UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA

Ana SLATNAR

AKTIVNOST ENCIMOV FENILPROPANOIDNE POTI IN IZBRANIH METABOLITOV V PLODOVIH IN LISTIH JABLANE (*Malus domestica* Borkh.) OKUŽENIH Z JABLANOVIM ŠKRLUPOM (*Venturia inaequalis* (Cooke) G. Winter)

DOKTORSKA DISERTACIJA

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DOCTORAL DISERTATION

Ljubljana, 2013

Doktorska disertacija je zaključek podiplomskega študija bioloških in biotehnoloških znanosti in se nanaša na znanstveno področje agronomije. Praktičen del poskusa je bil opravljen 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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Ana SLATNAR

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KZ SI-1000 Ljubljana, Jamnikarjeva 101
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AL V štirih ločenih poskusih, zastavljenih v več rastnih sezonah, smo poskusili odgovoriti na del odprtih vprašanj o biokemijskih obrambnih procesih jablane ob okužbi z jablanovim škrlupom (*Venturia inaequalis* (Cooke) G. Winter), ki predstavlja najpomembnejšega patogena v jablanovih nasadih. Izbrane primarne (sladkorji, organske kisline) in sekundarne metabolite (fenoli) smo analizirali s pomočjo tekočinske kromatografije visoke ločljivosti v kombinaciji z masnim spektrometrom (HPLC-MS). Osredotočili smo se predvsem na aktivnost nekaterih encimov fenilpropanoidne poti, ki vplivajo na vsebnosti fenolnih snovi oziroma njihovo sintezo. Encimsko aktivnost smo določali s pomočjo radiografskega skeniranja tankoplastne kromatografije, ki se uporablja za kvalitativno in kvantitativno analizo produktov encimske reakcije. Dokazali smo, da prihaja do velikih razlik tako v aktivnosti encimov kot tudi v vsebnosti fenolnih snovi med tkivi, ki so različno oddaljena od mesta okužbe s patogenom. Najbolj aktivni so bili encimi v sami pegi katerih aktivnost pa je z oddaljevanjem od mesta okužbe upadala. Ugotovili smo, da tudi tekom rastne dobe prihaja do sprememb v aktivnosti encimov in posledično do vsebnosti fenolnih snovi. V študijo encimske aktivnosti smo zajeli tudi odporne in občutljive sorte, ki pa niso kazale razlik v aktivnosti encimov fenilpropanoidne poti. Ker način pridelave značilno vpliva na vsebnost fenolnih snovi, ki so pomembni antioksidanti, smo preverili, kako na vsebnost primarnih in sekundarnih metabolitov v tkivih vpliva škropljenje z natrijevim in kalijevim bikarbonatom. Znano je, da sta ti dve snovi zelo uspešni pri zatiranju jablanovega škrlupa. Oba pripravka sta se izkazala kot uspešni sredstvi za zmanjšanje okužb z jablanovim škrlupom. Parametri kakovosti ploda so bili primerljivi s tistimi, kjer smo bolezen zatirali po navodilih za integrirano pridelavo (IP). Prav tako je bila vsebnost primarnih in sekundarnih metabolitov primerljiva s tistimi iz IP.

KEY WORDS DOCUMENTATION

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TI ENZYME ACTIVITY OF THE PHENYLPROPANOID PATHWAY AND SELECTED METABOLITES IN FRUITS AND LEAVES OF APPLE TREE (*Malus domestica* Borkh.) INFECTED WITH APPLE SCAB (*Venturia inaequalis* (Cooke) G. Winter)
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AB In four separate experiments set in several growing seasons we strived to answer some questions regarding plant's biochemical defence process induced by the pathogen infection. Selected primary (sugars, organic acids) and secondary (phenolic) metabolites were analyzed with the use of high performance liquid chromatography coupled with mass spectrometer (HPLC-MS). We mainly investigated the activity of selected enzymes of the phenylpropanoid pathway, which affect the synthesis and content levels of specific phenolic compounds. Enzymatic activity was measured with thin layer radiography, which is used for qualitative and quantitative analyzes of enzyme reactions. However, significant differences in enzyme activities and consecutively in phenolic content levels have been determined among the infected tissue and its surroundings. The highest activities were observed the spot and the enzyme activity decreased with the distance from the place of infection. Enzyme activity and phenolic content also changed through the growing season. No differences in enzymes activity have been observed between the susceptible and resistant cultivars included in the study. The impact of production systems on the content of phenolic compounds exhibiting strong antioxidant activity has been frequently reported. These findings lead us to further investigate the application of sodium and potassium bicarbonate salts and their influence on the content of selected primary and secondary metabolites. The experimental results supplemented the known facts reported by previous research studies. Both products proved very effective in controlling apple scab, which is the most important pathogen in apple orchards. External fruit quality parameters of bicarbonate treated trees were comparable with those managed according with the integrated pest management guidelines. Moreover, the content of primary and secondary metabolites in fruit treated with sodium and potassium bicarbonate were similar to the content of fruit from integrated production.

