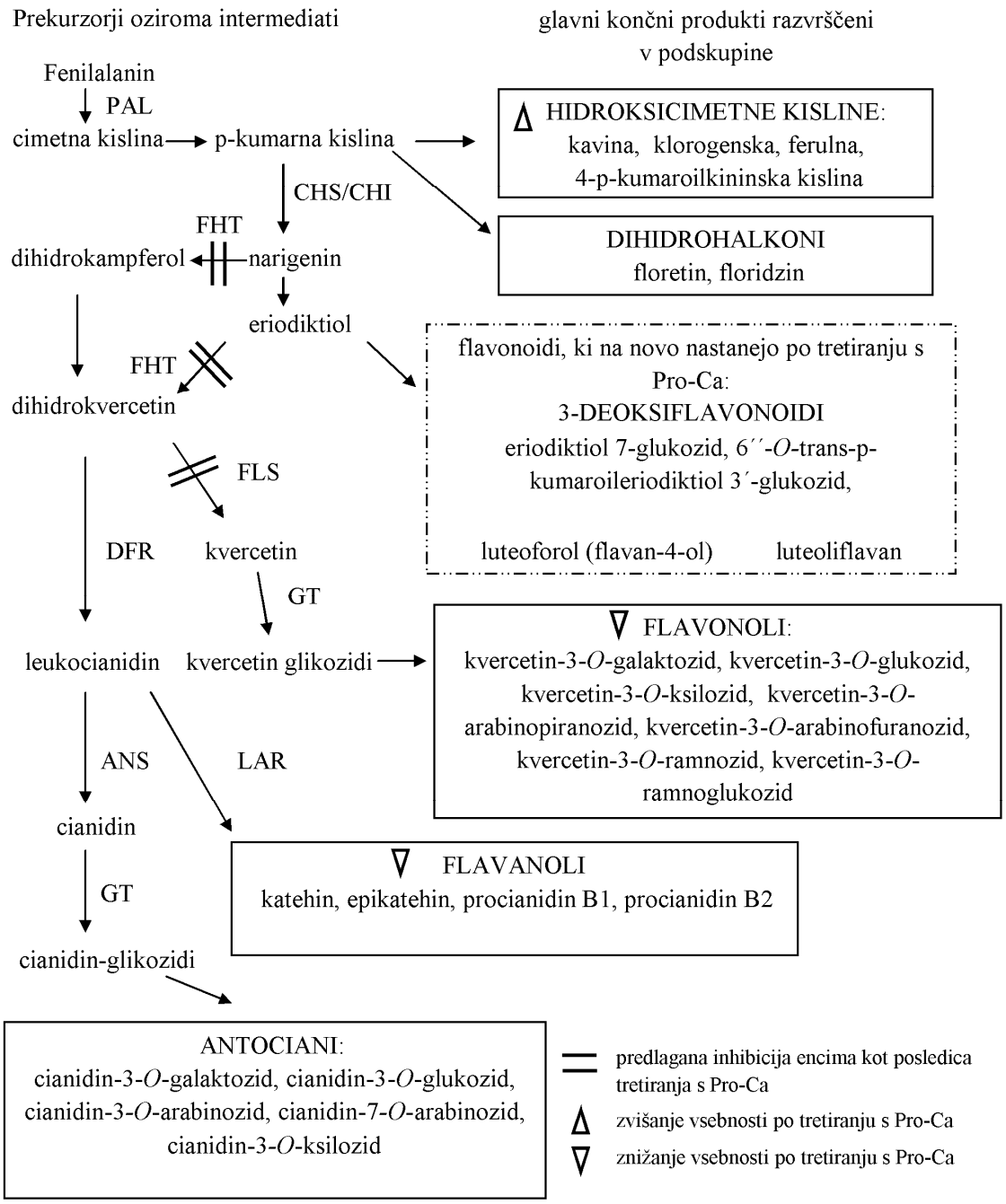
Flavonoidi in ostale fenolne spojine pa niso pomembni le za rastlino, ampak imajo vidno vlogo tudi v zdravi in uravnoteženi prehrani ljudi. Za številne fenolne spojine je bila namreč ugotovljena velika antioksidativna in antikarcenogena aktivnost (Lee in sod., 2003; Sun in Lu, 2008), ki lahko prepreči razvoj mnogih bolezni, med drugim raka, diabetesa, Alzheimerjeve, kardiovaskularnih in pljučnih bolezni (Hyson, 2011). Njihovo prehransko vrednost pa še dodatno povečuje tudi podatek, da jabolka, v primerjavi z ostalim sadjem, vsebujejo največji delež tako imenovanih prosto dostopnih fenolov, ki naj bi se lažje absorbirali v krvni obtok (Sun in sod., 2002).

Biosinteza antocianov v jabolkih se začne s primarnim prekurzorjem fenilalaninom in konča s končnim produktom glikozidi cianidina (Slika 1). V fenilpropanoidni metabolni poti poteka s pomočjo encimov vrsta kemijskih reakcij, katerih rezultat so številne skupine fenolnih spojin, med katerimi so najpomembnejše hidroksicimetne kisline, dihidrohalkoni flavonoli, flavanoli in antociani (Treutter, 2001). Biosinteza antocianov je genetsko kontrolirana s pomočjo strukturnih oziroma biosinteznih genov na eni ter regulatornih genov oziroma transkripcijskih faktorjev na drugi strani. Pri jablani je precej bolj raziskana prva skupina genov, ki kodira encime, ki so potrebni za sintezo antocianov (Telias in sod., 2011). Med regulatornimi geni je najpomembnejši MdMYB10, ki regulira izražanje mnogih encimov fenilpropanoidne poti (Takos in sod., 2006), medtem ko sta MdbHLH3 in MdbHLH33 s sintezo antocianov manj povezana (Telias, 2009). Tekom razvoja plodov naj bi bila s koncentracijo antocianov povezana predvsem raven prepisa genov encimov halkon sintaze (CHS), flavanon 3-hidroksilaze (FHT), dihidroflavonol reduktaze (DFR), antocian sintaze (ANS) in glikozil transfereze (GT) (Honda in sod., 2002; Takos in sod., 2006; Espley in sod., 2007). Ostali encimi, ki prav tako sodelujejo pri sintezi antocianov, so: fenilalanin amonijak-liaza (PAL), halkon izomeraza (CHI) in flavonol sintaza (FLS). Vpliv pomembnosti delovanja posameznih encimov še vedno ni točno pojasnjen, niti ni znano, kateri del fenilpropanoidne poti je za sintezo antocianov ključnega pomena (Ju in sod., 1995b; Lancaster, 1992).

PAL je encim, ki katalizira prvo reakcijo fenilpropanoidne poti, deaminacijo L-fenilalanina do transcimetne kisline in amoniaka ter povezuje primarni in sekundarni metabolizem rastlin (Lister in sod., 1996). Njegova aktivnost je največja v mladih plodičih, močno pade med rastjo plodičev in pri sortah, ki akumulirajo antociane, ponovno naraste med dozorevanjem plodov (Macheix in sod., 1990; Lister in Lancaster, 1996). Čeprav naj bila sinteza antocianov povezana s povečanjem aktivnosti encima PAL, pa je na voljo več raziskav, kjer so avtorji zabeležili njegovo aktivnost, akumulacije antocianov pa ni bilo (Lister in sod., 1996). Aktivnost PAL-a naj bi bila po mnenju Ju in sodelavcev (1995a) za sintezo antocianov ključna le v primeru, če med razvojem plodov primanjkuje substratov ali prekurzorjev, ki so potrebni za akumulacijo antocianov.

Halkon sintaza (CHS) je prvi encim v fenilpropanoidni poti, ki omogoča nastanek fenolnih spojin s petnajstimi ogljikovimi atomi, kamor uvrščamo tudi flavonoide. V številnih rastlinah naj bi bila njena aktivnost povezana s sintezo antocianov, pri jablanah pa je delovanje tega encima slabo raziskano (Ju in sod., 1995b). Slednji poročajo, da CHS ni imela regulatorne vloge oziroma njena aktivnost ni bila omejujoč dejavnik pri sintezi antocianov v jabolkih. Do podobnih zaključkov pa so prišli tudi Lister in sod. (1996) pri spremljanju aktivnosti halkon izomeraze (CHI).

Pomen aktivnosti flavanon-3-hidroksilaze (FHT), ki katalizira pretvorbo flavanonov (narigenina in eriodiktiola) v dihidroflavonole (dihidrokampferol in dihidrokvercetin) in flavonol sintaze (FLS), ki omogoča pretvorbo dihidrokvercetina v kvercetin (Slika 1), je slabo raziskan. Aktivnost FHT in FLS je bila spremljana predvsem v jablaninih listih v povezavi s proučevanjem vpliva tretiranja s proheksadion kalcijem na ta dva encima, katerih aktivnost Pro-Ca zavira (Halbwirth in sod., 2003).



Slika 1. Poenostavljeno prikazana biosintetska pot fenolnih snovi z najpomemnbenjšimi intermediati, encimi ter končnimi produkti pri jablani in vpliv tretiranja s proheksadion kalcijem. PAL, fenilalanin amonijak-liaza, CHS, halkon sintaza, CHI, halkon flavonon izomeraza, DFR, dihidroflavonol-4-reduktaza, FHT, flavanon-3-hidroksilaza, FLS, flavonol sintaza, GT, glikozil transferaza, LAR, leukocianidin reduktaza, ANS, antocianidin sintaza.

Figure 1: Simply presented flavanoid biosynthesis pathway and the influence of Pro-Ca treatment in an apple with the most important intermediates, enzymes and endproducts. PAL, phenylalanine ammonia lyase; CHS, chalcone isomerase; CHI, chalcone syntase; DFR, dihydroflavonol 4-reductase; FHT, flavanon 3-hydroxylase; FLS, flavonol synthase; LAR, leucoanthocyanidin reductase; GT, glucosyl transferase; ANS, anthocyanidin synthase.

Dihidroflavonol reduktaza (DFR) katalizira pretvorbo dihidrokvercetina v levkocianidin, ki predstavlja prekurzor za antocianidine in flavanole (katehin, epikatehin in procianidine). Med drugim ima DFR v listih jablane sposobnost katalizirati ključno reakcijo, ki pripelje do nastanka 3-deoksiflavonoidov (Fisher in sod., 2003). Aktivnost DFR se z dozorevanjem jabolk povečuje (Treutter, 2001), pomen njegove aktivnosti v povezavi s sintezo antocianov pa je prav tako slabo raziskan.

Antocianidin sintaza (ANS) katalizira pretvorbo leukocianidina (flavan-3, 4-ol) v cianidin, ki se nato pretvori v stabilnejšo obliko antocianin. ANS prav tako spada med deoksigenaze, katerih aktivnost v jablaninih listih tretiranje s Pro-Ca zmanjšuje (Halbwirth in sod., 2006).

Zadnji v vrsti ter eden od najpogosteje proučevanih in najpomembnejših encimov za sintezo antocianov pa je glikozil transferaza (GT) oziroma galaktozil transferaza (GalT), ki katalizira transformacijo nestabilnega cianidina v stabilni antocianin (Ju in sod., 1995a). Avtorji v svoji raziskavi predpostavljajo, da je pomen njegove aktivnosti za sintezo antocianov odvisen predvsem od količine razpoložljivega cianidina. V njihovi študiji je bila aktivnost GalT pozitivno povezana z akumulacijo antocianov, a le v obdobju dozorevanja plodov, zato sklepajo, da je njegova regulatorna vloga odvisna tudi od razvojnega stadija plodov. Ključni korak v biosintezi antocianov tako po njihovem mnenju predstavlja pretvorba dihidrokvercetina v levkocianidin oziroma cianidin. Podobnega mnenja so tudi Lister in sod. (1996), ki dodajajo, da je aktivnost encimov PAL, CHI in GT sicer potrebna za sintezo antocianov, vendar še ne zagotavlja, da bo ta res potekala. Hkrati domnevajo, da bi lahko pri sintezi flavonoidov pomembno vlogo imeli tudi izoencimi, ki so natančneje proučeni in poznani predvsem za PAL encim.

Akumulacija antocianov oziroma razvoj rdeče obarvanosti jabolk je odvisna od različnih dejavnikov, med katerimi je najbolj ključen svetloba oziroma delež osvetlitve (Saure, 1990). Njen pomen so v svoji študiji lepo ovrednotili Ju in sod. (1995a), ki so ugotovili, da je odstranitev vrečk z jabolk nekaj tednov pred njihovim obiranjem močno spodbudila sintezo flavonoidov, med katerimi pa so le antociani dosegli nivo, ki so ga imela jabolka, ki z vrečkami niso bila pokrita. Ostali pomembni dejavniki so še podlaga drevesa (Saure, 1990), temperatura (Arakawa, 1991), genetska struktura sorte (Treutter, 2001), razvojni stadij tkiva oziroma celic (Ju in sod., 1995a), izločanje etilena (Whale in Singh, 2007) in abscizinske kisline (Kondo in sod., 1991), mineralna prehrana rastlin oziroma razpoložljivost in dostopnost hranil (Awad in Jager, 2002a), razpoložljivosti ogljikovih hidratov v povezavi z obloženostjo dreves (Treutter, 2001) ter uporaba različnih agrotehničnih ukrepov (Whale in sod., 2008). Koncentracija antocianov v kožici jabolk kaže tekom razvoja plodov dva vrhova; prvi se pojavi med celično delitvijo v mladih nerazvitih plodičih (Mata in sod., 2006), drugi pa med pospešenim dozorevanjem jabolk, še posebno v zadnjem mesecu pred tehnološko zrelostjo oziroma obiranjem plodov (Lister in sod., 1994).

Najučinkovitejši agrotehnični ukrepi, ki jih raziskovalci navajajo kot zelo uspešne pri doseganju boljše obarvanosti plodov so: uporaba odbojnih folij (Jakopič in sod., 2010, Blanke, 2008), nadzorovano gnojenje z dušikom (Treutter, 2001), poškodovanje dreves (Chalmers in Faragher, 1977), prekrivanje plodov s papirnatimi vrečkami (Hudina in

Štampar, 2011), namakanje (Iglesias in sod., 2002) in uporaba različnih kemičnih pripravkov (Whale in sod., 2008).

Pri sortah, kjer prevladuje rdeča krovna barva kožice, je zaželeno, da ta pokriva čim večjo površino plodov. Večji delež rdeče krovne barve pri jabolkih so nekateri raziskovalci poskušali doseči s foliarno uporabo gnojil na osnovi kalcija in fosforja (senifos, Phostrade Ca,...). Senifos; zmes fosforja in kalcija, ki se najpogosteje uporablja v koncentraciji: 310 g/L P_2O_5; 40 g/L kalcija, se je uporabil za doseganje boljše obarvanosti že v več študijah, vendar pa njegov princip delovanja še vedno ni poznan (Li in sod., 2002). Kot najbolj verjeten mehanizem delovanja se omenja hipoteza, da naj bi na izboljšano obarvanost in kakovost plodov vplival preko spremenjene mineralne sestave jabolk, predvsem večjega deleža kalcijevih ionov (Larrigaudiere in sod., 1996). Njegov učinek je primerljiv z etefonom (2-kloroetil fosforna kislina). Slednji se v največji meri uporablja za pospeševanje dozorevanja plodov mnogih sadnih vrst, pogosto pa je bil uporabljen tudi v poskusih z namenom spodbuditve tvorbe antocianov in doseganja boljše obarvanosti jabolk (Gómez-Cordovés in sod., 1996; Larrigaudiere in sod., 1996; Awad in Jager, 2002b; Li in sod., 2002). Prednost senifosa v primerjavi z etefonom naj bi bila tudi, da prvi ne vpliva na poslabšanje skladiščnih sposobnosti plodov (Larrigaudiere in sod., 1996).

Med ostalimi pripravki za katere avtorji navajajo, da so prav tako vplivali na sintezo barve, najdemo: aminoetoksivinglicin (AVG), različne gibereline (GA_{4+7}, GA_3), sintetični avksin 2,4- diklorofenoksipropionsko kislino (2,4-DP), daminozid (Alar), cikocel (CCC), raztopino glukoze in galaktoze, pripravka vitamina E (25 % alfa-tokoferol) in uporabo šikiminske kisline (Wang in Dilley, 2001; Awad in Jager, 2002b, Stover in sod., 2003; Whale in sod., 2007).

Pri nekaterih sortah jabolk, kot je na primer 'Granny Smith', pa prisotnost rdeče krovne barve ni zaželena, zato bi bilo koristno poznati mehanizem, s katerim bi se nezaželeni rdeči obarvanosti lahko izognili. Ker je aktivnost encimov tudi eden od pomembnih dejavnikov, ki vpliva na sintezo antocianov, bi z blokado aktivnosti določenih encimov potencialno lahko uravnavali sintezno pot antocianov in ostalih fenolnih spojin ter s tem vplivali na barvo plodov. Ena od spojin, ki dokazano regulira biosintezno pot fenolnih spojin, je tudi proheksadion kalcij (Pro-Ca). Slednji se v prvi vrsti uporablja kot retardant z namenom zmanjšati rast vegetativnih poganjkov (Rademacher in Kober, 2003). Med drugimi pa raziskovalci poročajo tudi, da je Pro-Ca vplival na manjše pojavljanje nekaterih škodljivcev (Krawczyk in Greene, 2002), škrlupa (Bazzi in sod., 2003), hruševega ožiga (Spinelli in sod., 2005) ter nekaterih ostalih bakterijskih in glivičnih bolezni (Rademacher in Kober, 2003).

Pro-Ca je strukturno zelo podoben 2-oksoglutaratu, s čimer zmanjšuje delovanje 2-oksoglutarat odvisnih deoksigenaz (2-OODs); flavanon 3 hidroksilaze (FHT), flavonol sintaze (FLS) ter antocianidin sintaze (ANS). S tem uravnava biosintezno pot fenolnih spojin in povzroči značilne spremembe v fenolnem profilu jabolk (Halbwirth in sod., 2003; Halbwirth in sod., 2006; Fisher in sod., 2006). V jablaninih listih naj bi povzročil predvsem manjšo vsebnost flavonolov (Roemmelt in sod. 2003a; Mikulič-Petkovšek in sod., 2009). Na drugi strani pa blokada omenjenih encimov in zmanjšana sinteza nekaterih flavonoidov lahko povzroči kopičenje nekaterih ostalih fenolnih spojin oziroma intermediatov fenilpropanoidne poti. Za liste jablane avtorji poročajo predvsem o povečani

vsebnosti hidroksicimetnih kislin (Roemmelt in sod., 2003a; Mikulič Petkovšek in sod., 2009). S toksikološkega oziroma ekotoksikološkega vidika je uporaba Pro-Ca zelo sprejemljiva, saj se spojina po uporabi relativno hitro razgradi in se ne kopiči v tretiranih rastlinah (Rademacher in Kober, 2003). Učinek njegovega delovanja je bil v listih viden le 10 dni (Fischer in sod., 2006), v tkivih ostalih rastlin pa je trajal do 15 dni po tretiranju (Halbwirth in sod., 2003). Tretiranje s Pro-Ca pa poleg kopičenja oziroma zmanjšanja nekaterih fenolnih spojin na drugi strani privede tudi do nastanka novih flavonoidov, 3-deoksiflavonoidov (Roemmelt in sod., 2003a; Halbwirth in sod., 2006; Fisher in sod., 2006). Med njimi se v jablaninih listih po tretiranju s Pro-Ca najpogosteje pojavijo eriodiktiol 7-glukozid, 6´´-*O*-trans-p-kumaroileriodiktiol 3´-glukozid, luteoforol in luteoliflavan (Slika 1), ki drugače niso prisotni (Roemmelt in sod., 2003a).

Ker je bil vpliv tretiranja s Pro-Ca proučevan predvsem v jablaninih listih in v plodovih ni bilo narejenih še skoraj nobenih raziskav, ni znano, če je mehanizem delovanja Pro-Ca in njegov učinek na biosintezno pot fenolnih snovi v plodovih enak kot v listih. Kot je že bilo omenjeno, se Pro-Ca v nasadih jablan v prvi vrsti uporablja predvsem za umirjanje rasti vegetativnih poganjkov. Za ta namen tretiranje s Pro-Ca poteka v spomladanskem času na začetku rastne sezone, medtem ko o učinku tretiranja v jesenskem času ni na voljo nobenih podatkov. Tako Mata in sod. (2006) ter Medjoub in sod. (2005) pri jabolkih sorte ´Fuji´, ki so bila tekom razvoja plodov dvakrat tetirana s Pro-Ca, za tretirana jabolka poročajo o večjem deležu rdeče krovne barve in večji tvorbi antocianov v primerjavi z netretiranimi jabolki. Razlog za pozitiven učinek Pro-Ca na obarvanost so bili verjetno krajši poganjki z manjšimi listi, ki so omogočili prodor večje količine svetlobe do plodov in s tem povzročili večjo tvorbo antocianov oziroma boljšo obarvanost plodov. Kakorkoli, s pomočjo tretiranja s Pro-Ca v jesenskem času in blokade encimske aktivnosti določenih deoksigenaz, bi potencialno lahko uravnavali sintezno pot antocianov, flavonolov in nekaterih ostalih fenolnih spojin ter s tem zmanjšali oziroma preprečili tvorbo rdeče obarvanosti kožice jabolk, kar bi bilo koristno in zanimivo predvsem pri nekaterih sortah jabolk kot je na primer 'Granny Smith', kjer prisotnost rdeče krovne barve ni zaželena.

S pomočjo uporabe kemičnega pripravka, ki naj bi rdečo obarvanost kožice jabolk povečal (Phostrade Ca) in pripravka, ki bi jo potencialno lahko zmanjšal (proheksadion kalcij), smo v treh ločenih poskusih v treh rastnih sezonah želeli natančneje proučiti spremembe, ki se v fenilpropanoidni poti v kožici jabolk dogajajo v zadnjem stadiju dozorevanja plodov ter hkrati ugotoviti, ali lahko z njuno uporabo v jesenskem času vplivamo na razvoj rdeče krovne barve pri jabolkih. Vsi trije poskusi so bili opravljeni na jablanah sorte 'Braeburn', saj se je slednja že v prvem poskusu izkazala kot zelo primerna za raziskovanje in vrednotenje sprememb v obarvanosti kožice plodov. Z analizo encimatske aktivnosti smo želeli ovrednotiti tudi pomen encimov pri razvoju barve pri jabolkih ter ugotoviti, ali bi ti lahko bili ključni mehanizem, preko katerega pripravek na obarvanost vpliva.

<u>Poskus 1: Vpliv tretiranja z rastnim regulatorjem proheksadion kalcijem na vsebnost fenolnih spojin in obarvanost jabolk med dozorevanjem plodov</u>

V poskus je bila vključena sorta 'Braeburn'. V analizo vsebnosti fenolov in delovanja encimov sta bili vključeni kožica in pulpa plodov. Poskus se je izvajal na lokaciji Sadjarskega centra Maribor (Gačnik). Cilj poskusa je bil ugotoviti, kako jesensko tretiranje s proheksadion kalcijem vpliva na vsebnost fenolnih spojin v jabolkih in posledično na

obarvanost plodov. Prav tako smo s spremljanjem aktivnosti encimov želeli ugotoviti, kakšen je encimatski odziv fenilpropanoidne poti po tretiranju in na aktivnost katerih encimov tretiranje vpliva.

Poskus 2: Vpliv proheksadion kalcija na razvoj barve in vsebnost fenolnih spojin v jabolkih med dozorevanjem ter zunanje in notranje kakovostne parametre jabolk med skladiščenjem

V prvem poskusu smo ugotovili, da je Pro-Ca vplival na zmanjšano sintezo nekaterih flavonolov in antocianov ter posledično tudi na slabšo obarvanost plodov. Vendar pa je bil njegov učinek delovanja kratkotrajen, saj smo vpliv tretiranja zabeležili le v obdobju prvih petnajstih dni od samega škropljenja, medtem ko ga kasneje in ob sami tehnološki zrelosti plodov nismo več zaznali. Zato smo s sledečim poskusom želeli ugotoviti, kakšen bo vpliv tretiranja, če odmerek razdelimo na dva odmerka, med katerima naredimo 14-dnevni presledek. Prav tako smo tudi v tem poskusu proučevali encimatski odziv fenilpropanoidne poti po tretiranju s Pro-Ca, opravili pa smo tudi analizo ekspresije genov najpomembnejših encimov. Ob tehnološki zrelosti plodov smo del jabolk vključenih v poskus obrali in jih dva meseca skladiščili v hladilnici, s čimer smo želeli ugotoviti, če oziroma kako uporaba proheksadion kalcija med zorenjem jabolk vpliva na vsebnost primarnih in sekundarnih metabolitov in skladiščno sposobnost plodov.

Poskus 3: Vpliv jesenske uporabe Phostrade Ca na razvoj barve in vsebnost fenolnih spojin v jabolkih med dozorevanjem

V sledeči poskus smo vključili foliarno gnojilo na osnovi fosforja in kalcija (Phostrade Ca) ter ugotavljali, ali z njegovo uporabo med dozorevanjem plodov lahko pripomoremo k boljši obarvanosti jabolk. Prav tako smo tudi v tem poskusu proučevali encimatski odziv fenilpropanoidne poti po tretiranju z omenjenim pripravkom. Z analizo sladkorjev v plodovih in koncentracije fosforja in kalcija v listih pa smo želeli tudi ovrednotiti potencialno možne mehanizme delovanja samega pripravka.

Barvo plodov smo v vseh treh poskusih merili s pomočjo kolorimetra Minolta CR-200 b, ki smo ga pod pravim kotom prislonili na vedno isto mesto soncu izpostavljenemu delu ploda in odčitali vrednost parametrov L*, a*, b*, in h. Spremembe v obarvanosti smo opisali s pomočjo barvnega sistema L* a* b* (CIELab), kjer je barvni prostor definiran s svetlostjo L* in barvnima koordinatama a* in b*.

Postavili smo naslednje hipoteze:

- jesensko tretiranje s Pro-Ca med zorenjem jabolk preko regulacije fenilpropanoidne poti vpliva na zmanjšano sintezo antocianov in flavonolov ter posledično zmanjša delež in intenziteto rdeče krovne barve kožice plodov;
- učinek delovanja Pro-Ca je v kožici jabolk podoben, kot je že bil ugotovljen in dokumentiran v jablaninih listih;
- trajanje učinka oz. delovanja Pro-Ca na biosintezno pot fenolnih snovi v kožici jabolk je podoben trajanju delovanja, ki je že bil dokumentiran v jablaninih listih.
- v kožici jabolk aktivnost encimov PAL, CHS/CHI, FHT, FLS in DFR ni ključnega pomena za sintezo antocianov;

- jesenska uporaba pripravkov na osnovi fosforja in kalcija poveča sintezo antocianov in flavonolov ter posledično poveča delež krovne rdeče barve kožice plodov.

Rezultati analize sekundarnih metabolitov-fenolov in z njimi povezane encimatske aktivnosti in ekspresije genov bodo prispevali dodatne informacije o sintezni poti fenolnih spojin, ki bodo koristile nadaljnjim raziskavam s področja dinamike razvoja barve plodov. Razumevanje procesov fenilpropanoidne poti, pomena aktivnosti encimov in njihove vloge pri nastajanju intermediatov je ključnega pomena za možnost njihovega uravnavanja. Informacije o učinkovanju preučevanih kemičnih pripravkov pa bodo pridelovalcem jabolk lahko v veliko pomoč v prizadevanjih za doseganje boljše obarvanosti jabolk oziroma potencialno možni izognitvi rdeči obarvanosti plodov pri sortah, kjer ta ni zaželena.

2 ZNANSTVENA DELA

2.1 POZNA UPORABA PROHEKSADION KALCIJA VPLIVA NA VSEBNOST FENOLNIH SPOJIN IN ZMANJŠA VSEBNOST ANTOCIANOV V JABOLKIH SORTE 'BRAEBURN' MED DOZOREVANJEM

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V poskusu smo raziskovali vpliv jesenske uporabe pripravka na osnovi rastnega regulatorja proheksadion kalcija (Pro-Ca) na metabolizem fenolnih spojin in razvoj barve pri jabolkih sorte 'Braeburn' med njihovim dozorevanjem. Jablane smo poškropili 3 tedne pred tehnološko zrelostjo plodov ter nato v šestih terminih vzorčenja mesa in kožice ugotavljali spremembe v vsebnosti hidroksicimetnih kislin, dihidrohalkonov, flavanolov, flavonolov in antocianov. V kožici plodov smo dodatno analizirali tudi aktivnost encimov FHT, FLS, CHS in DFR, s čimer smo želeli dobiti jasnejšo sliko o poteku fenilpropanoidne poti in ugotoviti ali je učinek in mehanizem delovanja Pro-Ca v kožici jabolk podoben tistemu, ki je že bil ugotovljen v jablaninih listih. Vpliv Pro-Ca na obarvanost kožice jabolk smo dodatno ovrednotili s pomočjo beleženja barvnih parametrov a*, hº in L* ter sistema CIE L*a*b*. Pro-Ca je povzročil manjšo intenziteto in manjši delež rdeče krovne barve kožice in zmanjšal aktivnost encimov FHT in FLS, vendar pa je bil učinek le prehodnega značaja in je trajal manj kot 20 dni. Pri tretiranih jabolkih smo v kožici zabeležili značilno manjše vsebnosti dihidrohalkonov, flavonolov in antocianov, medtem je bila vsebnost skupnih fenolov in hidroksicimetnih kislin v tretiranih jabolkih v primerjavi z netretiranimi, značilno večja. V mesu plodov smo v skoraj vseh terminih vzorčenja v tretiranih jabolkih zabeležili večje vsebnosti večine fenolnih spojin kot v kontrolnih jabolkih. Rezultati poskusa so pokazali, da je Pro-Ca v kožici in mesu jabolk reguliral sintezno pot fenolnih spojin na podoben način kot je že bilo ugotovljeno za liste, ter da do neke mere ima potencial, da zmanjša rdečo obarvanost kožice, kar bi lahko bilo koristno pri zelenih sortah jabolk, kjer ta ni zaželena.

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Late Prohexadione-Calcium Application on Maturing Apple cv. 'Braeburn' Fruit Reduces Anthocyanins and Alters the Phenolic Content

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Summary

In this study the influence of prohexadione-Calcium (Pro-Ca) treatment on peel colour development and polyphenol metabolism of apples during advanced maturity was investigated. Apple trees were treated 3 weeks before technological maturity. Changes in the content of hydroxycinnamic acids, dihydrochalcones, flavonols, flavanols and anthocyanins were monitored six times during the advanced maturation until technological maturity of the fruits. To evaluate the effect of Pro-Ca on the coloration of apples, the changes in the chromaticy values a*, h° and the lightness coefficient L* were monitored. The parameters showed a significant difference in the intensity of red coloration between the treated and untreated apples. The application of Pro-Ca led to a decrease in the enzyme activities of the 2-oxoglutarate dependent dioxygenases flavanone-3-hydroxylase (FHT) and flavonol synthase (FLS), and the flavonoid pathway was negatively affected in general. Concomitantly, the concentrations of flavonols, anthocyanins and dihydrochalcones in the peel of treated apples decreased, whereas the contents of total phenolic compounds and hydroxycinnamic acids increased. Flavanol contents, however, remained unchanged. In apple pulp, slightly higher concentrations of all phenolic compounds were measured in the treated apples compared to the control on the majority of sampling dates. The results indicate that Pro-Ca modulates the polyphenol spectrum resulting in a pronounced decrease of red coloration during advanced maturation of apples. Pro-Ca therefore to some extent exhibits the potential to avoid undesired red coloration of apple fruit.

Key words. Anthocyanins – flavonoids – fruit colour – phenolic acids – prohexadione Calcium

Introduction

Prohexadione-Calcium (Pro-Ca) is a multi-functional plant bioregulator currently being used in a number of countries on apple and some other fruit trees (Rademacher and Kober 2003). Treating fruit trees with Pro-Ca resulted in the reduction of shoot growth (Medjoub et al. 2004), reduced incidence of insect pests (Krawczyk and Greene 2002), and reduced incidence of fire blight and other bacterial and fungal diseases (Rademacher and Kober 2003). The compound acts as a structural mimic of 2-oxoglutarate, thereby inhibiting 2-oxoglutarate-dependent dioxygenases. This leads to significant changes in the flavonoid spectrum of apple (Roemmelt et al. 2003a; Halbwirth et al. 2006). When applied in high dosage, anthocyanin formation and red coloration of fruits and flowers (Lancaster 1992; Lancaster et al. 1997) can also be inhibited (Rademacher et al. 1992). In apple fruits, Pro-Ca treatments caused a significant decrease in the formation of flavanols and flavonols, whilst hydroxycinnamic acid concentrations increased significantly (Mikulic-Petkovsek et al. 2009).

The accumulation of anthocyanins is influenced by many factors including light (Saure 1990), nitrogen fertilization (Treutter 2001), ethylene (Whale and Singh 2007) temperature and cultural practices (Lancaster 1992). Coloration of the apple peel is primarily based on the presence of anthocyanins, but other flavonoids, such as flavonols (quercetin 3-*O*-glycosides) and proanthocyanidins, also have some influence (Lister et al. 1994). Apple peel colour is of great commercial importance; hence different techniques have been used to improve coloration (Whale et al. 2008). However, in some green apple cultivars like 'Granny Smith', red coloration is not desirable and a deep green colour is usually preferred by the majority of its consumers.

Pro-Ca treatments can have divergent effects on peel colour development when applied once or twice in spring (MATA et al. 2006). An observed enhancement of red coloration in the case of cv. 'Fuji' might be the consequence of shoot growth inhibition or of the activation of the polyphenol metabolism by Pro-Ca as previously observed in apple leaves (FISCHER et al. 2006).

In the present study, the red-coloured cultivar 'Braeburn' was selected as a model plant to investigate the potential of Pro-Ca treatment to alter the levels of fruit coloration, total and individual phenolic content and related enzyme activities in treated fruit compared to the control. The objective was to determine if the application of Pro-Ca during the advanced ripening in autumn can reduce the red coloration of apples and if it alters the phenolic metabolism in a similar way as has previously been reported for apple leaves. This is the first study on autumn application of Pro-Ca on apple fruit. The results will be interesting for a further detailed research on apple phenolic metabolism as well as for fruit producers, which strive to effectively reduce the undesired red colour formation in certain apple cultivars.

Materials and Methods

Chemicals

For the quantification of phenolic compounds the following standards were used: chlorogenic acid (5-caffeoylquinic acid), phloretin and rutin (quercetin-3-*O*-rutinoside) from Sigma Aldrich Chemie (Steinheim, Germany), cyanidin-3-*O*-galactoside chloride, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, (−)-epicatechin, *p*-coumaric acid, procyanidin B2 and phloridzin dihydrate from Fluka Chemie (Buchs, Switzerland), quercetin 3-*O*-arabinofuranoside and quercetin-3-*O*-xyloside from Apin Chemicals (Abingdon, UK) and (+)-catechin from Roth (Karlsruhe, Germany). Methanol for the extraction of phenolics was acquired from Sigma. Chemicals for the mobile phases were the high performance liquid chromatography–mass spectrometry (HPLC-MS) grade acetonitrile and formic acid from Fluka Chemie. Water for mobile phase was bidistilled and purified with the Milli-Q system (Millipore, Bedford, MA, USA). For determination of the total phenolic content, Folin–Ciocalteu phenol reagent (Fluka Chemie GmBH), sodium carbonate (Merck, Darmstadt, Germany), gallic acid and ethanol (Sigma) were used. Substrates for enzyme assays were synthesised as described (GOSCH et al. 2003).

Plant materials

The trial was conducted in 2010 on 10-year-old trees of striped 'Braeburn' cultivar clone Hillwell grafted on M9 rootstock, grown according to the system of integrated production at the fruit growing center Maribor Gacnik location (latitude 46° 61', longitude 15° 68'). Full bloom (> 80 % of the opened flower buds) occurred on 22 April 2010. On 16 September 2010 (147 DAFB), approximately three weeks before technological maturity (169 DAFB), five trees were sprayed with 1 g (2.5 kg ha^{-1}) Regalis (BASF 125 10W-10 % Pro-Ca, BASF, Germany) per tree, whereas the control trees were sprayed with water.

Colour measurements

At each sampling date colour measurements on 50 randomly chosen fruit from the outside of the tree canopy (10 fruit per tree) up to a height of 2 m from the ground were performed. The peel colour was measured using a portable colorimeter (CR-200 b Chroma; Minolta, Osaka; Japan) and L*, a*, and b* values were recorded. The colorimeter was calibrated with a white standard calibration plate before use. In the CIE L*a*b* system of colour representation, the L* value corresponds to a dark-bright scale and represents the relative lightness of colours with a range from 0 to 100 (0 = black, 100 = white). Hue angle (h°), the most appropriate way of representing changes in colour, was also calculated ($\tan^{-1}$ b*/a*) and expressed in degrees from 0 to 360, where 0° = red, 90° = yellow, 180° = green and 270° = blue (MCGUIRE 1992).

Fruit sampling

Fruit sampling commenced five days after the treatment (152 DAFB) and was performed six times during the advanced fruit maturation until reaching technological maturity, which was evaluated using the starch iodine test. At the date of harvest the value of firmness was 60 N and the starch index was 2.8. The sampling dates were 5, 8, 12, 15, 19, and 22 d after the Pro-Ca treatment. At each sampling date 10 fruits were randomly harvested and combined in five samples (2 fruit per sample, n = 5). Immediately after harvest, apples have been transported to the laboratory, where peel was separated from pulp for the extraction of individual and total phenolic compounds. One part of the apple peel was kept frozen at −80 °C until enzymatic analysis.

Extraction and determination of phenolic compounds

The extraction of fruit samples (peel and pulp separately) 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. 5 g of pulp and 2 g of peel 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 in order to prevent oxidation. After extraction, the treated samples were centrifuged for 5 min at 12,000 g_n. The supernatant was filtered through a Chromafil AO-45/25 poly-

amide 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 flavanols were detected at 280 nm, flavonols at 350 nm and anthocyanins at 530 nm. For the separation of phenolic compounds Phenomenex (Torrance, CA) 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 (A) and 100 % acetonitrile (B). Samples were eluted according to the linear gradient described by Marks et al. (2007): 0–5 min, 3 % to 9 % B; 5–15 min, 9 % to 16 % B; 15–45 min, 16 % to 50 % B; 45–50 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 UV-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 (ESI) operating in the negative and positive ion mode. For the analyses full-scan data dependent MS^n scanning from m/z 115 to 2000 was used. Quantification was achieved according to the concentrations of a corresponding external standard. For the compounds lacking standards, similar 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*-xylosylglucoside in equivalents of phloridzin, quercetin-3-*O*-arabinopyranoside in equivalents of quercetin-3-*O*-arabinofuranoside and anthocyanins (cyanidin-3-arabinoside, cyanidin-3-glucoside, and cyanidin-3-xyloside) were quantified in equivalents of cyanidin-3-galactoside. Concentrations of the phenolic compounds were expressed in mg kg^{-1} of fresh weight (FW) for fruit peel and pulp separately.

Determination of the total phenolic content

Extracts of the samples were obtained in the same way as for the 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-Ciocalteau reagent were added; after resting between 8 s and 8 min at room temperature, 1.5 ml of sodium carbonate (20 % w/v) and 1.9 ml bidistilled water was 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, UV/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 (GAE) in mg kg^{-1} fresh weight (FW) of fruit peel and pulp. Absorption was measured in three replications.

Enzyme preparations

For determination of chalcone synthase and dihydroflavonol synthase activities, enzyme preparations were obtained according to Claudot and Drouet (1992). For FHT and FLS the protocol of Thill (2010) was used: 0.2 g plant material was ground with 0.2 g Polyclar AT, 0.2 g silica sand and 3 ml of extraction buffer and then centrifuged for 10 min at 4 °C and 10,000 g. The extraction buffer was a 0.1 M HEPES buffer pH 7.3 containing 1.5 g sucrose 1.5 g polyethylene glycol 20000, 2 g Na-ascorbate, 0.015 g dithioerythritol and 0.36 g $CaCl_2$ per 100 ml. Enzyme preparations were generally subjected to gel chromatography to remove low-molecular compounds with the exception of those used for the demonstration of the competitive FHT inhibition of Pro-Ca.

Analyses of enzyme activity

Enzyme assays were performed as described previously (Slatnar et al. 2010). FHT assays showing the competitive FHT inhibition of Pro-Ca were performed according to Fischer et al. (2006). All values represent an average of three independent biological repetitions.

Statistics

The data was analyzed using the Statgraphics Plus 4.0 program (Manugistics. Inc.; Rockville, Maryland, USA). Data from all the analyses was tested for any differences among treatments using one-way analysis of variance (ANOVA). Significant differences among treatments were determined independently for each sampling date using the least significant difference test (LSD), whereas the differences between sampling dates for each treatment were tested using the Duncan test. In both cases, the significance level was 0.05. In diagrams, the means as well as their standard errors are presented (mean ± SE). For the determination of the association between the hue angle and total anthocyanins and a* and total anthocyanins Spearman's rank correlation was calculated.

Results and Discussion

To evaluate the potential of Pro-Ca applications of preventing undesired red fruit coloration during advanced fruit maturity, the effect of Pro-Ca was determined as the altered levels of fruit coloration, polyphenol contents and related enzyme activities in treated fruit compared to the control.

The hue angle, which represents the most appropriate way of evaluating red colour changes (McGuire 1992), decreased during advanced fruit maturation (Fig. 1A), indicating an increasing colour intensity. This is in agreement with the results of Whale and Singh (2007), who monitored colour development of 'Pink Lady' apple. In our experiment, the measured control values were over 31 % lower than on the first sampling date. However, in the Pro-Ca treated apples the decline in hue angle was lower than 25 %. Pro-Ca significantly influenced the hue angle, indicated by significantly higher values of treated apples compared to the control 8 d after the treatment until technological maturity at all sampling dates. Similarly, an influence of Pro-Ca treatment on the a* parameter was also determined at the same sampling dates (Fig. 1B). The a* parameter increased from 15.97 to 25.90 in the Pro-Ca treated apples and from 17.46 to 28.78 in the controls. A higher a* value is in connection with the increasing red coloration of apples (McGuire 1992). The reduction of the hue angle and the increase of a* were significantly ($P \leq 0.001$) correlated with the increase in concentrations of total anthocyanins ($r_s = -0.72$, and 0.74 respectively) (Fig. 4), therefore the development of red colour during advanced maturity can be ascribed to the increased anthocyanin concentration.

The lightness coefficient L* decreased during advanced maturity. This indicates darker hues of the fruit peel. Unlike the parameters a* and h°, significant differences in L* value between the treatments were measured only three days before and at technological maturity (Fig. 1C). At that time the apples of the control were significantly darker than the apples treated with Pro-Ca. An increase in the peel darkness was probably the consequence of the increased anthocyanin concentration, due to a greater proportion of darker red vacuoles and several layers of red cells (Lancaster et al. 1997).

Anthocyanins were only present in the apple peel and the main anthocyanin was cyanidin-3-*O*-galactoside. The total anthocyanin content increased during advanced ripening (Fig. 1D) and varied between 93.74 and 193.84 mg kg^{-1} FW. The average value measured was comparable to earlier reports (Jakopic et al. 2007). Pro-Ca significantly reduced the content of anthocyanins 5, 12 and 15 d after treatment (Fig. 1D). Treated apple peels contained from 59.41 to 130.25 mg anthocyanins per kg FW. The biggest difference between means was

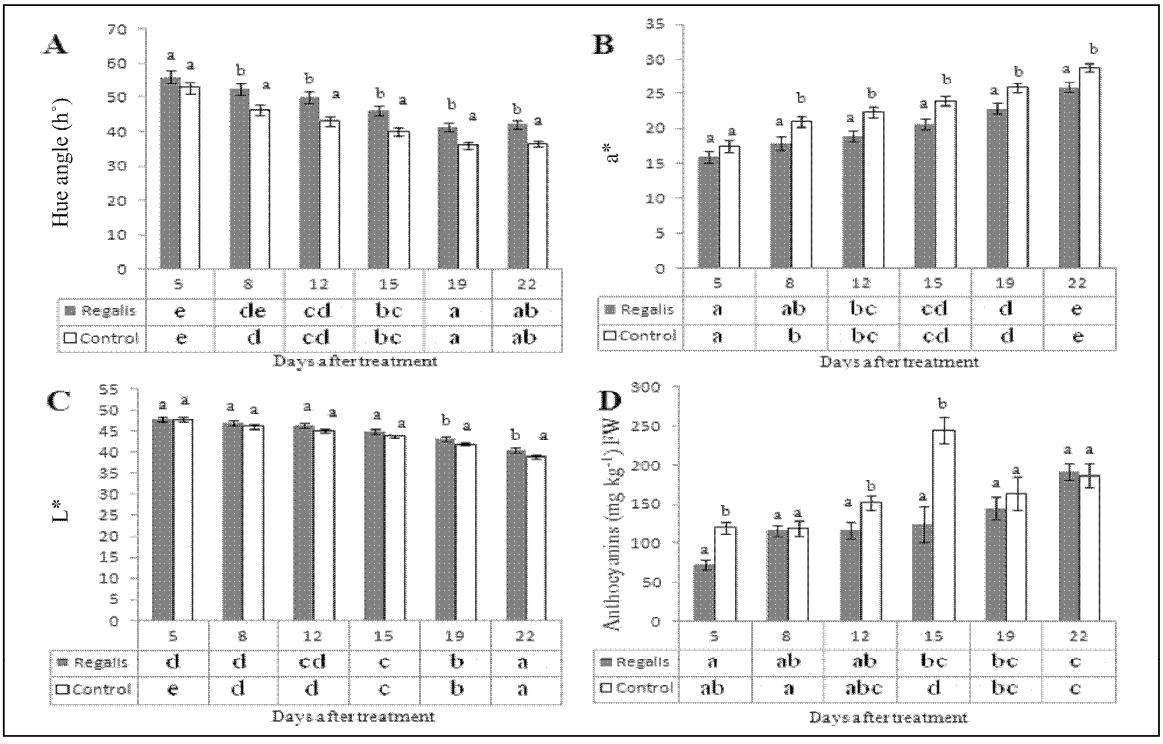

Fig. 1. Colorimetric parameters hue angle (h°) (A), a* (B), L* (C) and the content of anthocyanins of the 'Braeburn' apples (D) treated with prohexadione-Calcium ■ and control □ at different sampling dates.
Different letters above each column denote significant differences between treated and untreated apples on each sampling date (LSD test, $\alpha \leq 0.05$). Different letters in the table denote significant differences among sampling dates for each treatment (Duncan test, $\alpha \leq 0.05$). Vertical bars represent SE.

detected 15 d after treatment, when an almost 50 % lower content of anthocyanins was measured in the treated apples compared to the control. At technological maturity, however, there was no significant difference between the treated and untreated apples.

In our study 19 polyphenols were identified in apple peel belonging to five groups: hydroxycinnamic acids, flavonols, dihydrochalcones, flavanols and anthocyanins. In apple pulp, 13 polyphenols from the groups of hydroxycinnamic acids, dihydrochalcones, flavanols and flavonols were determined. Rutin (quercetin-3-*O*-rutinoside), quercetin-3-*O*-arabinofuranoside, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside, cyanidin-3-*O*-glucoside, and cyanidin-3-*O*-xyloside were detected and quantified only in the apple peel. In general, apple peel contained significantly higher amounts of phenolic compounds than apple pulp, which agrees with the results reported by Lata et al. (2009) and Veberic et al. (2010). The content of flavonols in the peel was as much as 1000 fold higher compared to pulp.

Total phenolic content in apple peel was quite constant during advanced fruit maturation, whereas a significant decrease was observed in the pulp regardless of the treatment (Fig. 2A, 2B). Also, in the study of Zheng et al. (2012) a decrease of total phenolics was noted during the final ripening stages. However, in their study whole apples where analyzed and apple pulp represents the major part of the fruit. Simultaneously, an increase in fruit weight and diameter has been reported, probably due to increased cell size. Therefore, the reduction of total phenolics could be due to the dilution effect which is probably much more pronounced in the apple pulp. Pro-Ca treated apples contained statistically higher amounts of total phenolic compounds in the pulp 8, 12, 15 and 19 d after the treatment and 19 and 22 d after the treatment in the peel. Our results are different from those reported by Mikulic-Petkovsek et al. (2009) who found lower total polyphenol contents in Pro-Ca treated apples compared to the control during the major part of the growing period and also at technological maturity. The reason for different results obtained in our study could be ascribed to a different influence of the Pro-Ca treatment on the total phenolics applied during the advanced ripening or early in the spring.

Among hydroxycinnamic acids, chlorogenic acid, caffeic acid and 4-coumaroylquinic acids were determined. A significantly higher content of total hydroxycinnamic acids in the peel of treated apples compared to the control was found 8 and 19 d after the treatment (Fig. 3A). In apple pulp, higher contents of hydroxycinnamic acids in the Pro-Ca treated apples were measured at the majority of sampling dates, however, the differences were significant only 8 d after treatment (Fig. 3B).

Our results are in agreement with Roemmelt et al. (2003a, b) who found higher content of hydroxycinnamic acids in Pro-Ca treated leaves. Since the effect of Pro-Ca on apple fruit in our study was visible for a longer period in comparison with those reported for the leaves, this could be the reason for the higher content of total hydroxycinnamic acids in the peel meassured 19 d after the treatment. Our results are similar to those of Mikulic-Petkovsek et al. (2009), who also found higher content of hydroxycinnamic acids in Pro-Ca treated fruits. However, at technological maturity they also did not find significant differences between the treated and untreated fruits. Hydroxycinnamic acid accumulation may be explained as a consequence of the FHT inhibition which on the one hand provokes an upstream accumulation of flavonoids and related precursors, while, on the other hand, the production of cinnamic acid precurors is accelerated to make up for the depletion of downstream flavonoids (Roemmelt et al. 2003a; Fischer et al. 2006). An increase of hydroxycin-

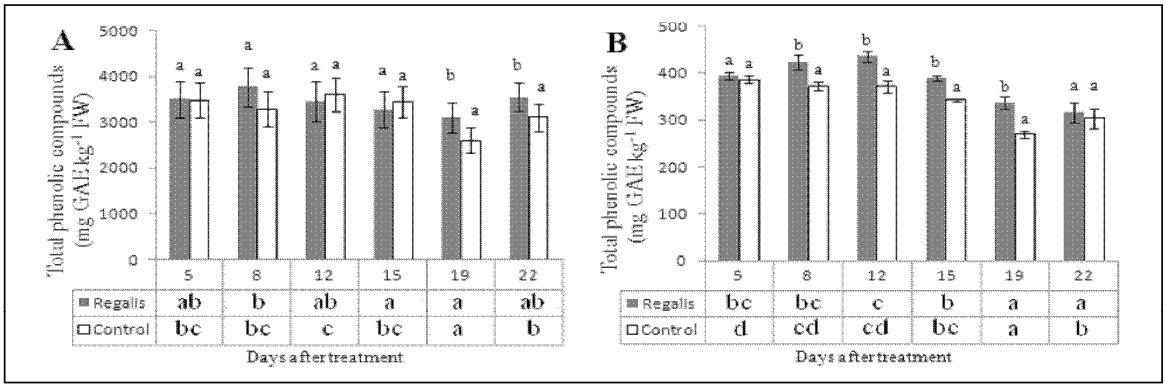

Fig. 2. Content of total phenolic compounds (mg GAE kg^{-1} FW) in the peel (A) and in the pulp of 'Braeburn' apples (B) treated with prohexadione-Calcium ■ and control □ at different sampling dates. Different letters above each column denote significant differences between treated and untreated apples on each sampling date (LSD test, $\alpha \leq 0.05$). Different letters in the table denote significant differences among sampling dates for each treatment (Duncan test, $\alpha \leq 0.05$). Vertical bars represent SE.