KAZALO VSEBINE

KAZALO SLIK

KAZALO PRILOG

OKRAJŠAVE IN SIMBOLI

Okrajšava/simbol	**Pomen**
ANR	antocianidin reduktaza
ANS	antocianidin sintaza
BF	Biotehniška fakulteta
C4H	cinamat 4-hidroksilaza
C4L	4-kumarat:CoA ligaza
CHI	halkon izomeraza
CHS	halkon sintaza
DFR	dihidroflavonol 4-reduktaza
F3'H	flavonoid 3-hidroksilaza
FHT	flavanon 3-hidroksilaza
FLS	flavonol sintaza
G2'TP	dihidrohalkon 2'-*O*-glukoziltransferaza
GalT	galaktozil transferaza
GT	glikozil transferaza
HPLC-MS	visoka tekočinska kromatografija-masnega spektra
IP	integrirana pridelava
KBK	kalijev bikarbonat
LAR	leukocianidin reduktaza
NaBK	natrijev bikarbonat
PAL	fenilalanin amonijak liaza
POD	peroksidaza
SM	sveža masa
SUM	suha masa
UV	ultravijolična

1 UVOD

Jablanov škrlup, ki ga povzroča gliva *Venturia inaequalis*, je najpogostejša in najnevarnejša glivična bolezen jablan. Z njegovim zatiranjem imamo v sodobnih nasadih največ dela. Okužba z jablanovim škrlupom zmanjša pridelek in kakovost plodov, okuženi plodovi s črnimi žametnimi pegami pa zato tržno niso zanimivi (Mikulič-Petkovšek in sod., 2009a). Jablanov škrlup okužuje liste, plodove in zelene poganjke. Spomladi se konec aprila ali v začetku maja na zgornji in spodnji strani listov pojavijo zeleno-črne žametne nazobčane pege, ki so na spodnji strani posebno izrazite. V začetku so ločene, pozneje pa se združijo. Povrhnjica (epidermis) pod pegami je uničena in se loči od listnega tkiva. Močno okuženo listje hira in prezgodaj odpade. V primeru hudih okužb lahko jablane, zlasti občutljive sorte, julija povsem ogolijo. Pri tem se mladi poganjki ne morejo normalno razvijati, les ne dozori, pa tudi cvetni nastavek za drugo leto je zmanjšan (Maček, 1990). Gliva spada v deblo zaprtotrosnic (Ascomycota). Njena spolna oblika (*V. inaequalis*) povzroča primarne okužbe spomladi. Razvije se čez zimo, v okuženem, jeseni odpadlem listju v vrčastih trosiščih (peritecijih), kjer so askusi, v njih pa askospore. Nespolna oblika (*Fusicladium dendriticum*) povzroča sekundarne okužbe med rastno dobo in skladiščenjem. Nespolni trosi (konidiji) se razvijajo na pegah iz micelija tik pod povrhnjico (Maček, 1990).

Pridelovalci skušajo na različne načine zmanjšati škodo, ki jo povzroča ta bolezen. V letih, ki so ugodna za razvoj glive, se nasade škropi 15 do 20-krat s preventivnimi in kurativnimi fungicidi. Eden izmed načinov za zmanjšanje števila potrebnih škropljenj je sajenje odpornih sort jablane. Slednje so rezultat križanja z samoniklimi sorodniki žlahtne jablane, ki so vir različnih genov za odpornost proti tej bolezni. Povečanje odpornosti jablan lahko dosežemo tudi s škropljenjem dreves s snovmi, ki stimulirajo mehanizme odpornosti rastlin (npr. prohexadione-Ca) (Mayr in sod., 1995; Hrazdina in sod., 1997; Rademacher in sod., 1998). Pri omejevanju razvoja te bolezni je smotrno poznati mehanizme, ki jih imajo rastline za večanje odpornosti na jablanov škrlup ter ugotoviti, ali se pri občutljivih sortah te mehanizme da na kakršenkoli način stimulirati. V ta namen je potrebno natančno poznavanje interakcij med glivo in rastlino ter mehanizmov, ki jih okužba s to glivo povzroči.

Strategije odpornosti rastlin na patogene so številne in temeljijo na konstitutivni in inducirani obrambi, ki je odvisna od fiziološkega stanja gostitelja. V konstitutivni obrambi so pomembne anatomske spremembe in strukturne posebnosti (npr. periderm, polifenolne celice, sklerenhim, oksalatni kristali), ki delujejo kot fizične prepreke in patogenu preprečijo vstop in širjenje po rastlini. V inducirani obrambi so ključne kvalitativne in kvantitativne spremembe v kemični sestavi gostitelja in predstavljajo trajnejši način obrambe. V biokemičnih reakcijah v rastlinskih celicah in tkivih nastajajo spojine, ki so ali toksične za patogena ali pa v rastlini ustvarijo pogoje ugodne za zaviranje rasti patogena (Agrios, 1997). Te snovi so lahko že v naprej formirane (prisotne v rastlinskem tkivu v večjih ali manjših količinah) ali inducirane z okužbo (*de novo* sintetizirane – fitoaleksini) (Grayer in Kokubun, 2001). Obe skupini snovi predstavljata predvsem produkte sekundarnega metabolizma. Hrazdina in sod. (1997) menijo, da tako imenovani malusfuran, ki nastane kot posledica okužbe z *V. inaequalis*, zavira kalitev spor in rast glive. Aglikon malusfurana kaže celo večjo toksičnost kot sam malusfuran in deluje kot

tipični fitoaleksin. Številni fitoaleksini ali druge vnaprej sintetizirane snovi, ki imajo anti-patogeni učinek, so značilne za posamezno rastlinsko vrsto in številne med njimi pripadajo različnim skupinam fenolov (Grayer in Kokubun, 2001).

Za fenolne spojine menijo, da prispevajo k odpornosti rastlin proti mehanskim stresom, ki so posledica mehanskih poškodb, poškodb zaradi insektov in okužb z glivami, bakterijami ali virusi. Znanstveniki pripisujejo fenolom tudi pomembno signalno vlogo pri cvetenju, oplojevanju in rastlinski simbiozi. Kot odgovor na stres naj bi rastline izkoristile v celici že prisotne fenolne spojine ali pa zaradi stresa nastale fenolne spojine – fitoaleksine. Običajen odgovor rastline na stres je povečanje vsebnosti fenolnih spojin, še posebno klorogenske kisline (Abram in Simčič, 1997). Fenoli so stresni presnovki, ki delujejo kot antihranilo in imajo protimikrobni učinek, saj za glive predstavljajo strupene snovi. Način antimikrobnega delovanja fenolnih snovi je močno odvisen od njihove koncentracije. V nižjih koncentracijah vplivajo na encime, ki so povezani z oskrbo celice z energijo, medtem ko višje koncentracije povzročajo obarjanje proteinov. Fenolne snovi so toksične za zelo širok spekter mikroorganizmov, le ti pa proti njim tudi težko postanejo odporni (Agrios, 1997; Usenik in sod., 2004a). Fenolne snovi imajo tudi pomembno strukturno vlogo v obrambi pred patogenimi glivami. Ključni strukturni fenol je lignin, ki v celični steni deluje kot prepreka in varuje rastlinsko celico pred infekcijo z glivo.