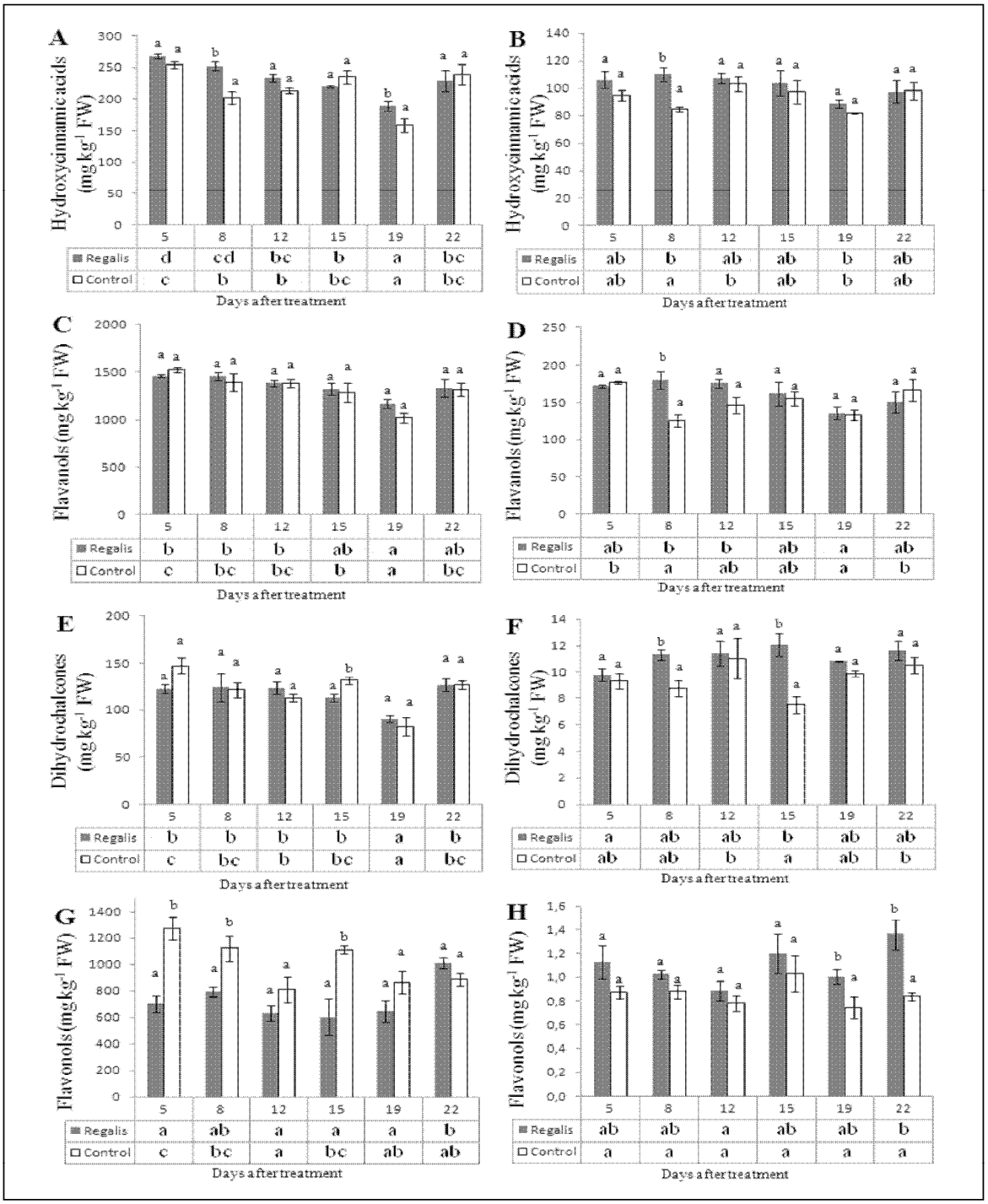

Fig. 3. Content of total hydroxycinnamic acids, flavanols, dihydrochalcones and flavonols (mg kg^{-1} FW) in the peel (A, C, E, G) and in the pulp (B, D, F, H) of 'Braeburn' apples treated with prohexadione-Calcium ■ and control □ at different sampling dates.
Different letters above each column denote significant differences between treated and untreated apples on each sampling date (LSD test, $\alpha \leq 0.05$). Different letters in the table denote significant differences among sampling dates for each treatment (Duncan test, $\alpha \leq 0.05$). Vertical bars represent SE.

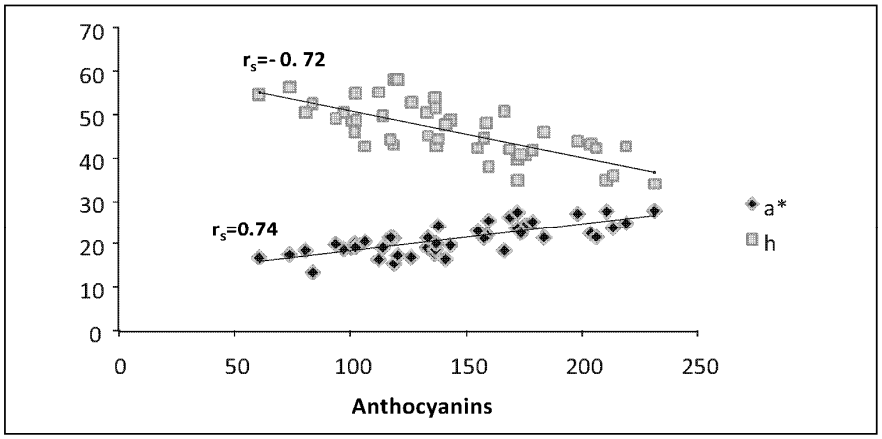

Fig. 4. Relationship between the colorimetric parameters hue angle (h°) and the content of anthocyanins and a* and the content of anthocyanins of 'Braeburn' apples during advanced ripening (r_S: Spearman's rank correlation coefficient ($P \leq 0.001$)).

namic acids caused by the Pro-Ca treatment was also found by Gosch et al. (2003) in *Actinidia arguta* leaves.

The following flavanols were determined in apple peel and pulp: catechin, epicatechin, procyanidin B1 and procyanidin B2. The content of total flavanols in the peel of the treated apples and controls were similar on the majority of sampling dates and no significant influence of Pro-Ca treatment was noted (Fig. 3C). In the pulp, significantly higher content of flavanols in the treated apples 8 d after the treatment was measured, whereas on the other sampling dates no significant differences were found (Fig. 3D). Our results differ from those reported by Mikulic-Petkovsek et al. (2009), who found a decrease in the flavanols content in apples treated with the Pro-Ca on the majority of sampling dates and also at technological maturity. It must be considered, however, that in their study the Pro-Ca application was done in the spring. Therefore, this might be the reason for different results of the influence of Pro-Ca on the content of flavonols compared to our study. Similarly Roemmelt et al. (2003a) also reported a significant decrease in oligomeric flavanols of apple leaves.

Among dihydrochalcones, phloretin and phloridzin (phloretin 2'-*O*-β-D-glucoside) were analyzed in apple peel and pulp. 15 d after the treatment, a statistically lower amount of total dihydrochalcones in the peel of the treated apples compared to the control was measured (Fig. 3E). At that sampling date, the treated apples contained statistically less phloridzin, whereas for phloretin no significant differences between means were noted (data not shown). However, at other sampling dates no significant differences in total dihydrocalchones content between the treated apples and the control were detected. In the pulp, on the contrary, a higher content of total dihydrocalchones in treated apples was detected. The difference between means was significant 8 and 15 d after the treatment with Pro-Ca (Fig. 3F). A decrease in phloridzin content was also observed in Pro-Ca treated fruits (Mikulic-Petkovsek et al. 2009); however, at technological maturity, no differences could be determined between treated and untreated fruits. Ruehmann and Treutter (2003) reported a significant reduction of phloridzin in treated apple leaves.

Among flavonols, hyperin (quercetin-3-*O*-galactoside), quercitrin (quercetin-3-*O*-rhamnoside), isoquercitrin (quercetin-3-*O*-glucoside) and quercetin-3-*O*-xyloside were identified in apple peel and pulp. In the peel, rutin (quercetin-3-*O*-rutinoside) and quercetin-3-*O*-arabinofuranoside were present as well. Quercetin-3-*O*-galactoside was the major flavonol in apple peel accounting for approx. 50 % of the total flavonols analyzed, whereas the content of rutin was lowest. Similar findings were also reported by Jakopic et al. (2010) in 'Fuji' apples. Pro-Ca caused a significant decrease of total flavonols in apple peel as early as five days after the treatment (Fig. 3G). The most pronounced differences in the contents of total flavonols were detected in the peel of the treated and untreated apples fifteen days after treatment. However, at technological maturity, no differences in the content of flavonols between the treatments were detected. In the pulp, on the other hand, 19 d after the treatment and at technological maturity, total flavonols in treated apples was higher than controls (Fig. 3H). Similary, Roemmelt et al. (2003a) also reported a significant decrease of the flavonols hyperin, isoquercetin, rutin and quercetin in Pro-Ca treated apple leaves. Similar effects were reported in other fruit species (Gosch et al. 2003) and rose leaves (Schlangen et al. 2003).

A decrease in the anthocyanin formation in treated apples is the consequence of the structural similarity between Pro-Ca and 2-oxoglutaric acids. This leads to a temporary blockage of the 2-oxoglutarate-dependent dioxygenases flavanone 3-hydroxylase (FHT), flavonol synthase (FLS) and anthocyanidin synthase (ANS) which play a key role in the biosynthesis of anthocyanins and other flavonoids (Halbwirth et al. 2006). The competitive inhibition by Pro-Ca was demonstrated in our study by measuring FHT activity in the absence of surplus 2-oxoglutarate and using enzyme preparations which were not

Table 1. Flavanone 3-hydroxylase, flavonol synthase, chalcone synthase/chalcone isomerase and dihydroflavonol 4-reductase activity of apples treated with prohexadione-Calcium (Pro-Ca) (as % of the respective control value) at different sampling dates.

		Days after treatment		
		5	15	22
FHT activity (% rel.)	Pro-Ca	59 %	53 %	98 %
FLS activity (% rel.)	Pro-Ca	87 %	68 %	97 %
CHS/CHI activity (% rel.)	Pro-Ca	100 %	84 %	84 %
DFR activity (% rel.)	Pro-Ca	98 %	60 %	75 %

subjected to gel chromatography as described previously (Fischer et al. 2006). The FHT inhibition in Pro-Ca treated apples could be observed until 15 d after the treatment. At technological maturity, however, there were no differences in the levels of anthocyanins between the treated and untreated apples (Table 1). This can be explained by the 10 to 15 d half-life of Pro-Ca and its rapid metabolisation in plant tissues (Halbwirth et al. 2003). However, the effect of Pro-Ca on apple fruit was visible for a longer period than on the leaves, where the inhibition was completely suspended 10 d after treatment (Fischer et al. 2006). Pro-Ca did not result in a complete blockage of FHT in the fruits, but in an approx. 50 % inhibition. This was also observed for leaves and creates a bottleneck leading to the observed changes in the polyphenol spectrum. In contrast to the leaves (Fischer et al. 2006), Pro-Ca treated fruits generally showed a universal reduction of all enzymes of the flavonoid pathway tested (Table 1). This is in agreement with the changes observed in the polyophenol spectrum, which was characterized by a strong decrease of anthocyanin and flavonol content and an increase of early intermediates such as hydroxycinnamic acids.

Conclusions

In the present study we were able to demonstrate, that prohexadione-Calcium significantly reduced the content of flavonols, anthocyanins and the red coloration of apple peel, when applied during advanced maturation of the apples. Contrary to that, total phenolic content in apple peel and pulp increased. The impact on the content of other polyphenol groups was only slightly noticeable and differed in apple peel and pulp. Moreover, it has been determined that the physiological response of the treated apples in our experiment was quite different from the response which was noticed in young developing leaves and fruits by the researchers, who applicated Pro-Ca in two replications in spring time. Thus it was demonstrated, that Pro-Ca to some extent has the potential to avoid undesired red coloration of apple fruit.

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2.2 VPLIV PHOSTRADE CA NA VSEBNOST ANTOCIANOV IN RAZVOJ BARVE JABOLK SORTE 'BRAEBURN' (Malus *domestica* Borkh.)

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Influence of Phostrade Ca on color development and anthocyanin content of 'Braeburn' apple (Malus *domestica* Borkh.)

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V raziskavi smo želeli raziskati vpliv foliarne uporabe Phostrade Ca (Pho Ca) na vsebnost primarnih in sekundarnih metabolitov jabolk sorte 'Braeburn' in ugotoviti, ali pripravek na osnovi fosforja lahko poveča sintezo antocianov in flavonolov ter posledično izboljša rdečo obarvanost jabolk med njihovim dozorevanjem. Pho Ca smo v koncentraciji 0,5 % prvič aplicirali pet tednov pred tehnološko zrelostjo plodov ter nato tedensko vzorčili jabolka za analizo vsebnosti hidroksicimetnih kislin, dihidrohalkonov, flavanolov, flavonolov in antocianov. Drugo škropljenje smo v enakem odmerku opravili 14 dni kasneje. Ob vsakem vzorčenju smo beležili tudi meritve parametrov a*, hº in L* ter s pomočjo sistema CIE L*a*b* dodatno opisali spremembe obarvanosti plodov. Da bi ugotovili mehanizem delovanja samega pripravka, smo spremljali in analizirali tudi spremembe v aktivnosti encimov PAL, CHS, FHT, FLS in DFR, vsebnostih posameznih in skupnih sladkorjev v plodovih ter spremembe v vsebnostih fosforja in kalcija v listih. Parametri obarvanosti so pokazali značilno razliko v intenziteti in deležu rdeče krovne barve med tretiranimi in netretiranimi jabolki. Tretiranje s Pho Ca se je odrazilo v značilno večjih vsebnostih posameznih in skupnih sladkorjev, flavonolov in antocianov v kožici ter značilno večjo vsebnost fosforja v listih. Pho Ca je značilno povečal DFR in rahlo FHT aktivnost, medtem ko ni vplival na aktivnost PAL in CHS. Prav tako uporaba Pho Ca ni vplivala na vsebnost skupnih fenolov, hidroksicimetnih kislin, dihidrohalkonov in flavanolov. Rezultati poskusa so pokazali, da dvakratna jesenska uporaba Phostrade Ca približno pet oziroma tri tedne pred tehnološko zrelostjo plodov lahko občutno poveča sintezo antocianov in flavonolov, s čimer veliko pripomore k lepši obarvanosti in večjemu deležu rdeče krovne barve jabolk sorte 'Braeburn' ob njihovem obiranju.

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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 (FHT) 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 (Treutter, 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 (Lueangprasert 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 coloration, 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 (Gómez-Cordovés 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 P_2O_5, 4.3% w/w CaO, and 3% w/w nitrogen) was applied at a concentration of 0.5% (company recommendation). Control trees were sprayed with water. The second spraying was done 14 d later on 23 Sept.

Fruit color. The skin color was measured using a portable colorimeter (CR-200 b Chroma; Minolta, Osaka, Japan) and recording L^*, a^*, and b^* color coordinates on the fruit surface (McGuire, 1992). Before measuring, the colorimeter was calibrated with a white standard calibration plate. The data were expressed also

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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 of phenolic substances and stored at –20 °C until the preparation of the samples. For the determination of enzyme activity, one part of the apple skin was stored frozen at –80 °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, Stauden, Germany). Samples were left for 30 min at room temperature and stirred frequently. After extraction, the homogenate was centrifuged (Eppendorf 5810 R Centrifuge, 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 RezexROA-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-caffeoylquinic acid), phloretin, and rutin (quercetin 3-*O*-rutinoside) from Sigma Aldrich Chemie (Steinheim, Germany); cyanidin 3-*O*-galactoside chloride, quercetin 3-*O*-rhamnoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, (–)-epicatechin, *p*-coumaric acid, procyanidin B2, and phloridzin dihydrate from Fluka Chemie (Buchs, Switzerland); quercetin 3-*O*-arabinofuranoside and quercetin 3-*O*-xyloside from Apin Chemicals (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 acetonitrile 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 used. 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*-xylosylglucoside in equivalents of phloridzin, quercetin 3-*O*-arabinopyranoside in equivalents of quercetin 3-*O*-arabinofuranoside, and anthocyanins (cyanidin 3-arabinoside, cyanidin 7-arabinoside, cyanidin 3-glucoside, and cyanidin 3-xyloside) were quantified in equivalents of cyanidin 3-galactoside. Concentrations of the phenolic compounds were expressed in mg·kg^{-1} of 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–Ciocalteau 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–Ciocalteau reagent were added; after resting between 8 s and 8 min at room temperature, 1.5 mL of sodium carbonate (20% w/v) and 1.9 mL 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. 2011 (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 molybdovanadate 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 H_3BO_3 + 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 (KH_2PO_4/K_2HPO_4) + 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 6.8.

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).

Analyses 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 least significant difference test, whereas the differences between sampling dates were tested using the Duncan test. In both cases, the significance level was 0.05. The relationships were tested at $P \leq 0.05$. For the determination of the association between the sucrose/glucose and total anthocyanins and enzyme activity and total anthocyanins, Pearson's correlation coefficient was calculated.

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

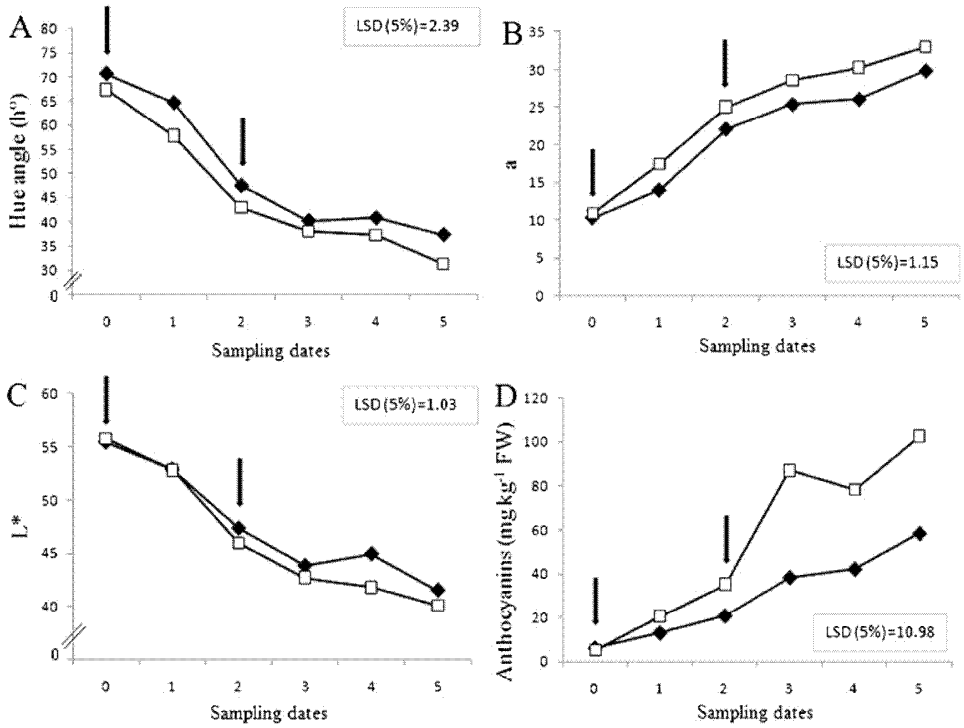

Fig. 1. Colorimetric parameters hue angle (*h*°) (**A**), *a** (**B**), *L** (**C**), and the concentration of anthocyanins of the 'Braeburn' apples (**D**) treated with Phostrade Ca (□) and control (♦) at different sampling dates (n = 20). Least significant differences (LSD) (5%) of the treatment are presented. Arrows indicate treatment application dates but measurements have been done before the respective application. FW = fresh weight.

the treated apple skin contained 102.57 mg anthocyanins per kg FW, whereas the concentration in the control was only 58.52 mg per kg FW.

Before the first application (sampling date 0), no significant differences in the total anthocyanin concentration between the treated and untreated apples were observed. Pho Ca significantly increased anthocyanin accumulation. During the 5 weeks of ripening, the anthocyanin level in the treated apples increased almost 20-fold, whereas the control increased only nine times. The average value for the treated apples was 64.72 mg·kg^{-1} FW, whereas average of the control was 34.66 mg·kg^{-1} FW (P = 0.00001). Cyanidin 3-*O*-galactoside was the main cyanidin glycoside in the apple skin, representing 80% to 86% of the total anthocyanins. Concentrations at commercial harvest were 82.02 in the treated apples and 47.33 mg·kg^{-1} FW in the control. In addition, cyanidin 3-*O*-arabinoside, cyanidin 7-*O*-arabinoside, cyanidin 3-*O*-xyloside, and cyanidin 3-*O*-glucoside, which were present in smaller amounts, were also analyzed. Pho Ca significantly increased accumulation of all cyanidin glycosides and the power impact of treatment on all of them was very similar (Table 1).

Regarding the flavonols, quercetin 3-*O*-galactoside (hyperin), quercetin 3-*O*-rutinoside (rutin), quercetin 3-*O*-rhamnoside (quercitrin), quercetin 3-*O*-arabinofuranoside, quercetin 3-*O*-arabinopyranoside, quercetin 3-*O*-glucoside (isoquercitrin), and quercetin 3-*O*-xyloside (reynoutrin) were analyzed. Among them, quercetin 3-*O*-galactoside represented the largest share, accounting for ≈40% of the total amount analyzed, whereas the concentration of quercetin 3-*O*-arabinopyranoside was the lowest. Similar findings were previously reported by Bizjak et al. (2012) with the difference that the concentrations were lower in the present study.

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 ($P \leq$ 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 FW) in the skin of apples treated with the Phostrade Ca and control.[z]

	Phostrade Ca	Control	P Value
Cyanidin 3-galactoside	49.59	28.03	0.000002
Cyanidin 3-arabinoside	5.08	2.59	0.000002
Cyanidin 3-glucoside	0.91	0.45	0.000001
Cyanidin 3-xyloside	2.14	1.13	0.000001
Cyanidin 7-arabinoside	4.75	2.45	0.000002
Quercetin 3-galactoside	166.83	124.25	0.0133
Quercetin 3-glucoside	39.77	28.48	0.0031
Quercetin 3-rutinoside	14.66	8.90	0.0004
Quercetin 3-arabinofuranoside	53.95	44.17	0.0339
Quercetin 3- arabinopyranoside	4.85	3.07	0.0007
Quercetin 3-xyloside	75.17	59.23	0.0154
Quercetin 3-rhamnoside	68.28	67.59	0.90
Hydroxycinnamic acids	33.27	31.75	0.57
Flavonols	362.89	336.84	0.35
Dihydrochalcones	72.71	68.35	0.27
Total phenolic content	1197.08	1213.38	0.70

[z]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.

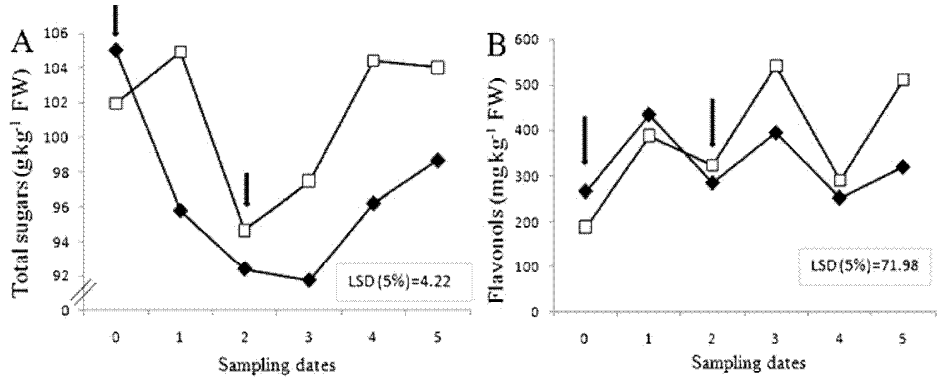

Fig. 2. The concentration of total sugars (**A**) and flavonols (**B**) in apples treated with Phostrade Ca (□) and control (♦) at different sampling dates (n = 5). Least significant differences (LSD) (5%) of the treatment are presented. Arrows indicate treatment application dates but samples have been 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 ($P < 00001$). 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).

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.[z]

	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

[z]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.

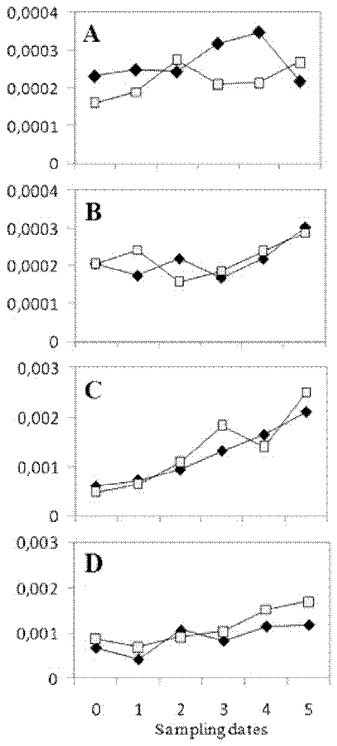

Fig. 3. Changes in enzyme activity (nkat·g^{-1} FW) of PAL (**A**), CHS (**B**), FHT (**C**), and DFR (**D**) in 'Braeburn' apples treated with Pho Ca (□) and control (♦) at different sampling dates. FW = fresh weight; PAL = phenylalanine ammonia lyase; CHS = chalcone synthase; FHT = flavanone-3-hydroxylase;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

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 P_2O_5, 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 flavonols 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

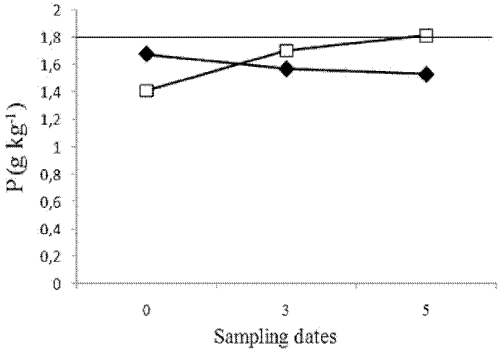

Fig. 4. Phosphorus (P) levels in the leaves of apples treated with the Phostrade Ca (□) and control (♦) at different sampling dates. Horizontal line presents the critical content for phosphor (according to Bergmann, 1992).

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 (Reay, 1999). 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. Mahajanaka) 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.

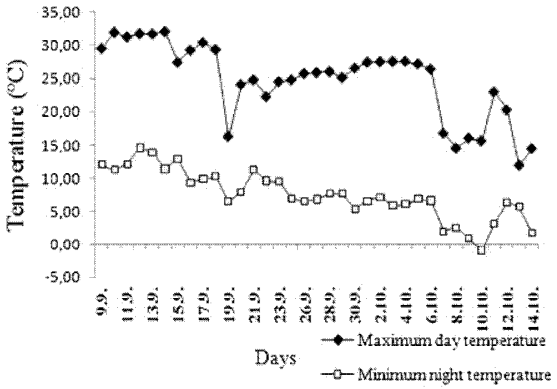

Fig. 5. Maximum day and minimum night temperatures during advanced fruit ripening recorded in the orchard of the Biotechnical faculty in Ljubljana in 2011.

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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2.3 EKSPRESIJA GENOV IN SPREMEMBE V VSEBNOSTI ANTOCIANOV TER POLIFENOLOV V KOŽICI JABOLK SORTE 'BRAEBURN' PO JESENSKI UPORABI PROHEKSADION KALCIJA

BIZJAK Jan, WEBER Nika, MIKULIČ-PETKOVŠEK Maja, ALAM Zobayer, THILL Jana, STICH Karl, HALBWIRTH Heidi, VEBERIČ Robert
Polyphenol gene expression and changes in anthocyanins and polyphenols in the skin of 'Braeburn' apples after the autumn application of prohexadione-calcium
Plant Growth Regulation, 2013, 71: 225–233
Prejeto: 20.2.2013, sprejeto: 7.5.2013

V prvem poskusu smo ugotovili, da jesensko tretiranje jablan s proheksadion kalcijem (Pro-Ca) začasno zmanjša aktivnost deoksigenaz FHT in FLS s čimer povzroči značilne spremembe v metabolizmu fenolnih spojin v jabolkih ter zmanjša obarvanost plodov. S sledečo študijo smo želeli ugotoviti, ali je, če polni odmerek Pro-Ca razdelimo na dve škropljenji, učinek na sintezno pot fenolnih spojin enak, kot smo ga ugotovili ob uporabi enkratnega odmerka. Poleg analiz sprememb v vsebnosti hidroksicimetnih kislin, dihidrohalkonov, flavonolov, flavanolov in antocianov, smo v kožici plodov v zadnjih petih tednih dozorevanja jabolk spremljali tudi aktivnost encimov PAL, CHS, FHT in DFR ter ekspresijo genov antocianidin sintaze (ANS), antocianidin reduktaze (ANR), flavonoid 3-O-glikoziltransferaze (FGT) in MYB10. Tudi pri tem poskusu smo tedensko spremljali parametre obarvanosti a*, hº in L*, ob obiranju pa smo del jabolk za dva meseca postavili v hladilnico in v mesečnem intervalu ugotavljali spremembe v vsebnosti glavnih skupin fenolnih spojin. Podobno kot v prejšnjem so tudi v tem poskusu parametri obarvanosti pokazali na začasen vpliv Pro-Ca na zmanjšanje intenzitete rdeče obarvanosti kožice jabolk, ki pa je izzvenel do tehnološke zrelosti plodov. V kožici tretiranih jabolk smo v primerjavi z netretiranimi zabeležili značilno večje vsebnosti hidroksicimetnih kislin, dihidrohalkonov, flavanolov in manjše vsebnosti antocianov. Tretiranje s Pro-Ca je rahlo zmanjšalo aktivnost vseh analiziranih encimov ter zmanjšalo ekspresijo (ANS), (ANR), (FGT) in transkripcijskega faktorja MYB10. Rezultati poskusa so pokazali, da je bil učinek jesenskega tretiranja jablan s Pro-Ca v dveh polovičnih odmerkih v nekaterih aspektih različen od učinka, ki smo ga ugotovili v poskusu, kjer smo jablane s Pro-Ca poškropili v enkratnem polnem odmerku. Vendar pa so bile tudi v tem poskusu spremembe kratkotrajnega značaja in so v celoti izzvenele do tehnološke zrelosti plodov.