Dosedanje raziskave kažejo značilno povezavo med odpornostjo na jablanov škrlup in vsebnostjo določenih fenolov (Schwalb in Feucht, 1999; Mayr in sod., 1995; Usenik in sod., 2004b; Mikulič-Petkovšek in sod., 2007; Mikulič-Petkovšek in sod., 2009a, 2009b; Mikulič-Petkovšek in sod., 2008). Pri proučevanju na škrlup odpornih in občutljivih sort so ugotovili, da imajo odporne sorte večje vakuole, kjer se skladiščijo fenolne snovi. Vendar na podlagi velikosti vakuol ni mogoče sklepati na vsebnost fenolnih snovi. Pri proučevanju fenolne slike med občutljivimi in na škrlup odpornimi sortami, se je pri odpornih pokazala značilno višja vsebnost posameznih fenolov (npr. klorogenske kisline, *p*-kumarne in flavanolov) (Picinelli in sod., 1994; Mikulič-Petkovšek in sod., 2007). Sorta 'Florina', na jablanov škrlup odporna sorta, je vsebovala dvakrat večjo koncentracijo klorogenske kisline kot sorta 'Melrose', ki spada med na jablanov škrlup občutljive sorte. Po infekciji z glivo *V. inaequalis* so bile ugotovljene visoke vsebnosti flavanolov (Mayr in sod., 1995; Mikulič-Petkovšek in sod., 2008). Treutter in Feuct (1990) poročata, da se ob okužbi z glivo *V. inaequalis* sintetizirajo predvsem flavan 3-oli.

Predhodna študija Mikulič-Petkovšek in sod. (2009b) je pokazala, da med rastno sezono obstaja možna povezava med vsebnostjo fenolnih snovi v listih jablane in njihovo občutljivostjo oziroma odpornostjo na jablanov škrlup. Odporne sorte vsebujejo več fenolnih snovi v primerjavi z občutljivimi sortami. V odpornih sortah je večjo odpornost na jablanov škrlup mogoče pojasniti s povečanjem fenolnih snovi skozi celo rastno dobo, medtem ko pri občutljivih sortah vsebnost teh snovi ostaja konstantna. Mikulič-Petkovšek in sod. (2008) so v svoji raziskavi potrdili že nekatere prejšnje raziskave, ki poročajo, da je vsebnost skupnih fenolov v listih, ki so zdravi, občutno manjša kot v listih okuženimi z glivo *V. inaequalis*. Kadar primerjamo vsebnost fenolnih snovi v zdravih in okuženih listih, se izkaže, da okužba z glivo *V. inaequalis* poveča vsebnost fenolnih snovi.