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ORIGINAL PAPER

Polyphenol gene expression and changes in anthocyanins and polyphenols in the skin of 'Braeburn' apples after the autumn application of prohexadione-calcium

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Abstract Prohexadione-calcium (Pro-Ca) transient inhibits 2-Oxoglutarate-dependent dioxygenases and causes significant changes in the flavonoid spectrum of apple. In the present study the influence of two autumn preharvest applications of Pro-Ca on the polyphenol metabolism in apple peel during the advanced maturation was investigated. Pro-Ca was sprayed in two doses, approximately five and 3 weeks before the technological maturity. Changes in the concentrations of hydroxycinnamic acids, dihydrochalcones, flavonols, flavanols and anthocyanins as well as their related gene expression and enzyme activities in the apple peel were monitored six times during the advanced maturation until the technological maturity of the fruits. To evaluate its influence on red coloration differences in the chromatic values a*, h° and L* between the treated and untreated apples were monitored. The parameters showed a temporary effect of Pro-Ca on the intensity of red coloration, which was not detected anymore at the technological maturity of apples. The application of Pro-Ca decreased the flavanone 3-hydroxylase activity and slightly inhibited activities of all the enzymes analyzed. Concomitantly, the concentrations of anthocyanins in the peel of the treated apples decreased, whereas the concentrations of hydroxycinnamic acids, dihydrochalcones and flavan 3-ols increased. Flavonol concentrations, however, remained unchanged. The expression of *ANS, ANR, FGT* and *MYB10* was downregulated after the Pro-Ca treatment. The results indicate that the autumn application of Pro-Ca modulates the biosynthetic pathway resulting in distinct changes in the flavonoid composition in the apple peel of 'Braeburn' apples. However, the changes are temporary and are generally suspended during apple storage.

Keywords Flavonoids · Anthocyanins · Gene expression · Phenolic acids · Prohexadione-calcium

Abbreviations

Pro-Ca	Prohexadione-calcium
BHT	2,6-di-tert-butyl-4-methylphenol
FW	Fresh weight
PAL	Phenylalanine ammonia-lyase
CHS/CHI	Chalcone synthase/chalcone isomerase
FHT	Flavanone 3-hydroxylase
DFR	Dihydroflavonol 4-reductase
ANS	Anthocyanidin synthase
ANR	Anthocyanidin reductase
FGT	Flavonoid 3-O-glycosyltransferase
EF	Elongation factor

Introduction

Prohexadione-calcium (Pro-Ca) is a multi-functional plant bioregulator registered in a number of countries and currently being used on various fruit species. On apple trees it is commonly used as a plant growth inhibitor as derived from inhibition of oxoglutarate-dependent dioxygenases in gibberellin biosynthesis (Medjdoub et al. 2005), however, its multiple biochemical effects result in a range of benefits,

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including a reduced incidence of insect pests (Krawczyk and Greene 2002) and apple scab (Bazzi et al. 2003), the suppression of fire blight (Yoder et al. 1999; Spinelli et al. 2005) and other bacterial and fungal diseases as well (Rademacher and Kober 2003). Moreover, a high dosage of Pro-Ca inhibits the accumulation of anthocyanins (Rademacher et al. 1992) and reduces the coloration of apples (Bizjak et al. 2012).

Anthocyanin accumulation is affected both by structural genes encoding enzymes required for pigment biosynthesis and by regulatory genes which influence the intensity of anthocyanin accumulation generally throuh controlling expression of many structural genes (Telias et al. 2011). In apples it has been shown that five genes encoding the anthocyanin biosynthesis enzymes; chalcone synthase (CHS), flavanone 3-hydroxylase (FHT), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) and flavonoid 3-O-glycosyltransferase (FGT) are coordinately expressed during the advanced fruit ripening and their transcription levels are positively correlated with anthocyanin concentration (Honda et al. 2002).

Prohexadione-calcium (Pro-Ca) is primarily used as a plant growth regulator and plant protectant and therefore, treatments are mainly carried out in spring, however, when applied in autumn, Pro-Ca modulates the polyphenol spectrum resulting in a pronounced decrease of red coloration during the advanced maturation of apples (Bizjak et al. 2012). The transient inhibition of 2-oxoglutarate-dependent dioxygenases of the flavanoid pathway (2-ODDs); FHT, flavonol synthase (FLS) and ANS, lead to significant changes in the flavonoid spectrum of apple (Roemmelt et al. 2003; Halbwirth et al. 2006). However, its mechanism of action has been well investigated and documented in apple leaves (Roemmelt et al. 2003; Fischer et al. 2006; Halbwirth et al. 2006), whereas just Bizjak et al. (2012) observed it in the apple fruits.

The objectives of the present study were (1) to find out if the application of Pro-Ca in two half-doses during the advanced ripening influences red coloration and alters the phenolic concentration in a similar way as has previously been reported for a single application at full dose, (2) to evaluate the changes in polyphenol composition comprising enzyme activities and related gene expression of the treated apples compared to the control and (3) to determine if the preharvest Pro-Ca application has any measurable effects on the phenolic concentration of apples during their storage. To our knowledge, this is the first comprehensive study of the complex polyphenol biosynthesis in the apple peel comprising gene expression, enzyme activities and polyphenol composition and the first study in which an impact of Pro-Ca on storage capacity of apples has been evaluated.

Materials and methods

Plant materials

The experiment was carried out in 2011 on 10-year-old trees of the striped 'Braeburn' cultivar clone Hillwell grafted on M9 rootstock, grown according to the system of integrated production at Ljubljana location (latitude 46°2′N, longitude 14°28′E). On September 9th, approximately 5 weeks before the technological maturity, five trees were sprayed with 0.5 g (1.25 kg ha^{-1}) Regalis (BASF 125 10 W–10 % Pro-Ca, BASF, Germany) per tree, whereas the control trees were sprayed with water. The second spraying at the same dose was done 14 days later on September 23rd.

Color measurements

The skin color was measured using a portable colorimeter (CR-200 b Chroma; Minolta, Osaka; Japan) and recording L^*, a^*, and b^* color coordinates on the fruit surface. Before measuring, the colorimeter was calibrated with a white standard calibration plate. The data were expressed also 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 September 9th (sampling date 0) and continued weekly until commercial harvest of the fruits (sampling date 5). At each sampling date color measurements were made on 20 fruits from the outside of the tree canopy (4 fruits per tree) up to the height of 2 m from the ground. Fruits were randomly selected before the first spraying with Pro-Ca (sampling date 0).

Fruit sampling

Fruit sampling started on September 9th (before the first spraying) and was performed weekly (16th, 23rd, 30th September, 7th, 14th October) until the technological maturity, which was determined using the starch iodine test. At that time the value of firmness was 9.3 kg cm^{-2} and the starch index was 2.6. At each sampling date 15 fruits were randomly harvested and combined in five samples, with 3 fruit per sample (n = 5). Immediately after the harvest, apples were transported to the laboratory, where the tissue of samples was frozen in liquid nitrogen to prevent oxidation of phenolic substances and stored at −20 °C until the preparation of the samples. For the determination of enzyme activities one part of the schock-frozen apple skin was stored frozen at −80 °C until the enzymatic analysis. At technological maturity, in addition 30 kg of fruit per treatment were harvested and stored in a

chamber at 5 °C in air for 2 months. Fruit sampling during storage was performed 1 and 2 months after the harvest.

Chemicals

For the quantification of phenolic compounds the following standards were used: chlorogenic acid (5-caffeoylquinic acid), phloretin and rutin (quercetin-3-*O*-rutinoside) from Sigma Aldrich Chemie (Steinheim, Germany), cyanidin-3-*O*-galactoside chloride, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, (−)-epicatechin, *p*-coumaric acid, procyanidin B2 and phloridzin dihydrate from Fluka Chemie (Buchs, Switzerland), quercetin 3-*O*-arabinofuranoside and quercetin-3-*O*-xyloside from Apin Chemicals (Abingdon, UK) and (+)-catechin from Roth (Karlsruhe, Germany). For the extraction of phenolics methanol was acquired from Sigma. Chemicals for the mobile phases were the high performance liquid chromatography–mass spectrometry (HPLC–MS) grade acetonitrile and formic acid from Fluka Chemie. Water for the mobile phase was bidistilled and purified with the Milli-Q system (Millipore, Bedford, MA, USA). Substrates for enzyme assays were synthesised as described (Gosch et al. 2003).

Extraction and determination of phenolic compounds

The extraction of fruit samples was done as described by Mikulic-Petkovsek et al. (2010), with some modification. Apples were peeled with a mechanical peeler and 1 mm thick apple peel was ground to a fine powder in a mortar chilled with liquid nitrogen. 5 g of sample powder 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 in order to prevent oxidation. After the 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, 350 and 530 nm. The hydroxycinnamic acids, dihydrochalcones and flavanols were detected at 280 nm, flavonols at 350 nm and anthocyanins at 530 nm. For the separation of phenolic compounds Phenomenex (Torrance, CA) HPLC column C18 (150 × 4.6 mm, Gemini 3μ) 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): 0–5 min, 3–9 % B; 5–15 min, 9–16 % B; 15–45 min, 16–50 % B; 45–50 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 UV–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 (ESI) operating in the negative and positive ion mode. For the analyses full-scan data dependent MS^n scanning from m/z 115–2,000 was used. 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*-xylosylglucoside in equivalents of phloridzin, quercetin 3-*O*-arabinopyranoside in equivalents of quercetin 3-*O*-arabinofuranoside and anthocyanins (cyanidin 3-arabinoside, cyanidin 7-*O*-arabinoside, cyanidin 3-*O*-glucoside, and cyanidin 3-*O*-xyloside) were quantified in equivalents of cyanidin 3-*O*-galactoside. Concentrations of the phenolic compounds were expressed in mg kg^{-1} of fresh weight (FW).

Extraction and assay of enzymes

Buffers used: Buffer A: 0.1 M H_3BO_3 + 0.4 % Na-ascorbate pH 8.5 (phenylalanine ammonia-lyase, PAL; EC 4.3.1.5). Buffer B: 0.1 M KPi (KH_2PO_4/K_2HPO_4) + 0.4 % Na-ascorbate, pH 7.0 (CHS/chalcone isomerase, CHS/CHI; CHS: EC 3.2.1.74; CHI: EC 3.2.1.14). Buffer C: 0.1 M Tris/HCl + 0.4 Na-ascorbate, pH 7.25 (FHT, EC 1.14.11.9). Buffer D: 0.1 M KPi + 0.4 % Na-ascorbate, pH 6.8 (DFR, DFR, DFR, EC 1.1.1.219).

Enzyme preparations

Shock-frozen apple skin was ground to powder with liquid nitrogen. A total of 1 g fine apple skin powder was homogenised 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 were passed through a gel chromatography column (Sephadex G25 medium).

Analyses of enzyme activity and gene expression

Enzyme assays were performed as previously described (Slatnar et al. 2012) using the assay conditions optimized for the apple skin. Values on each sampling date represent an average of four independent biological repetitions for each treatment. Activities of PAL, CHS/CHI, FHT and DFR were calculated and expressed as nkat g^{-1} of fresh weight (FW).

Gene expression studies

Total RNA was prepared according to Chang et al. (1993) used for the isolation of mRNA via the μMACS mRNA isolation kit (Miltenyi Biotech, Auburn, CA). cDNA was prepared using the RevertAid H Minus MuLV reverse transcriptase (Fermentas Life Science, St. Leon-Rot, Germany) with the oligo(-dT) anchor Primer GACCACGCGTATCGATGTCGAC$(T)_{16}$V. Gene expression was quantified using the StepOnePlus™ Real-Time PCR System with the SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, USA). The primers used are listed in Table 1. The efficiency of the PCR-reaction was determined on the basis of standard curves which were obtained by applying different DNA concentrations. Results were calculated in relation to the housekeeping gene Elongation factor *(EF)*. All values were calculated in comparison to the first sampling date. The product specificity was confirmed by the melt curve analysis and gel electrophoresis.

Statistics

The data was analyzed using the Statgraphics Plus 4.0 program (Manugistics. Inc.; Rockville, Maryland, USA). Data from the analyses was tested for any differences among treatments using one-way analysis of variance (ANOVA). Significant differences among treatments were determined independently for each sampling date using the least significant difference test (LSD). During the storage of apples the differences between sampling dates for each treatment were also tested using the LSD test. In both cases, the significance level was 0.05.

Results and discussion

The effect of Pro-Ca in the apple peel was investigated as the altered levels of fruit coloration, polyphenol concentrations, enzyme activities and the related gene expression in the treated fruit compared to the control.

The parameter a* increased during the advanced ripening (Fig. 1a), while the lightness coefficient L* (Fig. 1b) and hue h (Fig. 1c) decreased, indicating an increasing red coloration of apples (McGuire 1992). Regarding the parameter a* and L*, the significant effect of Pro-Ca was seen 2 weeks after the first and 1 week after the second treatment. At that time the apples of the control had a more intense red color and were significantly darker than the apples treated with Pro-Ca. The effect of Pro-Ca treatment was also seen by the hue angle, where a significantly higher value of the treated apples compared to the control was measured 1 week after the second treatment. At the technological maturity, however, we found no significant differences in any of the parameters between the treated and the untreated fruits. The results presented differ somewhat from those reported by Bizjak et al. (2012), who found differences in colorimetric parameters between the Pro-Ca treated and the control apples also at technological maturity. The reason for different results obtained in our study might be the distribution of a single recommended dose spray (2.5 kg ha^{-1}) in two half-doses and rapid metabolisation of Pro-Ca in plant tissues (Halbwirth et al. 2003).

In total 19 polyphenols were identified in the apple peel belonging to five groups: hydroxycinnamic acids, flavan 3-ols, dihydrochalcones, flavonols and anthocyanins.

Table 1 Sequence of primers used for the gene expression in 'Braeburn' apples

Primer ID	Sequence (5′ > 3′direction)	T_m (°C)	Size
Mal.EF.F	TACTGGAACATCACAGGCTGAC	60.3	22
Mal.EF.R	TGGACCTCTCATCATGTTGT	55.3	20
Mal.ANS.F	GTCATGCACATTGGGGATACACTT	61.0	24
Mal.ANS.R	CCATGAAATCCTCACCTTTTCCTTG	61.3	25
Mal.ANR.F	TTTTGGCTGAGAAAGAATCTGCATC	59.7	25
Mal.ANR.R	TGGGGGTATCTTTTGTTGAGGAACT	61.3	25
Mal.FGT.F	TGTACATAAGCTTCGGGACAGTG	60.6	23
Mal.FGT.R	TGATAGACCACAAGAAGGGTGCT	60.6	23
Mal.MYB10.F	TTAGACTTCACAGGCTTTTGGGAAA	59.7	25
Mal.MYB10. R	TCCGCAATCGAGTGTTCCAATAAT	59.3	24

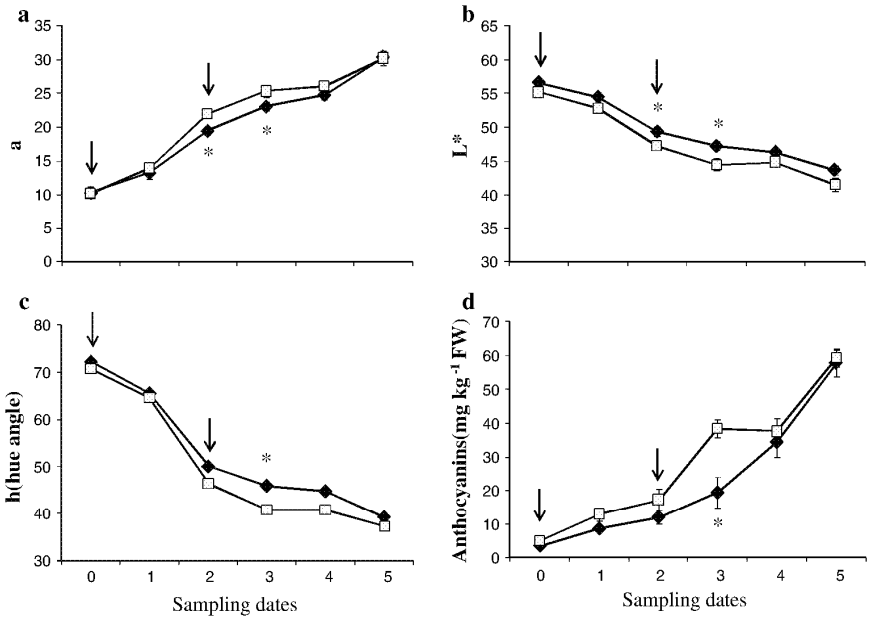

Fig. 1 Colorimetric parameters (mean ± SE; n = 20) a* (**a**), L* (**b**), hue angle (h°) (**c**) and the concentration of anthocyanins (mean ± SE; n = 5) of the 'Braeburn' apples (**d**) treated with prohexadione-calcium *filled diamond* and the control *white square* at different sampling dates. * denote statistically significant differences between treatments at each sampling date (LSD test; $\alpha \leq 0.05$). *Arrows* indicate treatment application dates but measurements were done before the respective application

The total anthocyanin concentration increased during the advanced ripening (Fig. 1d). For both treatments the highest concentration was measured at technological maturity, when the value of the total anthocyanins in the Pro-Ca treated apple peel (57.89 mg kg^{-1} FW) was almost the same as for the control (59.32 mg kg^{-1} FW).

Prohexadione-calcium (Pro-Ca) significantly reduced the concentration of anthocyanins 1 week after the second treatment when the value of the treated apples (19.49 mg kg^{-1} FW) was almost two fold lower compared to the control (38.53 mg kg^{-1} FW). At the next sampling date the value of the treated apples almost doubled and at technological maturity there was no significant difference between the treated and the untreated apples. Similar results were already found in the previous study (Bizjak et al. 2012), explaining this by the 10–15 days half-life of Pro-Ca and its rapid metabolisation in plant tissues. A decrease in the anthocyanin formation in the treated apples is the consequence of the temporary blockage of the 2-oxoglutarate-dependent dioxygenases FHT and ANS which play a key role in the biosynthesis of anthocyanins and other flavonoids (Halbwirth et al. 2006).

Among the hydroxycinnamic acids, chlorogenic acid, caffeic acid and 4-coumaroylquinic acids were determined. A significantly higher concentration of total hydroxycinnamic acids in the peel of the treated apples compared to the control was found 1 week after the first, 1 week after the second treatment and at technological maturity (Fig. 2a). Our results are similar to those of Mikulic-Petkovsek et al. (2009) and Bizjak et al. (2012); however, in the present study, the difference was significant also at technological maturity. Hydroxycinnamic acid accumulation was the consequence of the FHT inhibition which on the one hand decreases the synthesis of flavonoids and related precursors, while, on the other hand, accelerates the accumulation of cinnamic acid (Roemmelt et al. 2003; Fischer et al. 2006).

Significant influence of the Pro-Ca treatment was noticed also in the case of dihydrochalcones of which the concentration of phloretin and phloridzin (phloretin 2′-*O*-β-D-glucoside) were analyzed. The treated apples contained a higher amount of the dihydrochalcones compared to the control at sampling dates 1, 4 and also at technological maturity. Our results differ from those reported by Mikulic-Petkovsek et al. (2009) and Bizjak et al. (2012), who found a decrease in the phloridzin concentration in Pro-Ca of the treated fruits. However, at technological maturity, no differences could be determined between the treated and untreated fruits in their studies.

Among flavan 3-ols, the concentration of catechin, epicatechin, procyanidin B1 and procyanidin B2 was determined. A significantly higher concentration of flavan 3-ols was noticed in Pro-Ca treated apples 1 week after the first and 2 weeks after the second treatment (Fig. 2c). This is consistent with the results of Fischer et al. (2006), who found an increased formation of major flavan 3-ols in the Pro-Ca treated apple leaves but not within the previous study (Bizjak et al. 2012), where no significant influence of Pro-Ca treatment on the flavan 3-ol concentration in the apple peel was observed. The increase of flavan 3-ols could be of great importance, since they are the most important contributors to the in vitro antioxidant activity of apples (Tsao et al. 2005).

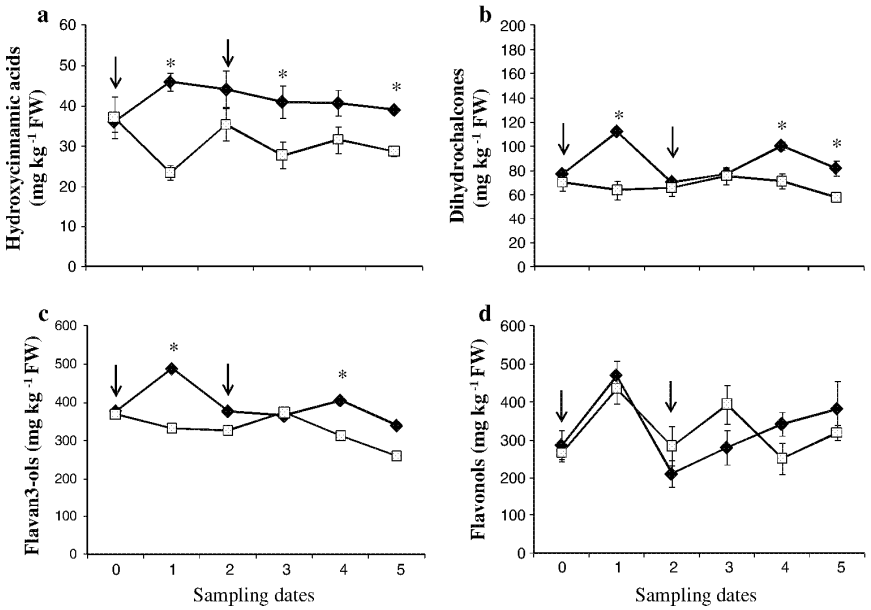

Fig. 2 The concentration (mean ± SE; n = 5) of hydrocinnamic acids (**a**), dihydrochalcones (**b**), flavan 3-ols (**c**) and flavonols (**d**) in the 'Braeburn' apples treated with prohexadione-calcium *filled diamond* and the control *white square* at different sampling dates. *Asterisk* denote statistically significant differences between treatments at each sampling date (LSD test; $\alpha \leq 0.05$). *Arrows* indicate treatment application dates but samples were taken before the respective application

The following flavonols were identified in the apple peel; rutin (quercetin-3-*O*-rutinoside), avicularin (quercetin-3-*O*-arabinofuranoside), hyperin (quercetin-3-*O*-galactoside), quercitrin (quercetin-3-*O*-rhamnoside), isoquercitrin (quercetin-3-*O*-glucoside) and reinutrin (quercetin-3-*O*-xyloside). No significant influence of Pro-Ca on total flavonols in apple peel was noted (Fig. 2d). These results differ from those reported in previous study (Bizjak et al. 2012), where a temporary decrease in the concentration of total flavonols in the peel of the treated apples was detected.

Activities of the four enzymes from the flavonoid pathway were investigated in the apple skin, PAL, CHS/CHI, FHT and DFR. For the late steps of the pathway, expressions of *ANS*, anthocyanidin reductase (*ANR*), and UDP-Glucose:flavonoid 3-*O*-glucosyltransferase (*FGT*), gene and the transcription factor *MYB10* were measured.

An increase of all the enzyme activities analyzed was observed during the 5 weeks of ripening (Fig. 3). Pro-Ca slightly inhibited the activity of all the enzymes analyzed; however a significant effect was found just in the case of FHT. FHT inhibition in the Pro-Ca treated apples was observed already 1 week after the first treatment (sampling date 1), where a decrease in FHT activity was detected (Fig. 3c). At that time activity was significantly lower in comparison with the control apples, where an increase in activity was found. The reduction of all the enzymes activities in the Pro-Ca treated fruits was very similar to the reduction observed in the previous study of Bizjak et al. (2012). However, in the present investigation the FHT inhibition in the Pro-Ca treated apples was detected through the whole sampling period, whereas in the previous one it was suspended 22 days after the treatment.

Prohexadione-calcium (Pro-Ca) treatments led to changes in the *ANS* expression (Fig. 4a). In the control fruits the *ANS* expression decreased at the first and the second sampling date but increased thereafter and peaked at the third sampling date. At the last two sampling dates, the relative gene expression of the control fruits was higher, when comparing to the zero sampling date, whereas by the treated apples it was much lower (Fig. 4a).

The effect of Pro-Ca was observed also in the *ANR* expression. In comparison to *EF*, the relative *ANR* expression by both treatments decreased steadily during the fruit development (Fig. 4b). However, the decrease was much more evident in the treated fruits. The inhibitory effect of Pro-Ca was seen also in the *FGT* expression (Fig. 4c) and was most pronounced 14 days after the first treatment (sampling date 2).

The transcription factor *MYB10* is a known activator of the apple anthocyanin pathway (Takos et al. 2006). The Pro-Ca treatment led to a decrease of relative *MYB10* expression at all sampling dates with the exception of the third sampling date (Fig. 4d). The decrease was detected also in the control apples, however it was much less pronounced.