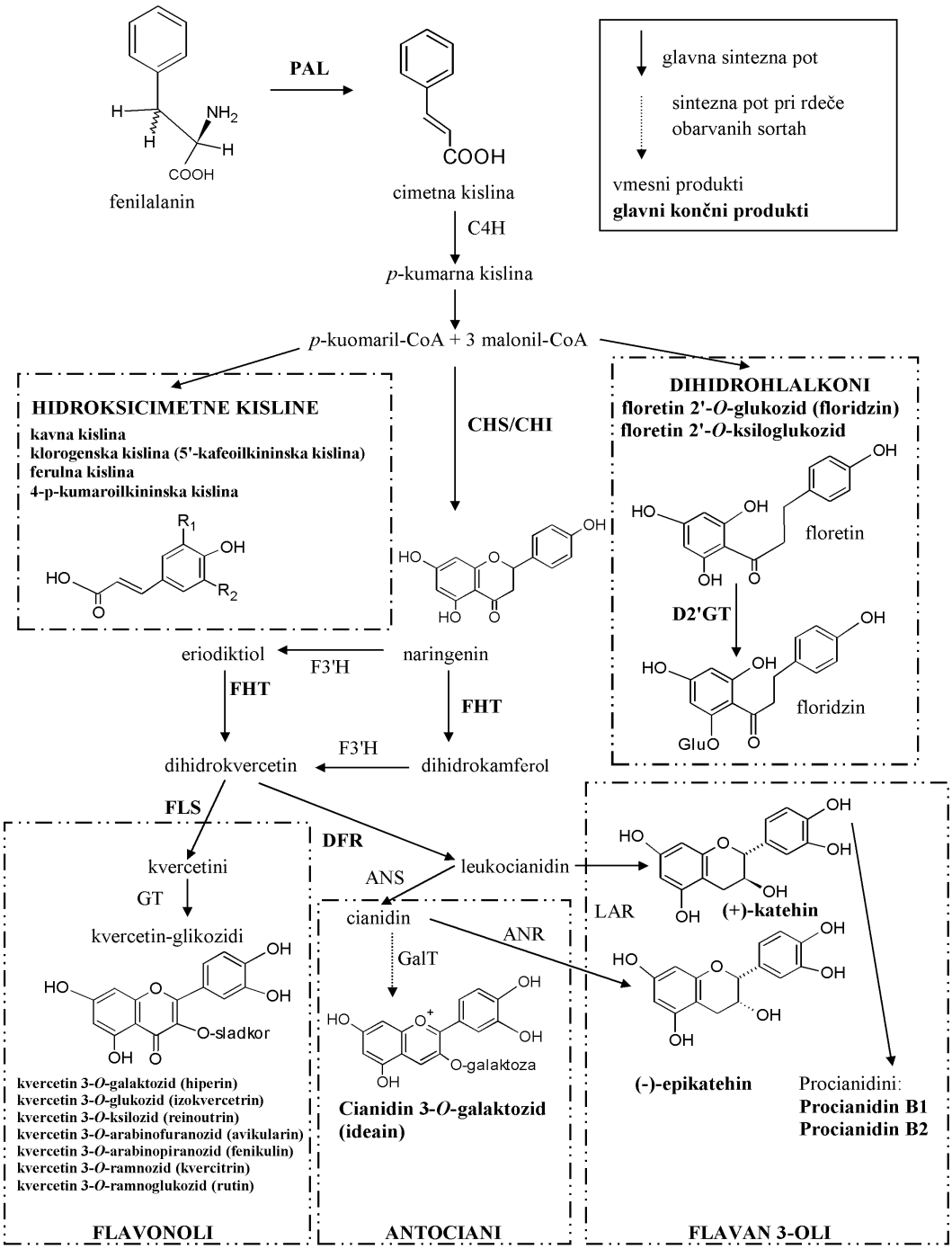
Sposobnost akumulacije fenolnih snovi v tkivih okrog mesta okužbe je lastnost tako odpornih kot občutljivih sort (Treutter in Feucht, 1990), kar ponovno dokazuje, da igrajo fenoli pomembno vlogo v obrambnih mehanizmih proti jablanovemu škrlupu (Mayr in sod., 1995). Sinteza fenolnih snovi pri odpornih sortah uspešno zadrži okužbo, medtem ko pri občutljivih sortah pride do kasnejšega odziva ali pa je ta odziv neučinkovit (Picinelli in sod., 1995; Treutter, 2005). Floridzin ima prav tako pomembno vlogo v razmerju odpornosti gostitelja do okužbe s škrlupom. Predvideva se, da ga *in vivo* različne glive kot npr. *V. inaequalis* hidrolizirajo v floretin, ki se naprej razgrajuje v floroglucinol in floretinsko kislino ter ostale produkte, ki naj bi bili za glivo toksični (Hamauzu, 2006).

Ugotovljeno je bilo, da ob okužbi z glivo med drugim pride do hitre sinteze fenolnih snovi, ki pri odpornih sortah skupaj z drugimi snovmi uspešno zadržijo infekcijo (Picinelli in sod., 1995; Hamauzu, 2006). Fenoli prispevajo tako h kemijskemu kot tudi strukturnemu obrambnemu odzivu na infekcijo z glivo, in sicer tako, da inaktivirajo encime in/ali se nalagajo znotraj celičnih sten, s čimer predstavljajo mehansko oviro za prodirajoče hife (Schwalb in Feucht, 1999). K takšnemu sklepanju nas napeljuje večja akumulacija določenih fenolov okrog mesta infekcije. Poleg do sedaj znanih mehanizmov in fenolov, ki naj bi bili vključeni v obrambni mehanizem rastline, pa obstajajo še številne druge fenolne in nefenolne snovi, ki pomagajo omejevati rast glive. Za uspešno varstvo pred patogenom je potrebna hitra biosinteza flavonoidov iz fenilalanina (Mayr in sod., 1997; Michalek in sod., 1999) (slika 1).