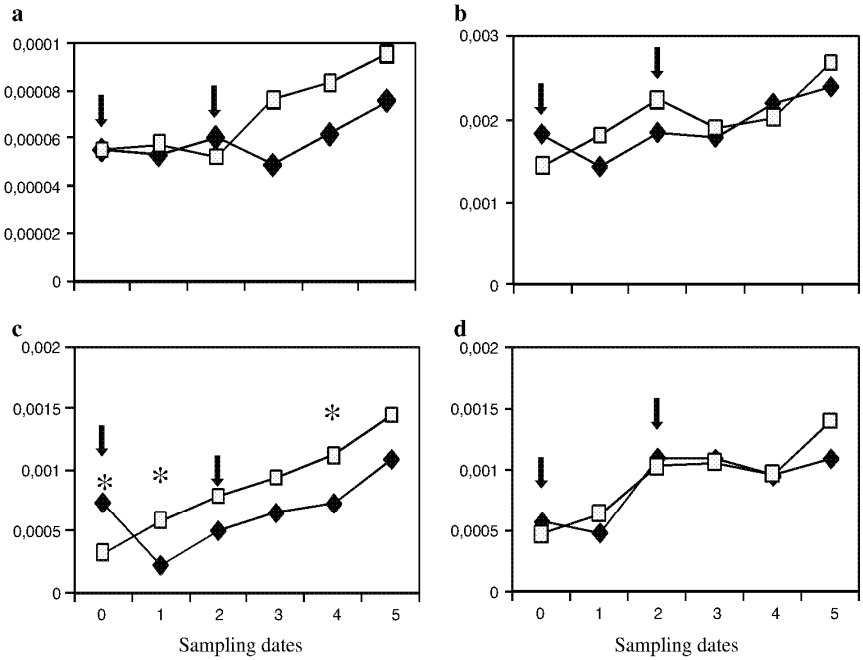

Fig. 3 Enzyme activity (nkat g^{-1} FW) of PAL (**a**), CHS (**b**), FHT (**c**) and DFR (**d**) in 'Braeburn' apples treated with prohexadione-calcium *filled diamond* and the control *white square* at different sampling dates. * denote statistically significant differences between treatments at each sampling date (LSD test; $\alpha \leq 0.05$; n = 4). *Arrows* indicate treatment application dates but samples were taken before the respective application

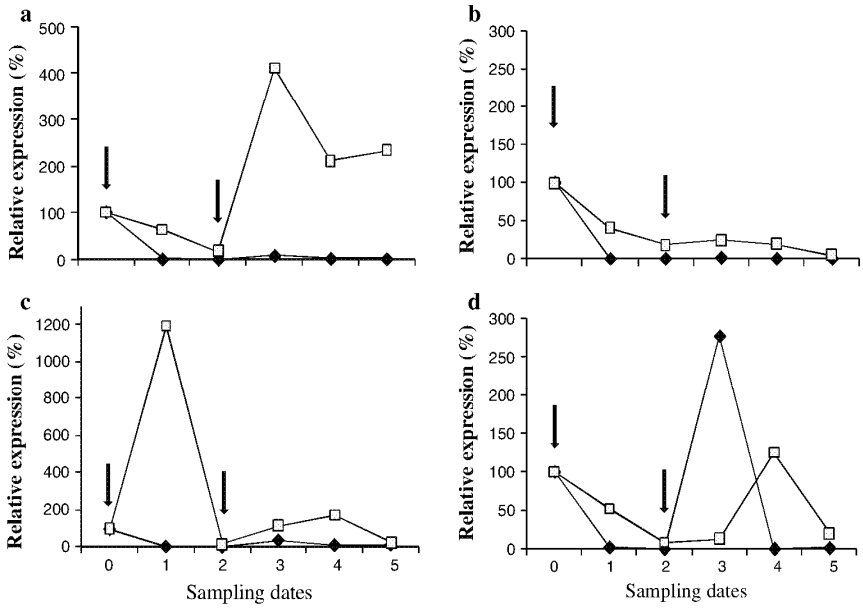

Fig. 4 *ANS* (**a**), *ANR* (**b**), *FGT* (**c**) and *MYB10* (**d**) expression normalized to *elongation factor (EF)* in apple skin of apples treated with prohexadione-calcium *filled diamond* and the control *white square* at different sampling dates. The relative gene expressions (%) are expressed as relative activity (%) based on the day of spray (sampling date 0) as 100 %. *Arrows* indicate the time of spraying but samples were taken before the respective application

To find out if the preharvest application of Pro-Ca has any effects on the phenolic concentration during the storage, the changes in the same phenolics during the ripening of apples were evaluated. In the control apples the concentration of hydroxycinnamic acids, flavan 3-ols and dihydrochalcones significantly increased after the 2 months of storage, whereas in the treated apples it remained almost unchanged (Table 2). In these phenolic groups significant differences between the treatments during the storage were detected just in the case of the hydroxycinnamic acids, where at the end of storage the control apples contained a significantly higher level of total hydroxycinnamic acids than the treated apples. However, it has to be taken into account that, before storage, the treated apples contained significantly higher values of hydroxycinnamic acids, dihydrochalcones and flavan 3-ols as well, thus it might be

Table 2 Changes in the sum of different phenolic groups (mg kg^{-1} FW) in the peel of apples treated with prohexadione-calcium (Regalis) and the control during two months of storage

	Months of storage		
	0	1	2
Hydroxyc. acids			
Regalis	39.16 ± 0.62* a	37.20 ± 2.57 a	37.08 ± 2.96 a
Control	28.81 ± 1.37 a	43.96 ± 3.41 b	47.17 ± 1.41* b
Dihydrochalcones			
Regalis	82.25 ± 6.30* a	82.84 ± 3.92 a	70.09 ± 6.68 a
Control	58.04 ± 1.02 a	91.94 ± 4.17 b	82.40 ± 3.34 b
Flavan 3-ols			
Regalis	340.71 ± 16.18* a	370.03 ± 20.75 a	362.02 ± 31.88 a
Control	259.28 ± 11.56 a	437.09 ± 28.85 b	432.61 ± 16.89 b
Flavonols			
Regalis	382.56 ± 71.98 b	434.58 ± 59.23 b	213.1 ± 8.33 a
Control	319.55 ± 19.41 ab	447.18 ± 68.54 b	273.33 ± 36.54 a
Anthocyanins			
Regalis	57.89 ± 4.01 b	58.39 ± 5.81 b	36.67 ± 3.01 a
Control	59.37 ± 2.58 a	85.37 ± 5.78* b	68.91 ± 3.00* b

Average values (n = 5) ± standard errors (SE) are presented

Asterisks denote statistically significant differences between treatments on each sampling date (LSD test; $\alpha \leq 0.05$)

[a] Different letters in rows (a-b) denote significant differences among sampling dates for each treatment

speculated that Pro-Ca prevented an increase of these phenolic groups, which has been observed in the control apples.

Similar observations were also found in the case of anthocyanins and flavonols. In terms of total anthocyanins their concentration significantly decreased in the treated apples after the 2 months of storage, while it increased in the control apples. In comparison with the treated apples the control one's contained a significantly higher level of anthocyanins already 1 month after and at the end of storage as well. Regarding flavonols, the concentration in the treated apples decreased after the 2 months of storage, while it remained unchanged in the control (Table 2). However, no significant difference in their level between the treatments after the 2 months of storage was detected. When analyzing firmness and soluble solids, no significant influence of the Pro-Ca treatment either during the ripening or during the storage of apples was recorded (data not shown).

Conclusions

In the present study a broad insight into the changes in the phenylpropanoid pathway in the apple skin after the two autumn applications of Pro–Ca during the advanced maturation of apples has been provided. Pro–Ca increased the concentration of hydroxycinnamic acids, dihydrochalcones and flavan 3-ols; while temporarily decreased anthocyanins and reduced red coloration of apples. The data obtained with housekeeping gene *EF* showed that the *ANS, ANR, FGT* and *MYB10* gene expression was down regulated after the Pro-Ca treatment which corresponded well with the observed decrease of all the enzyme activities in the treated apples. In conclusion, it has been shown that the physiological response of the apples to the treatment in two separate doses was in some respects different from the response which was noticed when Pro-Ca was applied in one full dose. Anyway, it has been shown that Pro-Ca modulates the polyphenol spectrum and to some extent has the potential to avoid red coloration of apple fruit. During the storage, however, Pro-Ca might have a negative effect on the phenolic concentration of 'Braeburn' apples.

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2.4 SPREMEMBE PRIMARNIH METABOLITOV IN POLIFENOLOV V KOŽICI JABOLK SORTE 'BRAEBURN'(Malus *domestica* Borkh.) MED DOZOREVANJEM

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The changes in primary metabolites and polyphenols in the peel of 'Braeburn' apples (Malus Domestica Borkh.) during the advanced maturation
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Na dveh različnih lokacijah smo v dveh ločenih sezonah ugotavljali, kako se v kožici jabolk sorte 'Braeburn' med njihovim dozorevanjem spreminjajo vsebnosti primarnih in sekundarnih metabolitov in kako se spreminjajo nekateri ostali parametri kakovosti plodov. Spremembe v trdoti, suhi snovi, masi plodov, vsebnosti sladkorjev, organskih kislin ter fenolnih spojin iz razredov hidroksicimetnih kislin, dihidrohalkonov, flavonolov, flavanolov in antocianov smo v kožici jabolk spremljali tedensko, in sicer v zadnjih petih tednih njihovega dozorevanja pa vse do obiranja plodov v njihovi tehnološki zrelosti. S pomočjo beleženja in analize sprememb smo želeli pridobiti sliko razvoja oziroma gibanja primarnih in sekundarnih metabolitov ter ugotoviti, kdaj točno se v kožici jabolk pojavijo največje vsebnosti primarnih in sekundarnih metabolitov in kakšno je razmerje med njimi. Med posameznimi sladkorji je bila saharoza edina, katere vsebnost se je med pettedenskim zorenjem jabolk značilno povečala v obeh rastnih sezonah, medtem ko sta se vsebnosti fruktoze in glukoze v enem letu zelo malo spreminjali, v drugem pa zmanjšali. V skladu z našimi pričakovanji, se je vsebnost jabolčne in citronske kisline z zorenjem zmanjševala. Vsebnosti hidroksicimetnih kislin, dihidrohalkonov in flavanolov se v kožici jabolk v zadnjih petih tednih zorenja niso kaj dosti spreminjale, v primerjavi s prvim terminom vzorčenja, pa so se nekoliko zmanjšale. Se je pa v zadnjih petih tednih zorenja jabolk pojavila akumulacija kvercetin glikozidov in antocianov, ki je bila intenzivnejša približno tri tedne pred tehnološko zrelostjo plodov. Vsebnost skupnih fenolov se v obeh letih med zorenjem ni kaj dosti spreminjala. Na podlagi rezultatov naše študije priporočamo, da se poletna rez, uporaba odsevnih folij, foliarna uporaba različnih elementov oziroma pripravkov ter podobni ukrepi, ki so namenjeni izboljšanju kvalitete in obarvanosti plodov, na jablanah sorte 'Braeburn' izvedejo približno 3-4 tedne pred pričakovano tehnološko zrelostjo plodov.

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Changes in Primary Metabolites and Polyphenols in the Peel of "Braeburn" Apples (*Malus domestica* Borkh.) during Advanced Maturation

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ABSTRACT: During the two growing seasons the evolution of primary metabolites and wide range of polyphenols in the "Braeburn" apple peel during advanced maturation were investigated. During the five weeks sucrose significantly increased, whereas fructose and glucose fluctuated around the same level in one season and decreased in another. Regarding malic and citric acids, an expected decrease was recorded. The concentrations of hydroxycinnamic acids, dihydrochalcones, and flavanols remained quite constant or slightly decreased during advanced apple ripening. On the contrary an intensive accumulation of quercetin glycosides and anthocyanins took place during this period, starting with the onset of rapid formation approximately 3 weeks before the technological maturity of apples. Total phenolic content was relatively constant or slightly increased. The present results suggest that measures designed to improve the apple color and quality of "Braeburn" apples should be performed approximately 3−4 weeks before the expected technological maturity of apples.

KEYWORDS: *flavonoids, quercetin-glycosides, anthocyanins, sugars, organic acids, advanced apple ripening*

INTRODUCTION

Apart from water, apples contain different amounts of primary (sugars and organic acids) and a wide range of secondary metabolites (polyphenols). Glucose, fructose, sucrose, and organic acids represent the most important taste components in apples, and their ratio has a marked influence on the sensory quality of the fruit.[1,2] Polyphenols, together with organic acids and sugars, make an important contribution to the inner quality as well as enhance the outer appearance of the apples.[3]

Due to the high content of phenolic compounds and widely consumption, apples represent an important share of consumed flavonoids in American and European diet.[4] Many of them have been found to have strong antioxidant activity and anticancer activity[5] and thus have the potential to modulate many processes in the development of diseases, including cancer, cardiovascular disease, diabetes, pulmonary disorders, Alzheimer's disease, and other degenerative disease states.[6] Moreover, when compared to other fruits, apples have the highest portion of free phenolics, which may be more available for eventual absorption into the bloodstream.[7]

Based on molecular structure, phenolics in apples are divided into five major groups including hydroxycinnamic acids, flavanols dihydrochalcones, flavonols, and anthocyanins, mainly presented in the peel of the red apple cultivars.[8] With the exception of hydroxycinnamic acids, their accumulation is concentrated in the apple peel which contains anywhere from 2−6 times more phenolic compounds than the flesh[9,10] and 2−3 times more flavonoids in the peels when compared to the flesh.[11]

Flavonoid accumulation in the apple peel is affected by numerous factors, including light,[12] variety,[13] plant nutrition,[14] stress situations,[15] temperature,[16] cultivation type, and conditions of the plant.[17] In addition, fruit development and maturity also significantly affect the polyphenol composition of apples. Awad et al.[18] found that quercetin glycosides, catechins, phloridzin and chlorogenic acid in "Jonagold" and "Elstar" apples were mainly accumulated during the fruit development until the onset of maturation, whereas the main accumulation of anthocyanins occurred during the maturation of the fruit. Lister et al.[19] reported that concentration of quercetin glycosides and procyanidins in the peel of "Splendour" apples decreased from early to mid season but increased during apple ripening.

Despite the fact that there are several reports on the developmental changes in the concentration of polyphenols in apples during development and ripening,[15,18−20] fewer details are known on the variation of primary metabolites and polyphenolic concentrations during the advanced maturation prior to technological maturity of apples. However, in some studies the changes in polyphenols during ripening in the apple flesh were observed.[21,22]

In the present study the changes of the concentrations of sugars, organic acids and a wide range of polyphenols as well as total phenolic compounds in the "Braeburn" apple peel during the advanced maturation of apples in two growing seasons are reported. All phenolic compounds were confirmed using a mass spectrometer with an electrospray interface (ESI) operating in negative ion mode. The results will contribute information about the evolution and maximal concentration of primary and secondary metabolites in the last stages of apple ripening and their relation as well. At the same time, by knowing the time of the most intensive changes in the primary and secondary metabolites, these results could be very helpful in determining

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the most appropriate time to carry out the agro-technical measures designed for improving the outer and inner quality parameters of apples, such as apple coloring and the like.

MATERIALS AND METHODS

Plant Materials. Trials were conducted in the central and eastern Slovenia on striped "Braeburn" apple cultivar clone Hillwell (*Malus domestica* Borkh.) at Ljubljana location (latitude 46°2′, longitude 14°28′) in 2011 and at at the fruit growing center Maribor Gacnik location (latitude 46° 61′, longitude 15° 68′) in 2012. At both locations apple trees were grafted on M9 rootstock and grown according to the system of integrated production.

At Ljubljana location apples for measurements were taken from the 10-year-old trees planted maintaining row distances of 3.75 m and tree-to-tree distance of 1.1 m. Full bloom (>80% of the opened flower buds) occurred on the 22nd of April 2011, whereas the technological maturity, which was determined using the starch iodine test occurred on the 14th of October (175 DAFB).

At the fruit growing center Maribor Gacnik, 13-year-old trees planted maintaining row distances of 3.5 m and tree-to-tree distances of 1 m were used in the experiment. Full bloom (>80% of the opened flower buds) occurred on the 15th of April 2012, while the technological maturity, occurred on the 7th of October, (177 DAFB). At the time of technological maturity the value of firmness was approximately 9.5 kg cm^{-2} and the starch index (on the scale of 1−5) was 2.7 in both years.

Fruit Sampling. At Ljubljana the fruit sampling started on September 9th whereas at Gacnik Maribor the first sampling was carried out on September 4th. At both locations sampling continued weekly until the technological maturity of the fruit which at both locations occurred five weeks after the first sampling. At each sampling date 15 fruits from five trees were randomly harvested and combined in five samples, with 3 fruit per sample (n = 5). Immediately after the harvest, the apples were transported to the laboratory, where the tissue of samples was frozen in liquid nitrogen to prevent oxidation of phenolic substances and stored at −20 °C until the preparation of the samples.

Firmness and Soluble Solid Content Measurements. Soluble solid content (SSC) was measured using the digital refractometer ATAGO WM-7. The firmness was measured on four peeled sides in the equatorial plane of the fruit with a penetrometer (TR Italy) equipped with an 11 mm tip. The depth of tip penetration was 8 mm.

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.[9] For the extraction of individual sugars and organic acids, 10 g of the fresh mass of each sample was homogenized in 50 mL of bidistilled water using Ultra-Turrax T-25 (Ika-Labortechnik, Stauden, Germany). Samples were left for 30 min at room temperature and stirred frequently. After extraction, the homogenate was centrifuged (Eppendorf 5810 R Centrifuge, Hamburg, Germany) at 10 000 rpm for 7 min at 4 °C. The supernatants were filtered through a 0.20-μm cellulose ester filter (Macherey-Nagel, Düren, Germany) and 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.[9]

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 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.[9] Organic acids were analyzed using a RezexROA-organic acid column (300 × 7.8 mm; Phenomenex, Torrance, CA) and a UV 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 (3-caffeoylquinic acid), phloretin, and rutin (quercetin 3-*O*-rutinoside) from Sigma Aldrich Chemie (Steinheim, Germany), cyanidin 3-*O*-galactoside chloride, quercetin 3-*O*-rhamnoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, (−)-epicatechin, *p*-coumaric acid, procyanidin B1, B2, and phloridzin dihydrate from Fluka Chemie (Buchs, Switzerland), quercetin 3-*O*-arabinofuranoside and quercetin 3-*O*-xyloside from Apin Chemicals (Abingdon, U.K.), and (+)-catechin from Roth (Karlsruhe, Germany). Methanol for the extraction of phenolics was acquired from Sigma. Chemicals for the mobile phases were the high performance liquid chromatography−mass spectrometry (HPLC-MS) grade acetonitrile and formic acid from Fluka Chemie. Water for mobile phase was bidistilled and purified with the Milli-Q system (Millipore, Bedford, MA, USA). For determination of the total phenolic content, Folin−Ciocalteu phenol reagent (Fluka Chemie GmbH), sodium carbonate (Merck, Darmstadt, Germany), gallic acid, and ethanol (Sigma) were used.

Extraction and Determination of Phenolic Compounds. The extraction of fruit samples for phenolic compounds was done as described by Mikulic-Petkovsek et al.[17] with some modification. Apple samples were ground to a fine powder in a mortar chilled with liquid nitrogen. A total of 5 g of apple peel was extracted with 10 mL of methanol containing 3% (v/v) formic acid and 1% (w/v) 2.6-ditert-butyl-4-methylphenol (BHT) in a cooled ultrasonic bath for 1 h. BHT was added to the samples in order to prevent oxidation. After extraction, the treated samples were centrifuged for 5 min at 10 000 rpm. The supernatant was filtered through a Chromafil AO-20/25 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, 350, and 530 nm. The hydroxycinnamic acids, dihydrochalcones, and flavanols were detected at 280 nm, flavonols at 350 nm, and anthocyanins at 530 nm. For the separation of phenolic compounds a Phenomenex (Torrance, CA) 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% acetonitril (A) and 100% acetonitrile (B). Samples were eluted according to the linear gradient described by Marks et al.[23] 0−5 min, 3% to 9% B; 5−15 min, 9% to 16% B; 15−45 min, 16% to 50% B; 45−50 min, 50% isocratic; this step was followed by the washing and reconditioning of the column. Compound identification was achieved by comparing the retention times and their UV−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 using a mass spectrometer (Thermo Scientific, LCQ Deca XP MAX) with an electrospray ionization (ESI) operating in the negative and positive ion mode. For the analyses full-scan data dependent MS scanning from m/z 115 to 2000 was used. 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*-xylosylglucoside in equivalents of phloridzin, quercetin 3-*O*-arabinopyranoside in equivalents of quercetin 3-*O*-arabinofuranoside or anthocyanins (cyanidin 3-arabinoside, cyanidin 7-arabinoside, cyanidin 3-glucoside, and cyanidin 3-xyloside) were quantified in equivalents of cyanidin 3-galactoside. Concentrations of the phenolic compounds were expressed in mg kg^{-1} of fresh weight (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.[24] To 100 μL of sample extracts were added 6 mL of bidistilled water and 500 μL

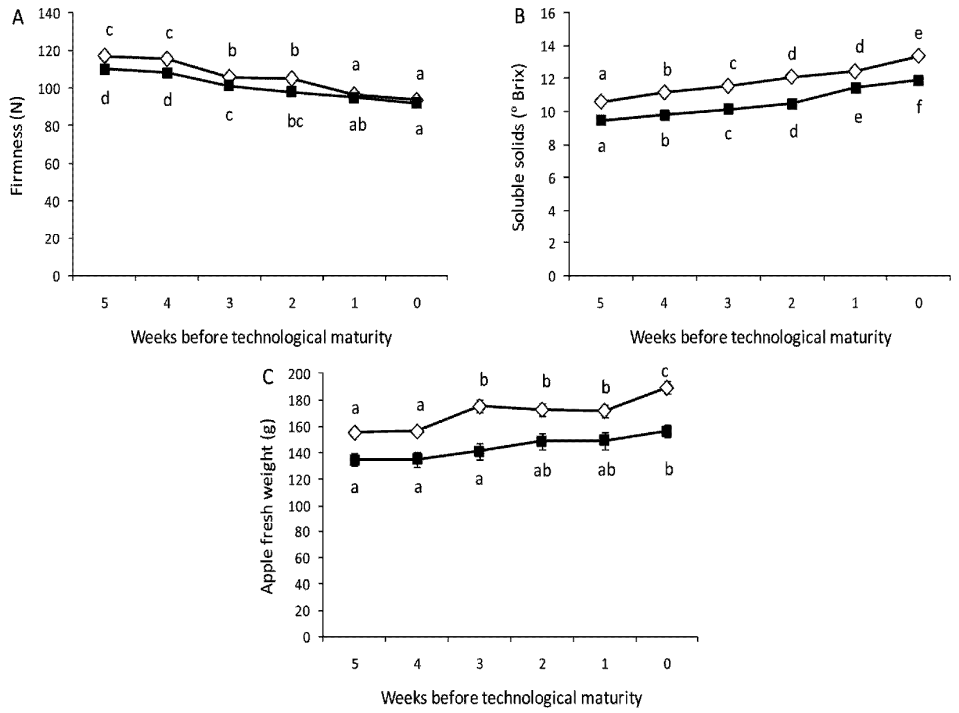

Figure 1. Mean (±SE) firmness (A), soluble solids (B), and fruit fresh weight (C) of "Braeburn" apples in 2011 (◊) and 2012 (■) at different sampling dates during the advanced maturation of apples. Different letters (a–f) denote statistically significant differences between sampling dates within each year (Duncan test, $p \leq 0.05$, $n = 15$).

of Folin-Ciocalteau reagent; after resting between 8 s 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, UV/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 (GAE) in mg kg^{-1} fresh weight (FW). Absorption was measured in three replications.

Statistics. Statistical analysis was conducted in order to evaluate the changes of primary and secondary metabolites in "Braeburn" apples during the advanced ripening. The data was analyzed using the Statgraphics Plus 4.0 program (Manugistics. Inc.; Rockville, Maryland, USA). Data from all of the analyses was tested for any differences among sampling dates within each year using one-way analysis of variance (ANOVA). The differences were tested using the Duncan test with the significance level 0.05. In diagrams, the means as well as their standard errors are presented (mean ± SE).

RESULTS

Firmness decreased gradually whereas soluble solids content (SSC) increased linearly during the advanced maturation of "Braeburn" apples in both years (Figure 1A,B). During the 5 weeks of ripening SSC increased by 26% in both years, while firmness decreased in 2011 by 20% and in 2012 by 16%. A similar percentage also increased the fruit weight, namely by 22% in 2011 and 16% in 2012 (Figure 1C).

The changes of sugars (sucrose, glucose, and fructose), sorbitol, and organic acids (citric and malic acid) are presented in Figure 2. At the technological maturity of "Braeburn" apples, fructose was the predominant sugar representing 48/51% of the total sugars in 2011/2012, followed by sucrose (39/33%), glucose (9/14%), and the main sugar alcohol sorbitol (4/2%). Our results are in agreement with those obtained on other apple varieties.[9,25−27] Sucrose increased during the advanced ripening, reaching the highest concentration at technological maturity of "Braeburn" apples in both years. In the five weeks of fruit ripening the concentration increased from 23% in 2011 to 51% in 2012. On the contrary, fructose and glucose in 2011 decreased by 20%, whereas in 2012 it decreased prior to 3 weeks before technological maturity and recovered afterward (Figure 2B,C). The concentration of sorbitol fluctuated during fruit ripening in 2012 and increased prior to 1 week before technological maturity in both years (Figure 2D). Regarding the total sugars their concentration decreased from 5 to 2 weeks before the technological maturity of apples and increased afterward, reaching the peak at the technological maturity of apples (data not shown). Regarding the organic acids, malic and citric acids were quantified. The level of shikimic and fumaric acids were of the order of 10 mg kg^{-1} of fresh weight, thus their concentrations are not reported. Malic acid was the main acid contributing about 82−85% of the total acid concentrations. Its concentration significantly decreased during the 5 weeks of advanced ripening in 2012, whereas no significant changes were observed in 2011 (Figure 2F). Significant changes were observed also in the case of citric acid (Figure 2E). When comparing the concentrations measured at the beginning of the advanced ripening and concentrations noted at technological maturity of apples, the concentration of citric acid in 2012 decreased while it remained unchanged in 2011.

A total of 21 phenolics (Table 1) were identified and quantified in "Braeburn" apple peels belonging to five groups: hydroxycinnamic acids, dihydrochalcones, flavonols, flavanols,

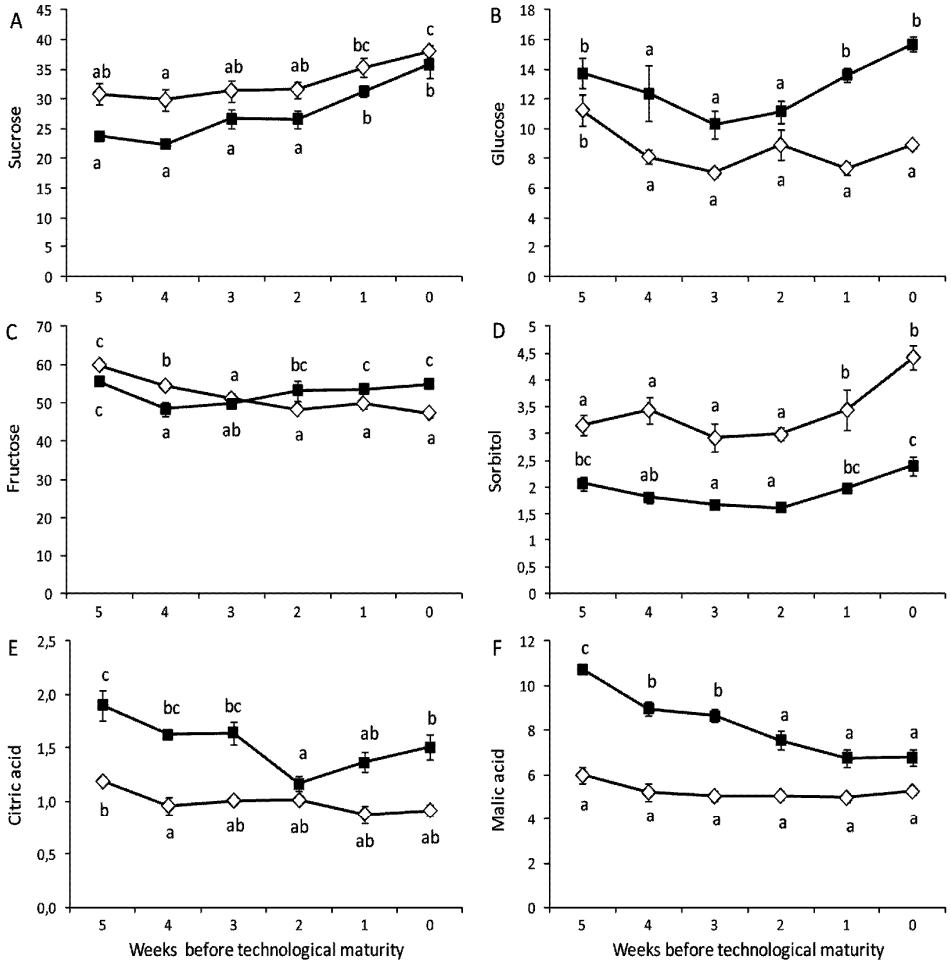

Figure 2. Concentration (g kg^{-1} FW) of sucrose (A), glucose (B), fructose (C), sorbitol (D), citric acid (E), and malic acid (F) in "Braeburn" apples in 2011 (◊) and 2012 (■) at different sampling dates during the advanced maturation of apples. Means and their standard errors are presented (n = 5). Different letters (a–c) denote statistically significant differences between sampling dates within each year (Duncan test, $p \leq 0.05$).

and anthocyanins. With the exception of flavonols the concentrations of all the phenolic groups was in 2012 much higher than in 2011. Great differences up to 70% in the phenolics between seasons have already been noted in the study of Lata et al.[13] in several other cultivars.

Among hydroxycinnamic acids chlorogenic, caffeic, and 4-coumaroylquinic acids were analyzed. Hydroxycinnamis acids were the lowest represented group of phenolics in "Braeburn" apple peels ranging from 6% of the total analyzed phenolic concentration at the beginning of ripening to 3% at the technological maturity of apples. During the 5 weeks of advanced ripening the total hydroxycinnamic acids concentration decreased by 27% in both years (Figure 3A). However, the decrease was significant just in 2012 and was mainly the consequence of the decrease of the 4-*p*-coumaroylquinic acid (Figure 4B). Viewed from the perspective of the individual acids significant changes during the ripening were found only in the case of chlorogenic acid in 2011 (Figure 4A).

Phloridzin was the main dihydrochalcone in "Braeburn" apple peels, accounting for approximately 65% of the total dihydrochalcones. Besides phloridzin, phloretin 2-xylosylglucoside was also identified and quantified. Since the beginning of ripening till the technological maturity of apples concentration of the dihydrochalcones decreased by 18% in both years; however, the decrease was not significant (Figure 3B). The decrease was more pronounced in the case of phloridzin than phloretin (data not shown).

The analysis of flavonols covered 7 types of quercetin glycosides. At technological maturity the major flavonol species was quercetin 3-galactoside, accounted for approximately 35–40% of total flavonols concentration in "Braeburn" apple peels, followed by quercetin 3-*O*-arabinofuranoside (15–21%), quercetin 3-*O*-rhamnoside (15–18%), quercetin 3-*O*-xyloside (12–14%), quercetin 3-*O*-glucoside (7–8%), quercetin 3-*O*-rutinoside (4%), and quercetin 3-*O*-arabinopyranoside (2%). Similar findings in "Braeburn" apples were already reported in the previous study of Bizjak et al.[28]

In both years 2011 and 2012, the developmental pattern of flavonols was very similar (Figure 3C). From 5 to 3 weeks before the technological maturity, a slight increase in the concentration was observed, followed by a significant increase which was completed approximately 1 week before the

Table 1. Identification of Phenolic Compounds in Apple Peels in Positive and Negative Ions With HPLC-MS and MS^2

peak no.	$[M-H]^-$ (m/z)	Rt[b]	MS^2 (m/z)	tentative identification	standard	expressed as	compound class	detection (nm)
1	577	6.33	425, 407, 289	procyanidin B1	yes	procyanidin B1	flavanols	280 nm
2	289	7.70	245	catechin	yes	catechin	flavanols	280 nm
3	353	7.84	191, 179	chlorogenic acid	yes	chlorogenic acid	hydroxycinnamic acids	280 nm
4	577	8.83	425, 407, 289	procyanidin B2	yes	procyanidin B2	flavanols	280 nm
5	179	9.64	135	caffeic acid	yes	caffeic acid	hydroxycinnamic acids	280 nm
6	289	10.13	245	epicatechin	yes	epicatechin	flavanols	280 nm
7	337	10.82	173	4-*O*-*p*-coumaroylquinic acid	no	*p*-coumaric acid	hydroxycinnamic acids	280 nm
8	567	19.2	273	phloretin-2′-*O*-xylosylglucoside	no	phloridzin	dihydrochalcones	280 nm
9	435	21.6	273	phloridzin	yes	phloridzin	dihydrochalcones	280 nm
10	609	16.44	301	quercetin-3-*O*-rutinoside	yes	quercetin-3-*O*-rutinoside	flavonols	350 nm
11	463	17.09	301	quercetin-3-*O*-galactoside	yes	quercetin-3-*O*-galactoside	flavonols	350 nm
12	463	17.54	301	quercetin-3-*O*-glucoside	yes	quercetin-3-*O*-glucoside	flavonols	350 nm
13	433	18.69	301	quercetin-3-*O*-xyloside	yes	quercetin-3-*O*-xyloside	flavonols	350 nm
14	433	18.19	301	quercetin-3-*O*-arabinopyranoside	no	quercetin-3-*O*-arabinofuranoside	flavonols	350 nm
15	433	19.86	301	quercetin-3-*O*-arabinofuranoside	yes	quercetin-3-*O*-arabinofuranoside	flavonols	350 nm
16	447	20.24	301	quercetin-3-rhamnoside	yes	quercetin-3-rhamnoside	flavonols	350 nm
17[a]	449	6.56	287	cyanidin-3-galactoside	yes	cyanidin-3-galactoside	anthocyanins	530 nm
18[a]	449	7.07	287	cyanidin-3-glucoside	no	cyanidin-3-galactoside	anthocyanins	530 nm
19[a]	419	7.65	287	cyanidin-3-arabinoside	no	cyanidin-3-galactoside	anthocyanins	530 nm
20[a]	419	9.29	287	cyanidin-7-arabinoside	no	cyanidin-3-galactoside	anthocyanins	530 nm
21[a]	419	9.56	287	cyanidin-3-xyloside	no	cyanidin-3-galactoside	anthocyanins	530 nm

[a]$[M+H]^+$ (m/z) anthocyanins were obtained in the positive ion mode. [b]Retention time.

technological maturity of apples. During the last week of ripening a decrease was found, which was more pronounced in 2011 (Figure 3C).

Among the individual quercetin glycosides, quercetin 3-*O*-galactoside contributed the largest share to the increase of total flavonols. During the 5 weeks of ripening its concentration increased by 35% in 2011 and almost 5-fold in 2012 (Figure 4E). However, it is necessary to take into account that in 2012 its concentration at the beginning of the advanced ripening (29.15 mg kg^{-1} FW) was very low in comparison with 2011 (93.14 mg kg^{-1} FW), thus increases were so different sizes. The significant increase from 3 to 1 week before the technological maturity was observed in the case of all the other individual quercetin glycosides, except quercetin 3-*O*-rutinoside (rutin) in 2011 (Figure 4D). In the case of quercetin 3-*O*-rhamnoside (Figure 4F) as well as quercetin 3-*O*-arabinofuranoside and quercetin 3-*O*-xyloside (data not shown), a significant decrease in the last week of the advanced ripening in 2011 was detected.

Within flavanols the monomeric catechin and epicatechin and dimeric flavanol procyanidin B1 and B2 were analyzed. Procyanidin B2 was the most abundant individual flavanol in the "Braeburn" apple peels, representing approximately 55% of the total flavanol concentration. Other flavanols listed in descending order, were epicatechin, catechin and procyanidin B1. With regard to quantity flavanols were the most represented phenolic group in "Braeburn" apple peels. Their concentration ranged from 50% and 65% of the total analyzed phenolic concentration at the beginning of the advanced ripening to 38% and 47% at the technological maturity of apples in 2011 and 2012, respectively. Flavanols have already been reported to be quantitatively the most abundant phenolic groups by numerous of authors in several other cultivars.[17,29,30]

No significant changes in flavanols were observed during the 5 weeks of the advanced ripening (Figure 3D), however flavanol concentration slightly decreased in both years. Among individual flavanols, significant changes during ripening were observed only in the case of procyanidin B1 in 2012, when a decrease was found (Figure 4C).

Among anthocyanins cyanidin 3-*O*-galactoside, cyanidin-3-*O*-arabinoside, cyanidin 7-*O*-arabinoside, cyanidin 3-*O*-xyloside, and cyanidin 3-*O*-glucoside were identified and quantified. Cyanidin 3-*O*-galactoside was the main cyanidin glycoside in the "Braeburn" apple peels, while the cyanidin 3-*O*-glucoside level was the lowest, findings which are in accordance with those obtained by other autors.[20,28,31] Cyanidin 3-*O*-galactoside ranged from 89% at the beginning of ripening to 81% of the total anthocyanin concentration at the technological maturity of apples. Cyanidin 3-*O*-glucoside with the concentration of 1% of total anthocyanins became detectable 4 weeks before technological maturity of the apples. Other cyanidin glycosides were each present at 8% or less of the total anthocyanin concentration and their percent increase during the advanced ripening. In 2012 anthocyanin synthesis probably started shortly prior to our first sampling, since at first sampling the measured concentration of total anthocyanin was just 0.89 mg kg^{-1} FW. However, the intensive onset of anthocyanin accumulation occurred in both years approximately at the same time somewhere between 3 and 2 weeks before the technological maturity of apples (Figure 3C,E).

Total phenolic content was relatively constant during advanced ripening, and no significant changes were observed neither in 2011 nor in 2012 (Figure 3F). The average content (mg GAE kg^{-1} FW) in 2012 ranged from 1578.33 to 1923.73 and was higher than in 2011 when varied between 1062.40 and

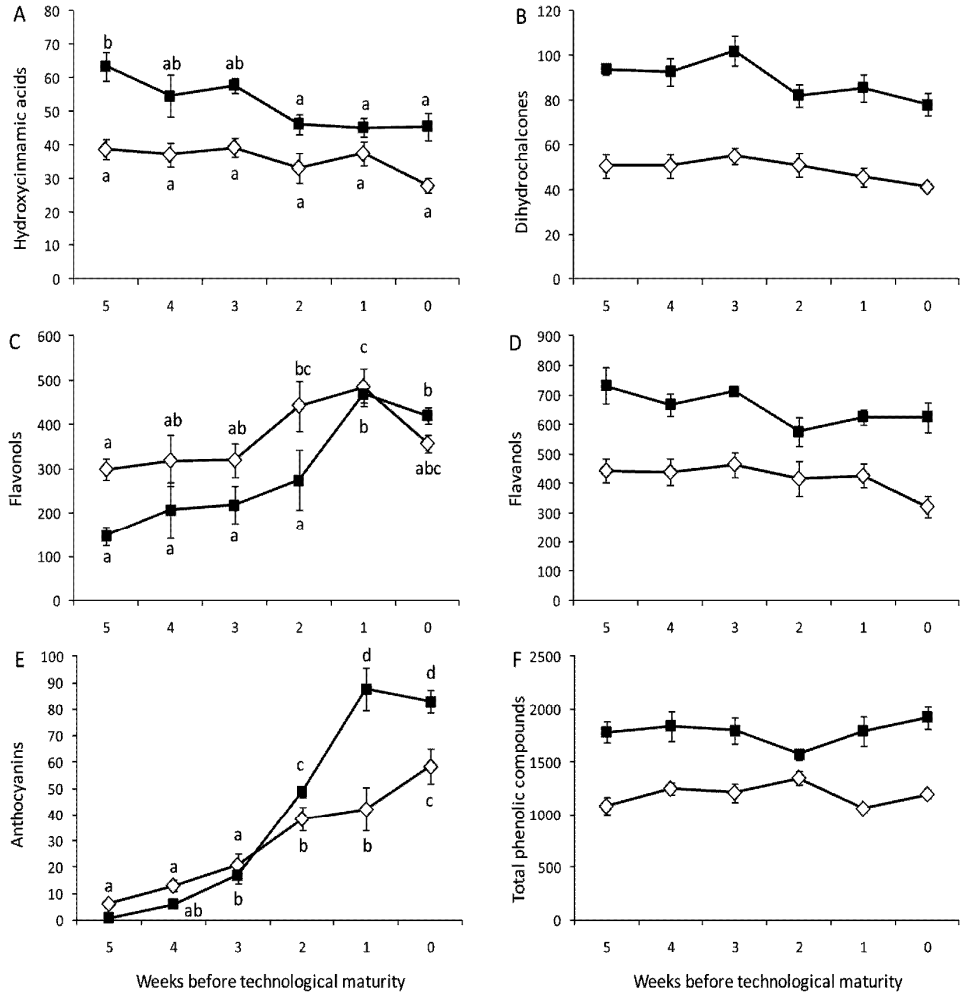

Figure 3. Concentration (mg kg^{-1} FW) of total hydroxycinnamic acids (A), dihydrochalcones (B), flavonols (C), flavanols (D), anthocyanins (E), and total phenolic compounds (mg GAE kg^{-1} FW) in "Braeburn" apple peels in 2011 (◊) and 2012 (■) at different sampling dates during the advanced maturation of apples. Means and their standard errors are presented (n = 5). Different letters (a–d) denote statistically significant differences between sampling dates within each year (Duncan test, $p \leq 0.05$).

1347.24. Differences in the TPC between the years were so similar to those previously observed in several phenolic groups. Great differences in the total phenolic content between the seasons have already been reported by Lata et al.[13] in many apple cultivars.

DISCUSSION

Firmness and soluble solids content (SSC) are two important quality parameters in determining fruit maturity and harvest time, and represent key parameters in assessing and grading the postharvest quality of apples.[32] The loss of fruit firmness during advanced ripening is a consequence of many cellular events which are influenced by external factors,[33] whereas the increases in soluble solids during the ripening would likely be attributed to the hydrolysis of the starch component in the unripe apples.[34] The increase of the fruit weight during the fruit development of the apples is due to the cell division and cell enlargement and its pattern shows a typical sigmoid pattern.[35] However, during the advanced maturation, the growth curve becomes much more flat, which is largely conditioned by apple cultivar, growth conditions and number of fruits harvested on the tree.

Sugars (glucose, sucrose, and fructose) and organic acids (citric and in particular malic acid) represent the most important taste components in apples.[1,2] The concentration of glucose and fructose in 2012 fluctuated during the 5 weeks of advanced ripening, whereas it decreased in 2011 (Figure 2B,C). Our results, especially those in 2012 are consistent with the findings of Ackermann et al.,[25] who also found a decrease of glucose and fructose during the advanced ripening, followed by an increase approximately 2 weeks before the technological maturity of apples. Sorbitol is the primary product of photosynthesis mainly present in apple leaves and is transported to sink tissues such as fruit where being converted to fructose.[36] The increase in the fructose concentration during the fruit ripening corresponded well with the decrease in sorbitol concentration,[21] which was not confirmed by our results where no interconnection between the concentration of

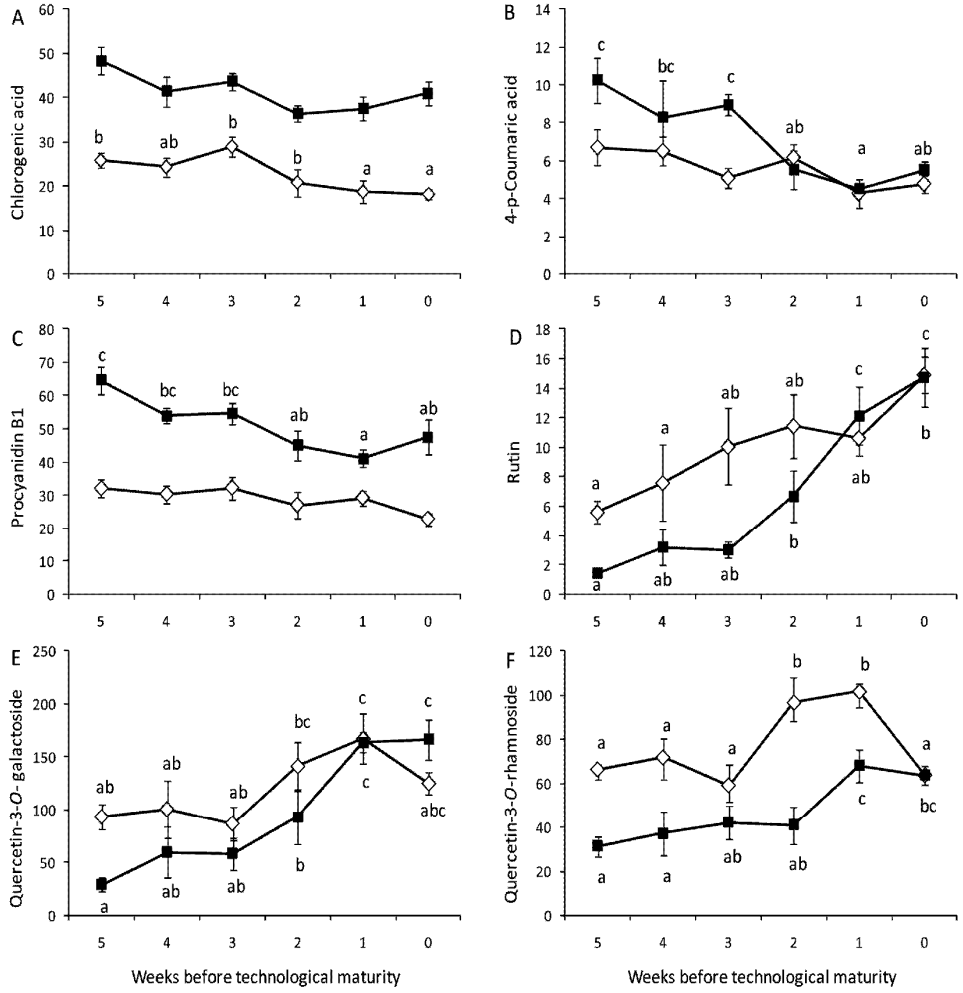

Figure 4. The concentration (mg kg^{-1} FW) of chlorogenic acid (A), p-coumaric acid (B), procyanidin B1 (C), rutin (D), quercetin-3-*O*-galactoside (E), and quercetin-3-*O*-rhamnoside in "Braeburn" apple peels in 2011 (◊) and 2012 (■) at different sampling dates during the advanced maturation of apples. Means and their standard errors are presented (n = 5). Different letters (a–c) denote statistically significant differences between sampling dates within each year (Duncan test, $p \leq 0.05$).

sorbitol and fructose was found. However, the continued increase in the sucrose concentration after the fructose accumulation stopped support the suggestion of Berüter[37] that part of the increase in sucrose before the fruit harvest might have been synthesized from fructose via sucrose synthase. In our study sucrose was the only individual sugar which increased during the advanced maturation and also the only one with the same developmental pattern in both years (Figure 2A). The accumulation of the sucrose during advanced ripening resulted most probably from sucrose that was transported from the leaves on the one hand and newly synthesized sucrose via sucrose phosphate synthetase and/or sucrose synthase on the other hand.[21] The sucrose increase during apple ripening has also been observed in some other apple varieties.[21,25] So it appears that among individual sugars sucrose is at least influenced upon many external factors, including apple cultivar, climate, and growth conditions.

Malic and citric acid concentrations decreased during advanced ripening in 2012, whereas they showed less significant changes in 2011 (Figure 2E,F). The decrease could be attributed to a dilution effect caused by the mass increase during the cell growth phase and/or increased respiration, since malic acid is the principal metabolic substrate used in this process.[25,38,39] The developmental pattern of malic and citric acids during advanced ripening was similar to those observed from other researchers.[21,25]

With respect to hydroxycinnamic acids, dihydrochalcones, and flavanols, no significant changes have been observed during advanced ripening of "Braeburn" apples (Figure 3B,D) with the exception of hydroxycinnamic acids in 2012 (Figure 3A), when a significant decrease was found. Developmental patterns of above-mentioned phenolic groups were very similar in both years, showing small fluctuations, but the general tendency was to slightly decrease during the advanced ripening of apples. A relatively constant level of total catechins (catechin plus epicatechin), phloridzin, and chlorogenic acid was observed also by Awad et al.[12] in the skin of "Elstar" and "Jonagold" apples. With respect to oligomeric procyanidins generally a

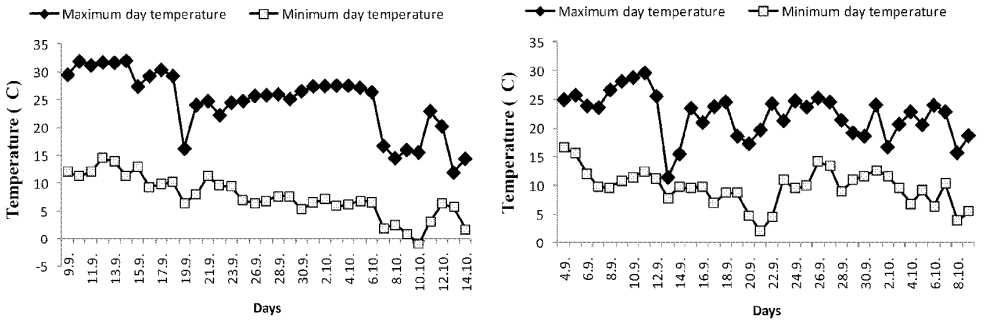

Figure 5. Maximum day and minimum night temperatures recorded during the advanced ripening of "Braeburn" apples in the orchard of the Biotehnical faculty in Ljubljana in 2011 (left) and growing center Maribor Gacnik in 2012 (right).

slight decrease was observed in our study, which is consistent with the findings of Macheix et al.,[40] but not in the accordance with the result of Lister et al.,[19] who found an increase of procyanidins during the advanced ripening of "Splendour" apples. Oligomeric procyanidins have attracted increasing attention in the fields of nutrition and medicine due to their potential health benefits observed in vitro and vivo.[41]

The concentration of anthocyanins and flavonols significantly increased during the advanced ripening of "Braeburn" apples. In both years the onset of rapid flavonol and anthocyanin formation occurred somewhere between 3 to 2 weeks before the technological maturity (Figure 3C,E). Similar to our findings Lister et al.[19] also reported that flavonol synthesis coincided with an increased accumulation of anthocyanins. Similar observations regarding the onset of anthocyanins were also reported by Bizjak et al.[28] and Awad and de Jager,[14] who also detected rapid anthocyanin accumulation in "Jonagold" apples and "Braeburn" apples around 20 days before the harvest. The accumulation of anthocyanins is rather dependent on the temperature.[42] Approximately three weeks before the harvest the night temperatures dropped, whereas the day temperatures still remained quite high (Figure 5). And just the drop in the night temperatures may have stimulated anthocyanins biosynthesis. Cold night temperatures followed by warm day temperatures have already been reported to stimulate anthocyanin synthesis in some apple cultivars.[43] Awad and de Jager[14] and Whale and Singh[44] suggested endogenous ethylene being the trigger for anthocyanin biosynthesis. Similar to our findings Lister et al.[19] also reported that the increased flavonol synthesis coincided with an increased accumulation of anthocyanins. Regarding the co-ordination of synthesis it can be assumed that during the advanced ripening the pool of hydroxycinnamic acids and dihydrochalcones decreased in favor with an increased synthesis of flavonols. A decrease in the pool of hydroxycinnamic acids in favor of flavonols has already been suggested by Mayr et al.[45] Since leucocyanidin (flavan-3,4-diol) is a common precursor for anthocyanidins and flavanols,[3] the slight decrease of flavanols found in our study could be on account of the increased accumulation of anthocyanins. Finally, a decrease of the astringent procyanidins coincided with an increase of flavonols and anthocyanins indicate people and fruit eating animals that an apple is suitable for use which makes an important contribution to the seed dispersal and spread of apple trees.

Within flavonoids, anthocyanins are the most important group for the production of the red color of the apple peel, which is of great commercial importance determining market acceptance of apples.[46] Although most of the phenolics are reported to have an antioxidant activity, quercetin, a major representative of the flavonol subclass, has been reported to have structural advantages as antioxidant[47] and contributes the largest share to the total antioxidant capacity of apples.[5] Recently, additional considerable attention has been paid to this phenol since quercetin and its sugar-bound, or glycosilated forms represent 60−75% of flavonoid intake.[48] In the present study we have demonstrated that in the "Braeburn" apple peel anthocyanins as well as quercetin glycosides are mainly accumulated during the last few weeks of advanced ripening until the onset of the technological maturity of apples. Therefore, viewed from marketing as well as from the health points of view it is very important to pick the apples at their proper technological maturity. Since accumulation of quercetin glycosides and anthocyanin are strongly light/tree position dependent,[12] it is additionally very important that trees are properly pruned and illuminated. Use of reflective foil represents one of the efficient agro-technical measures that can effectively increase light utilization in the apple orchard.[49] On the basis of our results, we suggest that summer pruning, orchard floor covering, applications of certain elements or plant regulators and similar measures that have been designed to improve the color and quality of the fruit should be performed approximately 3−4 weeks before the expected technological maturity of "Braeburn" apples, depending on the speed of an effect of each measure.

With respect to the total phenolic compounds (TPC) no significant changes during the advanced ripening were noticed in our experiment in both years (Figure 2F). The polyphenol content in the apples is strongly dependent upon their growth conditions; thus, it is not surprising that different results have been reported concerned with this issue. Similar to us, Zheng et al.[50] found out that TPC in the apples did not change significantly and remained nearly constant during the last weeks of apple ripening. On the other hand Blanco et al.[51] and Campo et al.[52] reported different behaviors, depending on the apple variety considered. However, when comparing and evaluating these results it must be taken into account that in the study of Blanco et al.[51] and Campo et al.[52] experiments have been done on the cider apple varieties which generally contain much higher levels of phenolics than table apples.[23]

In conclusion during the 5 weeks of the advanced ripening and technological maturity as well the monomeric and polymeric flavanols were the most abundant phenolic group in the "Braeburn" apple peel, followed by flavonols, dihydrochalcones, hydroxycinnamic acids, and anthocyanins,

the concentration of which in the last 2 weeks prior to technological maturity exceeded the concentration of dihydrochalcones and hydroxycinnamic acids. We demonstrate that during advanced ripening the concentrations of hydroxycinnamic acids, dihydrochalcones, and flavanols remain quite constant or slightly decrease. On the contrary an intensive accumulation of quercetin glycosides and anthocyanins takes place during this period, starting with the onset of rapid formation approximately 3 weeks before the technological maturity of apples. On the basis of our results, on summer pruning of "Braeburn" apples, the orchard floor covering and foliar applications of certain elements and similar measures designed to improve the color and quality of the fruit should be performed approximately 3−4 weeks before the expected technological maturity of apples. However, similar studies over a number of years should be performed on the basis of which it could be more accurately determined when during the advanced maturation of apples would be the best time that these technological measures should be carried out.