Ugotovljeno je bilo, da se aktivnost encima fenilalanin amonijak liaze (PAL) močno poveča, kadar obsevamo rastline z rdečo ali ultravijolično (UV) svetlobo, pod vplivom mikroorganizmov ter pri mehanskih poškodbah. Tako je inhibicija ključnega encima, ki povezuje primarni in sekundarni metabolizem, encima PAL, povzročila zmanjšanje akumulacije flavonoidov, kar je posledično vodilo v obširne simptomatične pege jablanovega škrlupa na listih. PAL katalizira reakcijo od fenilalanina do cimetne kisline (Roemmelt in sod., 2003). Cimetna kislina je pomemben prekurzor za širok spekter fenolov, med drugim flavonoide fitoaleksine in lignin, ki imajo tako strukturno kot tudi kemijsko obrambno funkcijo (Dixon in Pavia, 1995; Forkmann in Heller, 1999).

Derivati cimetne kisline so tako prekurzorji za številne fenolne spojine, zato aktivnost encima PAL nudi samo omejeno informacijo o sintezi posameznih fenolov. V eni sami rastlinski vrsti obstajajo številni fenoli in večina od njih lahko še konjugira z različnimi sladkorji (Forkmann in Heller, 1999).

Aktivnost encimov PAL, halkon sintaze (CHS)/halkon izomeraze (CHI) in dihidroflavonol 4-reduktaze (DFR) v kožici plodov je odvisna od svetlobe. Kakorkoli, med razvojem plodov jih za sintezo flavonoidov in antocianov nikoli ne primanjkuje (Ju in sod., 1997, 1995a, 1995b; Lister in sod., 1996). Biosinteze dihidrohalkonov še ne razumemo povsem natančno. Dihidrohalkoni najverjetneje izvirajo iz *p*-kumarne kisline. Lu in Foo (1997) sta izolirala 3-hidroksifloridzin, ki kaže vzorec zamenjave kvercetina in kavne kisline. Glikozil transferaze (GT) imajo visoko specifičnost za substrat glede na fenilpropanoidni ostanek, saj je glavni dihidrohalkon glukoziliran, medtem ko se glavni flavonol in glavni antocianidin pojavljata kot galaktozida. Galaktozil transferaze (GalT) kot substrate uporabljajo kvercetin in cianidin, biosintetske poti, ki dajejo aglikone, pa kontrolirajo



Slika 1: Sintezna pot fenolov pri jablani. PAL, fenilalanin amonijak liaza; C4H, cinamat 4-hidroksilaza; C4L, 4-kumarat:CoA ligaza; CHS, halkon sintaza; CHI, halkon izomeraza; G2'TP, dihidrohalkon 2'-*O*-glukoziltransferaza; FHT, flavanon 3-hidroksilaza; F3'H, flavonoid 3-hidroksilaza; FLS, flavonol sintaza; GT, glikozil transferaza; GalT, galaktozil transferaza; DFR, dihidroflavonol 4-reduktaza; ANS, antocianidin sintaza; ANR, antocianidin reduktaza; LAR, leukocianidin reduktaza.

Figure 1: Flavanoid biosynthesis pathway in apple. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; C4L, 4-coumarate:CoA ligase; CHS, chalcone isomerase; CHI, chalcone syntase; G2'TP, dihydrochalcone 2'-O-glucosyltransferase; FHT, flavanon 3-hydroxylase; F3'H, flavonoid 3- hydroxylase; FLS, flavonol synthase; GT, glucosyl transferase; GalT, galactosyl transferase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; LAR, leucoanthocyanidin reductase.

nivoje antocianinov oz. kvercetin-glikozidov (Ju in sod., 1995a). Lister in sod. (1997) predvidevajo, da so prisotni aloencimi.

DFR katalizira nastajanje levkocianidina (flavan 3,4-diola), ki je prekurzor za antocianidine in flavan 3-ole (katehin, procianidin). Poti, ki sta odgovorni za slednji skupini, med sabo 'tekmujeta' za levkocianidin. Levkocianidin je biosintetski intermediat nastanka 3-hidroksilflavanoid katehina; redukcijo pa katalizira leukocianidin reduktaza (LAR). Možno je tudi, da antocianidin sintaza (ANS) oksidira lekocianidin do cianidina, ki se potem lahko glikozilira do antocianina ali pa reducira z antocianidin reduktazo (ANR) do epikatehina. Oba flavan 3-ola, tj. katehin in epikatehin, najdemo kot monomerni podenoti procianidinov (Roemmelt in sod., 2003).

Ju in sod. (1997) so odkrili manjše vsebnosti vezanih procianidinov v kožici rdeče obarvanih plodov v primerjavi z rumenimi in zelenimi sortami, vendar tega opažanja Lister in sod. (1994) v svoji raziskavi ne potrdijo. Njihovi rezultati nakazujejo sočasno povečanje cianidinov, kvercetin glikozidov in proantocianidinov, iz česar so sklepali, da so encimi flavonoidne poti usklajeno uravnavani. Različna mnenja bi bila lahko posledica različnih zalog prekurzorjev, ki so odvisni od okoljskih pogojev in primarnega metabolizma plodov, tj. ogljika in energijskih rezerv (Bauer in Treutter, 1990).

Na vsebnost fenolnih spojin vplivajo tudi rastlinska vrsta, sorta, deloma pa tudi rastišče (vsebnost hranljivih snovi v tleh), podnebne razmere (temperatura, svetloba, količina padavin), agrotehnični dejavniki ter način pridelave (Häkkinen in sod., 1999). Količina fenolnih snovi ni odvisna samo od okužbe s patogeni, temveč na njihovo količino lahko vplivamo tudi z mehanskimi poškodbami in s tehnologijo pridelave. Pri vrednotenju vsebnosti fenolnih spojin v *in vivo* poskusih je potrebno upoštevati vplive multiplih dejavnikov na izbrana drevesa, kot so vpletenost različnih organizmov v napad, učinek podnebnih razmer (temperatura, svetloba, količina padavin), mehanske poškodbe, agrotehnični dejavniki ter način pridelave (Häkkinen in sod., 1999). Veberič in sod. (2005) poročajo, da so imela ekološko pridelana jabolka nekoliko višje vsebnosti fenolnih snovi v primerjavi z jabolki iz integrirane pridelave. Avtorji zaključujejo, da je to verjetno posledica stresa zaradi različnih tehnologij pridelave, saj je v ekološki pridelavi prepovedana tako uporaba lahko topnih mineralnih gnojil kakor tudi sintetičnih fitofarmacevtskih sredstev.

Pridelovalci skušajo na različne načine zmanjšati pojavnost jablanovega škrlupa. V letih ugodnih za razvoj glive, se nasade škropi 15 do 20-krat s preventivnimi in kurativnimi fungicidi. Množična uporaba sintetičnih fungicidov povzroča številne okoljske probleme (Ilhan in sod., 2006). Zaskrbljenost ljudi na svetovni ravni narašča zaradi vse večje uporabe sintetičnih kemikalij na živilih ter njihovih možnih negativnih učinkov na zdravje ljudi in okolje nasploh. Pojav odpornosti patogena je prav tako problematični dejavnik pri uporabi sintetičnih fungicidov (Jamar in sod., 2007). Bikarbonatne soli predstavljajo eno od številnih alternativnih možnosti nadzora bolezni. Te bikompatibilne kemikalije so zanimive zlasti zato, ker imajo fungicidne lastnosti in so hkrati zelo malo strupene za ljudi in okolje.

Bikarbonati so dokazano učinkoviti za zatiranje različnih vrst gliv, vključno z organizmi, ki povzročajo kvarjenje hrane, ter rastlinskimi patogeni (Jamar in sod., 2007). Te spojine najdemo povsod v naravi, prisotne so v prehrani ljudi ter na voljo za običajne funkcije v človeškem, živalskem in rastlinskem sistemu (Jamar in sod., 2007). Bikarbonati povečujejo pH in osmotski tlak celic na površju listov in s tem izboljšajo manj ugodne razmere za spore gliv (Palmer in sod., 1997). Visok pH na površju listov in plodov se je pokazal kot učinkovit način za zatiranje jablanovega škrlupa (Washington in sod., 1998). Natrijev bikarbonat zmanjšuje celični turgor v hifah gliv, kar vodi do kolapsa in krčenja micelija in spor. S tem zavira rast in razmnoževanje glive (Ilhan in sod., 2006). Učinkovitost nadzora bolezni z bikarbonati je mogoče izboljšati tako, da bikarbonate uporabimo v kombinaciji z rastlinskimi olji, ki izboljšajo pokritost listov tako omogočijo, da se bikarbonatni ioni dlje obdržijo na površju (Jamar in sod., 2007).