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Notes

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3 RAZPRAVA IN SKLEPI

3.1 RAZPRAVA

Jabolka vsebujejo širok spekter fenolnih spojin, med katerimi na obarvanost kožice jabolk najbolj vpliva prisotnost antocianov, klorofilov, karotenoidov, flavonolov in proantocianidinov. Čeprav je fenilpropanoidna pot fenolnih spojin v jabolkih (Slika 1) precej dobro raziskana, še vedno ni točno znano, kateri del poti je ključen za sintezo antocianov, ki največ prispevajo k tvorbi rdeče barve plodov. Prav tako še ni pojasnjen pomen aktivnosti encimov, ki pri tvorbi barve sodelujejo, oziroma se rezultati raziskav o pomembnosti posameznih encimov med avtorji precej razlikujejo.

Za proheksadion kalcij (Pro-Ca) je že bilo ugotovljeno, da regulira biosintezno pot fenolnih spojin in povzroči značilne spremembe v fenolnem profilu, ki so se v jablaninih listih odrazile v manjših vsebnostih flavonolov, dihidrohalkonov, flavanolov ter višjih vsebnostih hidroksicimetnih kislin (Halbwirth in sod., 2003; Halbwirth in sod., 2006; Fisher in sod., 2006; Roemmelt in sod. 2003a,b). Vpliv Pro-Ca pa je bil proučevan samo v jablaninih listih, medtem ko o vplivu v kožici plodov ni na voljo nobenih raziskav. Tako smo s prvim poskusom želeli izvedeti, ali podobno kot v listih Pro-Ca učinkuje tudi v kožici jabolk in, ali bi njegova jesenska uporaba preko modulacije biosintezne poti fenolnih spojin lahko zmanjšala sintezo antocianov in s tem delež rdeče krovne barve plodov. Približno tri tedne pred tehnološko zrelostjo plodov smo tretirana drevesa jablan sorte 'Braeburn' poškropili s pripravkom Regalis (BASF 125 10 W-10 % Pro-Ca, BASF, Germany) v koncentraciji 1 g na drevo (2,5 kg ha^{-1}), medtem ko smo kontrolna drevesa poškropili z vodo.

Spremembe v obarvanosti smo dodatno ovrednotili z merjenjem parametrov sistema CIE L*a*b*. Vrednost parametra a* se je z zorenjem plodov povečevala, medtem ko se je vrednost parametra hº zmanjševala, kar oboje nakazuje povečanje intenzitete rdeče obarvanosti plodov (McGuire, 1992). Vendar pa je bilo povečanje parametra a* in zmanjšanje parametra hº pri Pro-Ca tretiranih jabolkih precej izrazitejše kot pri netretiranih. Parameter hº je izračunan kot tan^{-1} (b*/ a*) in predstavlja najprimernejši način vrednotenja sprememb v obarvanosti plodov (McGuire, 1992). Značilno manjša intenziteta rdeče barve kožice jabolk je bila pri tretiranih jabolkih tako vidna že 8 dni po tretiranju in se je obdržala vse do tehnološke zrelosti plodov. Vpliv Pro-Ca smo zabeležili tudi pri spremljanju parametra L*, ki opisuje svetlost plodov, vendar pa je bil učinek pri tem parametru viden šele 19 dni po tretiranju, ko so bila tretirana jabolka značilno svetlejša od netretiranih.

Negativen vpliv Pro-Ca na razvoj rdeče krovne barve je potrdila tudi analiza vsebnosti antocianov. V primerjavi z netretiranimi smo značilno manjše vsebnosti antocianov pri tretiranih jabolkih zabeležili 5, 12 in 15 dni po tretiranju. Največjo razliko smo zabeležili 15 dni po tretiranju, ko smo v tretiranih jabolkih izmerili skoraj 50 % manjšo vsebnost antocianov v primerjavi s kontrolnimi jabolki. Z razliko od parametrov obarvanosti pa pri antocianih razlika v njihovi vsebnosti med tretiranimi in netretiranimi jabolki ob tehnološki zrelosti ni bila več statistično značilna. Zmanjšanje sinteze antocianov v tretiranih jabolkih je bilo najverjetneje posledica strukturne podobnosti Pro-Ca in 2-oksoglutaratnih kislin, ki je povzročila začasno blokado 2-oksoglutarat odvisnih deoksigenaz; flavanon 3-

didroksilaze (FHT), flavonol sintaze (FLS) in antocianidin sintaze (ANS), ki igrajo ključne vloge v sintezi antocianov in ostalih flavonoidov (Halbwirth in sod., 2006). To je potrdila tudi analiza encimatske aktivnosti, saj je bila 15 dni po tretiranju aktivnost FHT v tretiranih jabolkih za 47 %, in FLS za 32 % manjša kot aktivnost omenjenih encimov v kožici netretiranih plodov. Poleg deoksigenaz pa je bila v tretiranih jabolkih v primerjavi s kotrolnimi občutno manjša tudi aktivnost DFR, in sicer za 40 %. Ob tehnološki zrelosti jabolk sta bili aktivnosti FHT in FLS pri obeh obravnavanjih skoraj popolnoma enaki in tudi razlika v vsebnosti antocianov ni bila več prisotna.

Podobno kot pri antocianih smo negativen vpliv Pro-Ca na sintezo ugotovili tudi pri spremljanju in analizi flavonolov. Tudi pri tej skupini flavonoidov smo največjo razliko v vsebnosti med tretiranimi in netretiranimi jabolki zabeležili 15 dni po tretiranju, ko je le ta znašala skoraj 50 %, medtem ko ob tehnološki zrelosti ni bila več statistično značilna. Tudi Roemmelt in sod. (2003a,b) poročajo o zmanjšani sintezi flavonolov v Pro-Ca tretiranih jablaninih listih, o podobnih rezultatih pa poročajo tudi Gosch in sod. (2003) v nekaterih drugih sadnih vrstah in Schlangen in sod. (2003) v listih vrtnic. 15 dni po tretiranju smo v tretiranih jabolkih v primerjavi z netretiranimi zabeležili tudi manjše vsebnosti dihidrohalkonov, medtem ko v ostalih terminih značilnih razlik med obravnavanjema nismo opazili. Razlika v vsebnosti je bila posledica manjše vsebnosti floridzina, o kateri poročata tudi Ruehmann in Treutter (2003), ki sta vpliv tretiranja s Pro-Ca proučevala v jablaninih listih.

Vpliva tretiranja s Pro-Ca pa nismo ugotovili pri spremljanju flavanolov, saj je bila njihova vsebnost v kožici v vseh terminih vzorčenja zelo podobna pri obeh obravnavanjih. Naši rezultati se pri tej skupini flavonoidov nekoliko razlikujejo od rezultatov Roemmelta in sod. (2003a), ki poročajo o manjših vsebnostih oligomernih flavanolov v tretiranih listih jablane.

Poleg začasnega zmanjšanja sinteze antocianov in flavonolov na eni, pa je tretiranje s Pro-Ca povzročilo tudi povečano sintezo hidroksicimetnih kislin na drugi strani. Značilno večje vsebnosti hidroksicimetnih kislin smo v kožici tretiranih jabolk zabeležili 8 in 19 dni po tretiranju, ko je bila njihova vsebnost v primerjavi z netretiranimi jabolki večja za več kot 20 %. Naši rezultati se skladajo z ugotovitvami Roemmelta in sod. (2003a,b), ki so po tretiranju s Pro-Ca prav tako ugotovili povečane vsebnosti hidroksicimetnih kislin, le da so oni vpliv tretiranja proučevali v listih. Razlog za povečane vsebnosti hidroksicimetnih kislin po tretiranju s Pro-Ca bi lahko iskali v inhibiciji encima FHT, ki zmanjša sintezo določenih flavonoidov in njihovih prekurzorjev na eni, in poveča produkcijo cimetnih kislin in njihovih prekurzorjev na drugi strani (Roemmelt in sod., 2003a, Fisher in sod., 2006). Povečane vsebnosti po tretiranju s Pro-Ca so zabeležili tudi Gosch in sod. (2003) v listih aktinidije (*Actinidia arguta*).

Zmanjšanje aktivnosti encima FHT je v tretiranih jabolkih tako trajalo nekako do 15 dni po tretiranju s Pro-Ca, kar lahko razložimo z 10 do 15 dni dolgo dobo razgradnje Pro-Ca in njegovim hitrim metabolizmom v rastlinskih tkivih (Halbwirth in sod., 2003). Tako smo v kožici jabolk opazili daljše obdobje delovanja, kot je bilo poročano za liste jablane, kjer je inhibicija encima trajala le do deset dni po tretiranju (Fischer in sod., 2006).

V poskusu smo spremljali tudi spremembe vsebnosti fenolnih spojin v mesu plodov, kjer smo pri večini skupin fenolnih spojin dobili drugačne rezultate vpliva Pro-Ca kot v kožici

jabolk. Spremembe so bile podobne samo v primeru hidroksicimetnih kislin, medtem ko smo pri dihidrohalkonih, flavanolih in flavonolih v nekaterih terminih v mesu tretiranih jabolk zabeležili večje vsebnosti omenjenih skupin fenolnih spojin kot v mesu kontrolnih jabolk. Analize vsebnosti fenolnih spojin v mesu jabolk so nekoliko manj pomembne, saj se večina fenolnih spojin v jabolkih nahaja v kožici plodov (Lata, 2009; Mikulic-Petkovsek in sod., 2007).

V raziskavi smo tako ugotovili, da jesensko tretiranje jablan s Pro-Ca prehodno zmanjša sintezo antocianov in flavonolov v kožici jabolk, kar se odrazi v manjši intenziteti in deležu krovne rdeče barve plodov. Spremembe v fenilpropanoidni poti so bile podobne tistim, ki so že bile dokumentirane v listih (Roemmelt in sod., 2003a,b; Ruehmann in Treutter, 2003; Fischer in sod., 2006), le da so se v kožici jabolk obdržale nekoliko dlje, a vseeno ne do tehnološke zrelosti plodov.

Ker smo v prvem poskusu (Bizjak in sod., 2012) ugotovili, da je bil učinek Pro-Ca le začasen in je trajal le do 15 dni po tretiranju, smo z naslednjim poskusom, ko smo polni odmerek Pro-Ca razdelili na dva polovična, želeli ugotoviti, ali bo učinek tretiranja enak, kot smo ga zabeležili ob enkratnem tretiranju v polnem odmerku. Želeli smo raziskati tudi vpliv tretiranja na ekspresijo nekaterih genov. Tudi v tem poskusu so parametri obarvanosti pokazali na negativen vpliv Pro-Ca na razvoj rdeče krovne barve plodov, vendar pa je bil ta pri parametrih a* in L* viden le 2 tedna po prvem in en teden po drugem tretiranju, medtem ko so bile pri parametru hº značilne razlike med tretiranimi in netretiranimi jabolki vidne le en teden po drugem tretiranju. Rezultati se tako nekoliko razlikujejo od tistih, ki smo jih zabeležili v prejšnjem poskusu (Bizjak in sod., 2012), ko smo razlike v parametrih obarvanosti med tretiranimi in netretiranimi jabolki zabeležili tudi ob tehnološki zrelosti plodov. Razlog za to bi lahko bila razdelitev polnega priporočenega odmerka (2,5 kg ha^{-1}) na dva polovična odmerka ter hiter metabolizem oziroma razgradnja Pro-Ca v tkivih rastlin. Z rezultati parametrov obarvanosti so se lepo ujemali rezultati analize vsebnosti antocianov, kjer smo značilno razliko v njihovi vsebnosti med obravnavanjema zabeležili le en teden po drugem tretiranju, ko je bila vsebnost v kožici tretiranih jabolk (19,48 mg/kg^{-1} SM) skoraj enkrat manjša kot v kožici netretiranih jabolk (38,53 mg/kg^{-1} SM). Največje vsebnosti skupnih antocianov smo sicer zabeležili ob tehnološki zrelosti plodov, ko sta bili vrednosti pri tretiranih jabolkih (57,89 mg/kg^{-1} SM) in kontrolnih jabolkih (59,32 mg/kg^{-1} SM) skoraj enaki. Tudi v prejšnjem poskusu (Bizjak in sod., 2012) je bila vsebnost antocianov ob tehnološki zrelosti pri obeh obravnavanjih skoraj enaka, tako da se zdi, da ko se Pro-Ca razgradi, jabolka s povečano sintezo antocianov nadoknadijo primanjkljaj, ki ga je Pro-Ca povzročil z blokado deoksigenaz in modulacijo fenilpropanoidne poti fenolnih spojin.

V primerjavi s prvim poskusom so rezultati v drugem poskusu odstopali tudi pri nekaterih ostalih skupinah fenolnih spojin. Pri analizi hidroksicimetnih kislin smo značilno večje vsebnosti v tretiranih jabolkih v primerjavi s kontrolo zabeležili teden po prvem, teden po drugem škropljenju ter ob tehnološki zrelosti plodov. Učinek dvakratnega škropljenja s Pro-Ca je bil pri hidroksicimetnih kislinah tako podoben tistemu, ki smo ga opazili v prejšnjem poskusu, z razliko, da je bila v slednjem poskusu razlika med obravnavanjema značilna tudi ob tehnološki zrelosti plodov. So se pa rezultati bolj razlikovali pri dihidrohalkonih, kjer smo teden po prvem, dva tedna po drugem pa tudi ob tehnološki zrelosti plodov v tretiranih jabolkih izmerili značilno večje vsebnosti dihidrohalkonov kot

v kontrolnih plodovih. Do podobnih rezultatov smo prišli tudi pri spremljanju flavanolov, vendar pri tej skupini fenolov razlika med obravnavanjema ob tehnološki zrelosti plodov ni bila več statistično značilna. Pri analizi flavonolov smo 2 tedna po prvem in en teden po drugem škropljenju sicer opazili zmanjšanje njihove vsebnosti v tretiranih jabolkih, a v primerjavi s kontrolo razlika ni bila statistično značilna.

Razlog za večjo vsebnost dihidrohalkonov v tretiranih jabolkih bi lahko bila zmanjšana aktivnost encima FHT, ki lahko poveča produkcijo cimetnih kislin in njihovih prekurzorjev (Roemmelt in sod., 2003a, Fisher in sod., 2006). Tudi v tem poskusu smo namreč v tretiranih jabolkih zabeležili zmanjšanje aktivnosti encima FHT, do katerega je prišlo že teden po tretiranju. Razlika v aktivnosti FHT med tretiranimi in kontrolnimi jabolki se je tokrat obdržala vse do tehnološke zrelosti plodov, kar nakazuje na to, da aktivnost FHT ni ključni encim za sintezo antocianov. Pro-Ca je sicer rahlo zmanjšal aktivnost vseh analiziranih encimov, vendar pa pri encimih PAL, CHS/CHI, in DFR med obravnavanjema nismo zabeležili značilnih razlik v nobenem terminu vzorčenja.

Ekspresije genov, ki nastopajo v zadnjih delih fenilpropanoidne poti, smo izrazili kot relativno aktivnost v primerjavi z aktivnostjo, izmerjeno ob prvem vzorčenju, ki je tako predstavljala 100 %. Tretiranje s Pro-Ca je pripeljalo do zmanjšane ekspresije antocianidin sintaze (ANS), antocianidin reduktaze (ANR), flavonoid 3-O-glikoziltransferaze (FGT) in transkripcijskega faktorja MYB10. Pri tretiranih jabolkih so bile tako relativno izražene ekspresije genov (%) ANS, ANR, FGT in MYB10 v vseh terminih manjše kot v prvem terminu, razen pri transkripcijskem faktorju MYB10 smo en teden po drugem tretiranju zabeležili povečano transkripcijo. MYB10 je znan kot aktivator akumulacije antocianov (Takos in sod., 2006; Espley in sod., 2007), zato bi bila povečana ekspresija gena MYB10 lahko povezana ali celo razlog za povečano akumulacijo antocianov, ki smo jo pri tretiranih jabolkih zabeležili v naslednjem terminu. Zmanjšanje ekspresije večine genov smo sicer zabeležili tudi pri kontrolnih jabolkih, vendar pa je bilo le to manj izrazito.

Z namenom, da bi ugotovili, ali Pro-Ca vpliva na vsebnost fenolnih spojin tudi med skladiščenjem plodov, smo spremembe v vsebnosti enakih skupin fenolnih spojin kot že med dozorevanjem plodov, v mesečnem intervalu spremljali tudi med njihovim dvo-mesečnim skladiščenjem. Po dveh mesecih skladiščenja smo pri kontrolnih jabolkih ugotovili povečanje vsebnosti hidroksicimetnih kislin, flavanolov in dihidrohalkonov, medtem ko se vsebnosti omenjenih skupin fenolnih spojin pri tretiranih jabolkih skorajda niso spremenile. Do podobnih rezultatov smo prišli tudi pri merjenju antocianov, katerih vsebnost se je pri kontrolnih plodovih po dveh mesecih skladiščenja povečala, medtem ko se je pri tretiranih plodovih zmanjšala. Pri slednjih se je zmanjšala tudi vsebnost flavonolov, ki se pri netretiranih plodovih po dveh mesecih skladiščenja ni značilno spremenila. Na podlagi dobljenih rezultatov lahko domnevamo, da je Pro-Ca negativno vplival na vsebnost omenjenih fenolnih spojin.

Z drugim, nekoliko drugače zastavljenim poskusom smo tako potrdili, da jesenska uporaba s Pro-Ca začasno oziroma prehodno zmanjša intenziteto in nastajanje rdeče krovne barve plodov ter v kožici jabolk povzroči značilne spremembe v fenilpropanoidni poti fenolnih spojin. Spremembe so se tokrat nekoliko razlikovale od tistih, ki smo jih zabeležili v prejšnjem poskusu (Bizjak in sod., 2012), ko smo Pro-Ca aplicirali v enkratnem, polnem

odmerku. Med samim skladiščenjem plodov pa bi Pro-Ca v jabolkih sorte 'Braeburn' lahko imel negativen vpliv na vsebnost določenih skupin fenolnih spojin.

V splošnem so med potrošniki oziroma kupci jabolk pri nakupu rdečih sort bolj priljubljena bolje obarvana jabolka oziroma jabolka z večjo intenziteto in večjim deležem rdeče krovne barve. Dobra obarvanost jabolk ni pomembna le zaradi zunanjega izgleda sadežev, ampak preko večje vsebnosti antocianov pripomore tudi k večji prehranski vrednosti plodov. Eden od ukrepov, ki ga proizvajalci jabolk uporabljajo za doseganje boljše obarvanosti plodov, je tudi uporaba različnih kemičnih pripravkov (Whale in sod., 2008). V naslednjem poskusu smo želeli ugotoviti, ali jesenska uporaba pripravka na osnovi fosforja in kalcija (Phostrade Ca) lahko izboljša obarvanost jabolk med njihovim dozorevanjem. Hkrati smo z analizo primarnih metabolitov in encimatske aktivnosti želeli raziskati nekatere potencialno možne mehanizme delovanja samega pripravka. Phostrade Ca smo v 0,5 % koncentraciji aplicirali v dveh odmerkih, in sicer pet oziroma tri tedne pred tehnološko zrelostjo plodov. Pho Ca je značilno vplival na vse parametre obarvanosti plodov. Med pettedenskim zorenjem jabolk smo pri tretiranih jabolkih izmerili značilno večje vrednosti parametra a* ter značilno manjše vrednosti parametrov hº in L*, kar nakazuje na večjo intenziteto rdeče krovne barve plodov (McGuire, 1992). Boljšo obarvanost tretiranih plodov pa je potrdila tudi analiza antocianov. Vsebnost slednjih je bila pri obeh obravnavanjih največja ob tehnološki zrelosti oziroma obiranju plodov, ko je pri tretiranih znašala 102,57 mg kg^{-1} SM in pri kontrolnih le 58,52 mg kg^{-1} SM. Med pettedenskim zorenjem se je vsebnost antocianov pri tretiranih jabolkih tako povečala za 20-krat, medtem ko je v kontrolnih narasla le za 9-krat. Med posameznimi antociani je bil najbolj zastopan cianidin glalaktozid, ki je predstavljal 80-86 % skupne vsebnosti antocianov. Pho Ca je povečal sintezo vseh posameznih ciandin glikozidov in velikost povečanja je bila pri vseh zelo podobna. Pozitiven vpliv uporabe Pho Ca smo opazili tudi pri analizi flavonolov, med katerimi je največji, 40 % delež, predstavljal kvercetin galaktozid. Povečane vsebnosti smo v tretiranih jabolkih zabeležili pri vseh posameznih kvercetin glikozidih, razen pri kvercetin ramnozidu ne. V primerjavi z razliko pri antocianih je bila razlika med obravnavanjema pri flavonolih sicer manjša, a še vedno statistično značilna.

Pozitiven vpliv pripravkov na osnovi fosforja in kalcija na obarvanost jabolk je že bil dokumentiran pri številnih sortah, med drugim 'Starking Delicious' (Gómez-Cordovés in sod., 1996; Larrigaudiere in sod.,1996), 'Fuji' (Li in sod., 2002), 'Elstar' (Funke in Blanke, 2006), in 'Jonagold' (Wojcik in Wojcik, 2007). V prvih treh študijah je bilo izboljšanje obarvanosti ovrednoteno in izraženo z analizo antocianov, medtem ko je bila obarvanost v zadnjih dveh študijah ocenjena z deležem rdeče krovne barve plodov. Naši rezultati glede vpliva Pho Ca na sintezo antocianov in flavonolov se ujemajo z ugotovitvami Lia in sod. (2002), ki v jabolkih, ki so bila škropljena s Senifosom, poročajo o značilno večjih vsebnostih omenjenih fenolnih skupin, kot so jih imela kontrolna jabolka. Senifos, ki je bil uporabljen v njihovem poskusu, ima skoraj popolnoma enako sestavo kot Phostrade Ca, ki smo ga uporabili v našem poskusu. O pozitivnem vplivu tretiranja s senifosom na vsebnost antocianov pišejo tudi Gómez-Cordovés in sod. (1996) ter Larrigaudiere in sod.(1996) v jabolkih sorte 'Starking Delicious', medtem ko Awad in Jager (2002b) značilnega vpliva Senifosa na vsebnost antocianov in flavonolov v jabolkih sorte 'Jonagold' nista opazila. Kar se tiče analiz vsebnosti hidroksicimetnih kislin, dihidrohalkonov, flavanolov in skupnih fenolov (TPC), vpliva Phostrade Ca nismo ugotovili.

Mehanizem, preko katerega pripravki na osnovi fosforja vplivajo oziroma izboljšujejo obarvanost plodov, ostaja slabo raziskan. Li in sod. (2002) poročajo, da je uporaba Senifosa povečala aktivnosti encimov PAL in CHS. O povečani aktivnosti encima PAL, ki je bila povezana s povečano sintezo antocianov, pišejo tudi Larrigaudiere in sod. (1996). Na drugi strani nekateri avtorji navajajo, da med dozorevanjem jabolk PAL najverjetneje ni ključni encim za sintezo antocianov (Lister in Lancaster., 1996, Saure, 1990) in da CHS ni regulatorni encim v sintezi antocianov (Ju in sod., 1995b). V našem poskusu povečanih aktivnosti zgoraj omenjenih encimov nismo zabeležili, zato sklepamo, da aktivnost encimov PAL in CHI/CHS ni bila odločilna za povečano sintezo antocianov. Smo pa povečano aktivnost zabeležili pri spremljanju encimov FHT in DFR, ki sta bili značilno povezani s povečano sintezo antocianov. O večji aktivnosti encimov FHT in DFR v zadnjem stadiju dozorevanja plodov sorte 'Florina' pišejo tudi Slatnar in sod. (2012). Kakorkoli, značilen vpliv Pho Ca na večjo aktivnost smo v naši študiji zabeležili le pri encimu DFR. Na podlagi rezultatov o aktivnosti encimov predpostavljamo, da so encimi PAL, CHI/CHS, FHT in DFR sicer potrebni za sintezo antocianov, vendar pa večja aktivnost posameznega encima še ne zagotavlja tudi večje sinteze posamezne skupine fenolnih spojin. Podobnega mnenja so tudi Lister in sod. (1996), ki domnevo podkrepljujejo z dejstvom, da je aktivnost encima PAL pri zeleni sorti jabolk 'Granny Smith' dokaj velika, medtem ko je vsebnost antocianov v kožici plodov te sorte minimalna ali pa jih sploh ni možno zaznati. Tako bi bila sinteza antocianov lahko bolj odvisna od razpoložljivosti samega substrata dihidrokvercetina oziroma leukocianidina in pa aktivnosti encimov ANS in GT, katerih aktivnosti v naši študiji nismo uspeli analizirati. Da bi pretvorba dihidrokvercetina v cianidin lahko bila kontrolna točka sinteze antocianov predlagajo tudi Ju in sod. (1995b).

Sinteza antocianov je med drugim odvisna tudi od sladkorjev v rastlini (Lancaster, 1992; Lueangprasert in sod., 2010). V našem poskusu smo v Pho Ca tretiranih jabolkih zabeležili značilno večje vsebnosti sladkorjev kot v netretiranih. V primerjavi termina 0 (pred škropljenjem) in prvega termina (teden dni kasneje), smo pri primerjavi tretiranih in kontrolnih jabolk ugotovili značilno interakcijo pri saharozi ($P = 0,02$), medtem ko je bila interakcija pri glukozi ($P = 0,08$) in skupnih sladkorjih ($P = 0,06$) mejno značilna. Vsebnost sladkorjev se je teden po tretiranju pri tretiranih jabolkih povečala, medtem ko se je pri kontrolnih zmanjšala, kar bi lahko pomenilo, da je tretiranje s Pro-Ca povzročilo povečanje vsebnosti sladkorjev v tretiranih jabolkih. Med dozorevanjem jabolk sta bili tako vsebnost saharoze kot tudi vsebnost glukoze značilno povezani ($P < 0,0002$) s skupno vsebnostjo antocianov. Teden dni po prvem škropljenju in teden dni po drugem škropljenju se je vsebnost skupnih sladkorjev v tretiranih jabolkih povečala, medtem ko se je v kontrolnih zmanjšala, zato bi bilo povečanje sladkorjev, predvsem saharoze, mogoče vsaj delno lahko vzrok za povečano sintezo antocianov v tretiranih plodovih. Značilna interakcija vsebnosti saharoze oziroma glukoze s skupno vsebnostjo antocianov ($P < 0,0002$), ki je bila ugotovljena med dozorevanjem jabolk, potrjuje zgoraj napisano domnevo. Za saharozo je sicer že bilo poročano, da je spodbudila sintezo antocianov v številnih rastlinskih vrstah, med drugim pri žlahtni vinski trti (*Vitis vinifera*; Vitrac in sod., 2000), v hipokotilih redkvice (*Raphanus sativus*; Hara in sod., 2003), eksokarpu manga (*Magnifera indica* Linn. cv. Mahajanaka; Lueangprasert in sod., 2010) in semenih navadnega repnjakovca (*Arabidopsis thaliana*; Teng in sod., 2005). Vzrok za povečane vsebnosti sladkorjev v tretiranih plodovih pa bi lahko bila tudi povečana vsebnost fosforja

v listih tretiranih jablan, saj je transport sladkorjev preko ovojnice kloroplastov v citoplazmo neposredno povezan s koncentracijo anorganskega fosfata (Heldt in sod., 1977). Učinek Pho Ca na povečano sintezo antocianov je bil v naši študiji mogoče tako izrazit tudi zato, ker je bila pred škropljenjem s Pho Ca koncentracija fosforja v listih tako tretiranih (1,41 g kg^{-1}) kot tudi netretiranih dreves (1,67 g kg^{-1}) pod kritično mejo 1, 8 g kg^{-1}, ki jo predlaga Bergmann (1992). Uporaba Pho Ca je povečala koncentracijo P za 28 %. Ta je tako v listih tretiranih jablan ob tehnološki zrelosti dosegla kritično mejo 1,8 g kg^{-1}, medtem ko se je v listih netretiranih dreves koncentracija P ob tehnološki zrelosti v primerjavi s prvim terminom zmanjšala za 10 %.

V našem poskusu smo tako ugotovili, da lahko s pomočjo dvakratnega foliarnega škropljenja s Phostrade Ca med dozorevanjem jabolk sorte 'Braeburn' povečamo sintezo antocianov in flavonolov, s čimer izboljšamo obarvanost plodov ob obiranju v njihovi tehnološki zrelosti. V prihodnosti bi bilo smiselno podobne poskuse opraviti tudi na nekaterih zgodnejših sortah jabolk, pri katerih lahko, predvsem v letih s slabšimi razmerami za razvoj barve, prav tako prihaja do problemov z obarvanostjo plodov. Z vključitvijo analiz sladkorjev in aktivnosti encimov v plodovih ter analizo vsebnosti fosforja v listih in plodovih pa bi dobili boljši vpogled v mehanizem delovanja samega pripravka in hkrati ovrednotili vpliv posameznega dejavnika na sintezo antocianov pri jabolkih.

Z analizo sprememb, ki smo jih v kožici jabolk sorte 'Braeburn' beležili in analizirali v plodovih, vzorčenih na laboratorijskemu polju Biotehniške fakultete v Ljubljani v letu 2011 ter v sadjarskem centru Gačnik v letu 2012, smo želeli dobiti splošno sliko nastanka in gibanja vsebnosti primarnih in sekundarnih metabolitov v jabolkih v zadnjih stadijih njihovega dozorevanja. V obeh letih so si bile spremembe parametrov kakovosti jabolk ter primarnih in sekundarnih metabolitov v kožici jabolk zelo podobne med seboj, pa čeprav so bila jabolka vzorčena v dveh različnih letih na dveh različnih lokacijah. Padec trdote jabolk je bil posledica različnih dogajanj v celicah plodov, na katere v veliki meri vplivajo zunanji dejavniki (Rato in sod., 2012), medtem ko se je vsebnost suhe snovi povečala na račun hidrolize škroba v nezrelih plodovih (Bowen in sod., 1997). Med posameznimi sladkorji smo pri saharozi zabeležili povečanje vsebnosti v obeh letih. Povečanje je bilo najverjetneje posledica transporta saharoze iz listov na eni ter na novo sintetizirane saharoze s pomočjo encimov saharoze fosfat sintaze oziroma saharoze sintaze na drugi strani (Zhang in sod., 2010). Vsebnost glukoze in fruktoze je v letu 2012 nihala, medtem ko se je v letu 2011 med pettedenskim zorenjem zmanjšala. Tako se zdi, da je med posameznimi sladkorji sinteza saharoze najmanj odvisna od rastnih in vremenskih dejavnikov. Zmanjšanje jabolčne in citronske kisline v jabolkih bi lahko bila posledica razredčitvenega učinka, povzročenega z večanjem celic in povečanjem mase jabolk, drugi razlog pa bi lahko bila povečana respiracija plodov (Ackermann, 1992; Sturm in Stampar, 1997; Roth in sod., 2007).

Vsebnost skupnih fenolnih spojin se v zadnjih petih tednih dozorevanja plodov v nobenem letu ni bistveno spreminjala, o čemer v svoji študiji poročajo tudi Zheng in sod. (2012). Pri spremljanju in analizi hidroksicimetnih kislin, dihidrohalkonov in flavanolov smo v zadnjih petih tednih zorenja ugotovili zelo majhna nihanja njihovih vsebnosti, v splošnem pa so se le-te v zadnjih petih tednih zorenja v primerjavi s prvim terminom nekoliko zmanjšale. Izjema so bile le hidroksicimetne kisline v letu 2012, katerih vsebnost se je v

omenjenem letu značilno zmanjšala. Podobno tudi Awad in sod. (2001a) v jabolkih sort 'Jonagold' in 'Elstar' poročajo o relativno konstantnih vsebnostih skupnih katehinov, floridzina in klorogenske kisline. Rahlo zmanjšanje oligomernih procianidinov, ki smo ga zabeležili v naši študiji, pa se ujema z rezultati, ki jih za to skupino fenolnih spojin poročajo Macheix in sod. (1990). Vsebnosti procianidinov je pomembna tudi iz zdravstvenega vidika in prehranske vrednosti jabolk, saj procianidini skupaj s flavanoli prispevajo zelo pomemben delež k antioksidativnem potencialu jabolk (Tsao in sod., 2005).

Če pri analizi hidroksicimetnih kislin, dihidrohalkonov in flavanolov v kožici jabolk nismo opazili bistvenih sprememb, pa to ne velja za skupino flavonolov in antocianov, pri katerih je v zadnjih petih tednih zorenja jabolk prišlo do bistvenih sprememb v njihovi vsebnosti. Povečana sinteza flavonolov je sovpadala s povečano akumulacijo antocianov, o čemer v svoji študiji pišejo tudi Lister in sod. (1994). V obeh letih se je pospešena akumulacija antocianov in flavonolov pojavila nekako 2 do 3 tedne pred tehnološko zrelostjo plodov. Do podobnih rezultatov kot mi, sta prišla tudi Awad in de Jager (2002b) pri jabolkih sorte 'Jonagold', ki sta povečano akumulacijo antocianov prav tako opazila okoli 20 dni pred tehnološko zrelostjo oziroma obiranjem plodov. Avtorja skupaj z Whalom in Singhom (2007) začetek povečane biosinteze antocianov povezujeta z endogenim izločanjem etilena, ki naj bi akumulacijo antocianov sprožil.

Z vidika regulacije biosintezne poti bi lahko predvidevali, da je do rahlega zmanjšanja vsebnosti hidroksicimetnih kislin in dihidrohalkonov v naši raziskavi prišlo na račun povečanja vsebnosti flavonolov, kar so v svoji študiji že predlagali Mayr in sod. (1995). Z ozirom na to, da je levkocianidin (flavan-3,4-diol) prekurzor tako za antociane kot flavanole (Treutter, 2001), bi rahel padec flavanolov, ki smo ga zabeležili v naši raziskavi, mogoče lahko razložili kot posledico povečane akumulacije antocianov. Zmanjšanje vsebnosti adstrigentnih procianidinov skupaj s sočasnim povečanjem vsebnosti flavonolov, antocianov in obarvanosti plodov pa ljudem in frugivorom nakazuje, da so jabolka primerna za uporabo in zaužitje, kar pomembno pripomore k razširjanju pečk in vrste.

Čeprav v jabolkih večina prisotnih fenolnih spojin deluje antioksidativno, pa ima kvercetin kot oksidant določene strukturne prednosti, s čimer prispeva največji delež k antioksidativnem potencialu jabolk (Lee in sod., 2003). V človeški prehrani naj bi skupaj s svojimi glikoziliranimi oblikami predstavljal kar 60-75 % delež dnevno zaužitih flavonoidov (Bouktaib, 2002). V naši raziskavi je na obeh lokacijah v obeh letih akumulacija antocianov in flavonolov pospešeno potekala v zadnjih tednih zorenja plodov, zato je tako z marketinškega kakor tudi zdravstvenega vidika zelo pomembno, da jabolka oberemo v njihovi pravi tehnološki zrelosti. Ker je sinteza flavonolov, še posebno pa antocianov, pogojena z njihovo pozicijo v krošnji oziroma deležem osvetlitve (Awad in sod., 2001a,b), je zelo pomembno, da so drevesa pravilno rezana in s tem primerno osvetljena. Na podlagi naših rezultatov in analiz predlagamo, da se poletna rez, prekrivanje tal z odsevno folijo, foliarna uporaba določenih elementov oziroma pripravkov in ostali ukrepi, ki so namenjeni izboljšanju obarvanosti in kvalitete plodov, na jabolkih sorte 'Braeburn' izvedejo okoli 3 do 4 tedne pred tehnološko zrelostjo plodov, odvisno od hitrosti učinkovanja posameznega ukrepa.

V študiji smo s pomočjo uporabe Pro-Ca in Pho Ca ter analize širokega spektra sekundarnih metabolitov-fenolov in encimatske aktivnosti določenih encimov prispevali koristne informacije o sintezni poti fenolnih spojin ter vlogi posameznih encimov pri razvoju barve jabolk. Obenem smo prikazali, da bi uporaba Pho Ca pridelovalcem jabolk lahko predstavljala učinkovit in poceni ukrep za izboljšanje obarvanosti plodov.

3.2 SKLEPI

Na podlagi rezultatov poskusov, ki so bili vključeni v našo študijo, bi lahko podali naslednje zaključke:

- sinteza antocianov se je pri jabolkih sorte 'Braeburn' v povprečju začela oziroma pojavila približno pet tednov pred tehnološko zrelostjo plodov, medtem ko je pospešena akumulacija antocianov začela potekati približno tri tedne pred tehnološko zrelostjo plodov.

- Jesensko tretiranje jablan s proheksadion kalcijem v odmerku 2,5 kg ha^{-1} je preko modulacije fenilpropanoidne poti prehodno zmanjšalo sintezo antocianov in flavonolov v jabolkih, kar se je odrazilo v manjši intenziteti in deležu krovne rdeče barve plodov.

- spremembe, ki jih je Pro-Ca v fenilpropanoidni poti povzročil, so bile v kožici jabolk podobne tistim, ki so že bile dokumentirane v jablaninih listih.

- v kožici jabolk so bile spremembe fenilpropanoidne poti vidne od 7 pa do 15 dni, kar je sicer nekoliko dlje kot je poročano za liste, a se v kožici vseeno niso obdržale do tehnološke zrelosti plodov.

- spremembe, ki jih je v fenilpropanoidni poti fenolnih spojin kožice jabolk povzročilo tretiranje s Pro-Ca, so se ob uporabi Pro-Ca v dveh polovičnih odmerkih nekoliko razlikovale od tistih, ki smo jih zabeležili, ko smo Pro-Ca aplicirali v enkratnem, polnem odmerku. Prav tako je bil učinek tretiranja pri polovičnem odmerku viden krajši čas, kar je bilo najverjetneje posledica hitrega metabolizma in razgradnje Pro-Ca v tkivih rastlin.

- encima PAL in CHS/CHI sta sicer potrebna za sintezo antocianov, vendar pa njuna aktivnost ni omejujoč dejavnik pri njihovi sintezi. Prav tako višja aktivnost encimov PAL, CHS/CHI, FHT in DFR še ne zagotavlja tudi večje sinteze posameznih fenolov oziroma skupin fenolnih spojin.

- jesensko foliarno tretiranje jablan s Phostrade Ca, izvedeno približno pet oziroma tri tedne pred tehnološko zrelostjo plodov, poveča sintezo antocianov in flavonolov med njihovim dozorevanjem ter izboljša obarvanost jabolk sorte 'Braeburn' ob obiranju v njihovi tehnološki zrelosti.

Razvoj obarvanosti oziroma tvorba barve pri jabolkih je zapleten proces in veliko raziskav bo še potrebnih, da bomo natančno razumeli procese in mehanizme, ki so v razvoj barve vključeni. In prav razumevanje procesov, vloge encimov, primarnih metabolitov in posameznih hranil bo omogočilo razvijanje tehnik s pomočjo katerih bi, predvsem v letih s slabimi vremenskimi razmerami za razvoj barve, pri problematičnih sortah jabolk obarvanost plodov lahko občutno izboljšali.

4 POVZETEK (SUMMARY)

4.1 POVZETEK

Obarvanost jabolk predstavlja enega od najpomembnejših parametrov zunanje kakovosti plodov. Razvoj barve je odvisen od mnogih dejavnikov, med katerimi enega od najvplivnejših predstavljajo vremenske razmere. Predvsem v letih s slabim razmerami za razvoj barve se tako pogosto zgodi, da se plodovi slabo obarvajo, kar zmanjšuje tržno vrednost rdečih sort jabolk. Pri slednjih je namreč zaželjeno, da je rdeča krovna barva čimbolj intenzivna, in da pokriva čimvečji delež plodu. Na drugi strani pa si pri zelenih sortah jabolk ('Granny Smith', 'Golden Delicious'…) želimo, da rdeča krovna barva ni prisotna oziroma je prisotna v čimmanjšem deležu. Delež krovne rdeče barve je v jabolkih večinoma pogojen z razmerjem, količino in vrsto antocianov, na njihovo akumulacijo pa poleg vremena vpliva še vrsta drugih dejavnikov, med drugim tudi aktivnost encimov.

S pomočjo foliarne uporabe rastnega regulatorja proheksadion kalcija in foliarnega gnojila Phostrade Ca smo želeli raziskati vlogo aktivnosti encimov za razvoj barve plodov ter ugotoviti, ali lahko s pomočjo teh pripravkov reguliramo biosintezno pot fenolnih spojin v kožici jabolk in s tem vplivamo na obarvanost plodov. Proheksadion kalcij je večstransko-uporaben bioregulator rastlin, ki se na sadnem drevju v prvi vrsti uporablja kot retardant z namenom zmanjšati rast vegetativnih poganjkov, vendar pa je bilo že v več študijah dokazano, da zmanjšuje delovanje 2-oksoglutarat odvisnih deoksigenaz, s čimer povzroči značilne spremembe v fenolnem profilu in regulira biosintezno pot fenolnih spojin. V naši raziskavi je jesenska uporaba proheksadion kalcija zmanjšala aktivnost encimov FHT in FLS v kožici jabolk, vendar pa je bil učinek prehodnega značaja in je trajal manj kot 20 dni. Pri tretiranih jabolkih smo v kožici zabeležili značilno manjše vsebnosti dihidrohalkonov, flavonolov in antocianov, medtem ko je bila vsebnost skupnih fenolov in hidroksicimetnih kislin v tretiranih jabolkih v primerjavi z netretiranimi, značilno večja. Jesensko tretiranje jablan s proheksadion kalcijem je tako v kožici jabolk prehodno zmanjšalo sintezo antocianov in flavonolov, kar se je odrazilo v manjši intenziteti in deležu krovne rdeče barve plodov. Spremembe v fenilpropanoidni poti pa so bile le začasne in se niso obdržale do tehnološke zrelosti oziroma obiranja plodov.

Z drugim, nekoliko drugače zastavljenim poskusom, ko smo proheksadion kalcij aplicirali v dveh polovičnih odmerkih, smo potrdili, da jesensko tretiranje s proheksadion kalcijem začasno oziroma prehodno zmanjša intenziteto in nastajanje rdeče krovne barve plodov ter v kožici jabolk povzroči značilne spremembe v fenilpropanoidni poti fenolnih spojin, ki pa so se tokrat nekoliko razlikovale od tistih, ki smo jih zabeležili v poskusu, ko smo proheksadion kalcij aplicirali v enkratnem, polnem odmerku. Pri analizi ekspresije genov smo ugotovili, da je tretiranje s proheksadion kalcijem povzročilo zmanjšano ekspresijo genov ANS, ANR, GT in transkripcijskega faktorja MYB10.

V naslednjem poskusu smo želeli raziskati vpliv dvakratne foliarne uporabe pripravka na osnovi fosforja in kalcija; Phostrade Ca (Pho Ca), na razvoj barve in vsebnost primarnih in sekundarnih metabolitov v kožici jabolk sorte 'Braeburn' med njihovim dozorevanjem. Z analizo primarnih metabolitov in encimatske aktivnosti pa smo želeli raziskati nekatere potencialno možne mehanizme delovanja samega pripravka. Pho Ca je občutno povečal

sintezo antocianov, kar so potrdili tudi parametri obarvanosti plodov a*, hº in L*. Pozitiven vpliv uporabe Pho Ca smo opazili tudi pri analizi flavonolov, medtem ko pri skupinah hidroksicimetnih kislin, dihidrohalkonov, flavanolov in skupnih fenolov (TPC), vpliva Pho Ca nismo ugotovili. V tretiranih listih jablan smo zabeležili povečane vsebnosti fosforja, v tretiranih jabolkih pa povečane vsebnosti posameznih in skupnih sladkorjev, ki bi torej lahko bila eden od možnih razlogov za povečano akumulacijo antocianov v kožici jabolk tretiranih dreves. Na drugi strani se aktivnost encimov PAL, CHS/CHI, FHT in DFR v kožici tretiranih in netretiranih jabolk ni razlikovala. V našem poskusu smo tako ugotovili, da lahko med dozorevanjem jabolk sorte 'Braeburn' s pomočjo dvakratne foliarne uporabe Phostrade Ca povečamo sintezo antocianov in flavonolov ter tako izboljšamo obarvanost plodov ob obiranju v njihovi tehnološki zrelosti.

Pri proučevanju in analiziranju sprememb aktivnosti encimov in vsebnosti sekundarnih metabolitov v dozorevajočih jabolkih smo v ločenih poskusih v dveh ločenih rastnih sezonah prišli do podobnih rezultatov. Vsebnost hidroksicimetnih kislin, dihidrohalkonov in flavanolov se je v zadnjih petih tednih zorenja v kožici jabolk le malo spreminjala in se je v splošnem nekoliko zmanjšala, opazneje pa se ni spreminjala niti vsebnost skupnih fenolnih spojin. Se je pa v tem času pojavila intenzivna sinteza flavonolov, ki je sovpadala s povečano akumulacijo antocianov. Ta se je pri sorti 'Braeburn' pojavila približno pet tednov pred tehnološko zrelostjo plodov, medtem ko se je pospešena akumulacija flavonolov in antocianov začela približno tri tedne pred tehnološko zrelostjo plodov. Spremljanje razvoja barve v povezavi z beleženjem vremenskih podatkov bi bilo lahko pri posamezni sorti jabolk v veliko pomoč pri določanju najprimernejšega časa za izvajanje določenih agrotehničnih ukrepov, namenjenih izboljšanju kvalitete in obarvanosti plodov. Na podlagi naših rezultatov in analiz svetujemo, da se poletna rez, prekrivanje tal z odsevno folijo, foliarna uporaba določenih elementov oziroma pripravkov in ostali ukrepi, ki so namenjeni izboljšanju obarvanosti in kvalitete plodov, na jabolkih sorte 'Braeburn' izvedejo okoli 3 do 4 tedne pred tehnološko zrelostjo plodov, odvisno od hitrosti učinkovanja posameznega ukrepa.

Glede spremljanja aktivnosti encimov bi upoštevajoč naše rezultate lahko zaključili, da sta encima PAL in CHS/CHI sicer potrebna za sintezo antocianov, vendar pa velikost njune aktivnosti pri akumulaciji antocianov ni omejujoč dejavnik. Prav tako večja aktivnost encimov PAL, CHS/CHI, FHT in DFR še ne zagotavlja tudi večje sinteze posameznih fenolov oziroma skupin fenolnih spojin. Ob predpostavki, da bi pretvorba dihidrokvercetina v cianidin lahko bila kontrolna točka sinteze antocianov, bi se bilo pri raziskovanju pomembnosti aktivnosti encimov za akumulacijo antocianov v prihodnosti smiselno osredotočiti na aktivnost encimov ANS in GT, katerih v naši študiji nismo uspeli analizirati.

4.2 SUMMARY

Apple peel color is one of the most important parameters of the external quality of the fruit. Color development is dependent on many factors, among which the weather conditions are one of the most influential. Especially in years of bad weather conditions for the development of red color it often happens that the fruits do not color well, which reduces the market value of the red varieties of apples. On the other hand, by the green apple varieties ('Granny Smith', 'Golden Delicious'…) we want that the red color of apple

peel is not present or is present in a proportion as narrow as possible. In apple fruits, red color is mainly the consequence of the presence of anthocyanins and in addition to weather factors; their accumulation is affected by a number of other factors, including the activity of the enzymes.

Using the foliar application of growth regulator Prohexadione calcium (Pro-Ca) and foliar fertilizer Phostrade Ca we wanted to investigate the role of enzymatic activity on the development of apple color and find out whether the autumn application of these products regulates biosynthetic pathway of phenolic compounds in apple peel and thereby influence the red coloration of apples. Prohexadion calcium is a multi-functional plant bioregulator primarily being used to reduce the growth of vegetative shoots; however it has been shown in several studies that its application reduces the activities of 2-oxoglutarate dependent dioxygenases, resulting in significant changes in the polyphenol spectrum and flavonoid composition of the biosynthetic pathway.In our study the application of Pro-Ca led to a decrease in the enzyme activities of FHT and FLS in the apple peel, but the effect was transient and lasted less than 20 days. Comparing to the control in the peel of the treated apples we recorded significantly lower levels of dihydrochalcones, flavonols and anthocyanins, while the content of total phenols and hydroxycinnamic acids was significantly higher. Autumn application of Pro-Ca on apple trees transient decreased the synthesis of anthocyanins and flavonols, which resulted in a lower intensity and percentage of red coloration of apples. However, changes in the phenylpropanoid pathway were only temporary and did not retain till the technological maturity and harvest of the fruit.

In the second, slightly differently designed experiment, when Pro-Ca was applied in two half-doses, we confirmed that autumn application of Pro-Ca transiently decreased the intensity and the formation of red coloration of apples and caused significant changes in the flavonoids' composition in apple peel, which were this time a little bit different from those we had observed in the experiment, when prohexadione calcium had been applied in a single application at one, full dose. In the analysis of gene expression we found out that the expression of ANS, ANR, GT and transcription factor MYB10 were downregulated after the prohexadione calcium treatment.

In the next experiment, the influence of two foliar applications of Phostrade Ca (Pho Ca), which contains high concentrations of phosphorus and minor amounts of calcium and nitrogen, on color development and selected primary and secondary metabolites in 'Braeburn' apple peel during the advanced maturation of apples was investigated. To get insight into the action of Pho Ca in apple peel, changes in sugars and enzyme activity of several different enzymes were recorded. Pho Ca significantly increased the synthesis of anthocyanins, which was also confirmed by the chromatic values a *, h ° and L*. Positive impact of Pho Ca was also observed in the analysis of flavonols, while the total phenolic content and accumulation of hydroxycinnamic acids, dihydrochalcones and flavanols were not influenced. In the leaves of the treated apple trees an increased amount of phosphorous (P) was detected, whereas in the treated apple fruits an increase in individual and total sugars was recorded. Increased amount of P and sugars could therefore be one of the possible reasons for the increased accumulation of anthocyanins in the peel of apples harvested on the treated trees. On the other hand, the activity of the enzymes PAL, CHS / CHI, FHT and DFR in the peel of treated and untreated apples did not differ significantly.

In our study we demonstrated that two foliar applications of Phostrade Ca late in the growing season increased the synthesis of anthocyanins and flavonols, and thus represented an effective way to improve the color of 'Braeburn' apples at harvest in their technological maturity.

In examining and analyzing changes in activity of enzymes and secondary metabolites content in maturing apples it has been shown that the changes in the course of two years in two separate experiments were quite similar. In the apple peel the concentrations of hydroxycinnamic acids, dihydrochalcones, flavanols and total phenolic content remained quite constant or slightly decreased during the advanced apple ripening. On the contrary an intensive accumulation of flavonols and anthocyanins took place during this period, with the onset of rapid formation approximately 3 weeks before the technological maturity of apples. Monitoring the color development in relation to the recording of weather data could be for certain varieties of great help in determining the most appropriate time to carry out agro-technical measures designed to improve the color and quality of apple fruit. On the basis of our results, we suggest that summer pruning, orchard floor covering, and foliar applications of certain elements and similar measures designed to improve the color and quality of the fruit on the ´Braeburn´ apples should be performed approximately 3-4 weeks before the expected technological maturity of apples.

According to the observed changes of enzymatic activity in the apple peel found in our study it could be concluded that enzymes PAL and CHS / CHI are otherwise needed for the synthesis of anthocyanins, but their level of activity for the accumulation of anthocyanins is not a limiting factor. Also the increased activity of enzymes PAL, CHS / CHI, FHT and DFR does not guarantee a higher level of synthesis of individual phenols or phenolic groups. Assuming that the conversion of cyanidin to dihydroquercetin could be a checkpoint for the synthesis of anthocyanins, it would make sense in the future to focus, when exploring the importance of enzymatic activity for the accumulation of anthocyanins, on the enzyme activity of ANS and GT, which in our study we could not analyze.

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Med sodelavci in sodelavkami na Katedri za sadjarstvo, vrtnarstvo in vinogradništvo največjo zahvalo posvečam sodelavki Maji MIKULIČ-PETKOVŠEK. Hvala Maja, da si mi bila vselej pripravljena priskočiti na pomoč in mi pomagala vedno, ko sem pomoč potreboval. Hvala ti za vse pogovore in vzoren zgled, ki si mi ga dala, tako v poslovnem kot družinskem življenju. Hvala Niki in Ani za pomoč pri praktičnih poskusih in analizah, Valentini za pomoč pri razumevanju angleških izrazov ter Primožu za vso računalniško pomoč. Ana tebi še posebej hvala za vse nasvete in pomoč pri zaključnih korakih doktorske disertacije. Hvala šefu Franciju, da sem se od njega lahko veliko naučil. Hvala Jeleni za vedno čiste prostore na Biotehniški fakulteti. Zahvaljujem se tudi dr. Katarini Košmelj za prijaznost in pomoč pri statistični obdelavi rezultatov tretjega poskusa. Valentini, Nini, Greti, Zori, Tadeju, Dragu, Primožu, Gregorju in Draganu pa hvala za sproščene in zabavne pogovore, ki so mi polepšali marsikateri dan službovanja na Biotehniški fakulteti. Drago tebi še posebej hvala za vse znanje o rezi sadnega drevja, ki si mi ga nesebično predal.

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