Ilhan in sod. (2006) poročajo, da je uporaba natrijevega bikarbonata v 10-dnevnih presledkih pri 90 % vzorčenih listov pomembno zmanjšala pojav jablanovega škrlupa. V poskusu na sorti 'Mutsu' so ugotovili, da je škropljenje z 1 % vodno raztopino natrijevega bikarbonata znatno zmanjšalo pojav bolezni na listih in plodovih; tudi ko so bile vremenske razmere za razvoj bolezni zelo ugodne. Ilhan in sod. (2006) v svoji študiji pišejo, da je bilo zaviranje razvoja jablanovega škrlupa, ki so ga dosegli s tretiranjem z 1-% natrijevim bikarbonatom, lahko glavni razlog za povečanje velikosti in teže plodov tretiranih dreves. Hkrati ugotavljajo, da tretiranje z 2-% natrijevim bikarbonatom že lahko povzroči fitotoksičnost (Ilhan in sod., 2006). Navkljub spoznanjem, da je natrijev bikarbonat uspešno sredstvo za zatiranje jablanovega škrlupa, njegovega učinka na vsebnost primarnih in sekundarnih metabolitov ne poznamo.

Kljub intenzivnim raziskavam v zadnjih 50 letih je biokemijski princip odpornosti na škrlup še vedno neznan. Med fenolnimi snovmi zlasti flavonoidi igrajo pomembno vlogo pri odpornosti jablan na jablanov škrlup. Sinteza fenolnih snovi pri odpornih sortah uspešno pomaga zadržati infekcijo, medtem ko pri občutljivih sortah pride do kasnejšega odziva ali pa je ta odziv neučinkovit. Fenoli se lahko oksidirajo in reagirajo s proteini ter tako povzročijo inaktivacijo encimov, s čimer zmanjšajo življenjsko sposobnost patogena. Njihovo drugo funkcijo predstavlja nalaganje znotraj celičnih sten, ki pomeni mehansko oviro za prodirajoče hife. Encim PAL katalizira spremembo aminokisline fenilalanin v cimetno kislino, ki je pomemben prekurzor za širok spekter fenolov, vključno s flavonoidi, fitoaleksini in ligninom. Derivati cimetne kisline so tako prekurzorji za številne fenolne skupine, in zato aktivnost encima PAL nudi samo omejeno informacijo o sintezi posameznih flavonoidov (Forkmann in sod., 1999).

Z znanjem o sintezni poti fenolov in njihovem odzivu na okužbo z jablanovim škrlupom postajamo vedno bolj izkušeni v različnih pristopih in orodjih, ki jih imamo na razpolago za ciljno spreminjanje točno določenih metabolnih reakcij z namenom doseganja določenega fenolnega profila, ki lahko pripelje k večji odpornosti na jablanov škrlup. Za popolnejšo sliko sprememb v metabolizmu fenolov nastalih kot posledica odziva rastline na okužbo je potrebno določiti aktivnost encimov tudi na drugih stopnjah sinteze. Halkon sintaza (CHS) in halkon izomeraza (CHI) sta encima fenilpropanoidne poti, ki vodita v nastanek naringenina. Slednji se kasneje s pomočjo flavanon 3-hidroksilaze (FHT) in dihidroflavonol 4-reduktaze (DFR) pretvori v prekurzorje za nastanek antocianov in flavan

3-olov. Flavonol sintaza (FLS) katalizira tvorbo flavonolov. Dihidrohalkon 2'-*O*-glukoziltransferaza (G2'TP) pa je povezan z nastankom floridzina (Forkmann in sod., 1999). Encim peroksidaza (POD), ki s pomočjo oksidacije fenolne snovi spreminja v kinone, pa je eden od encimov, ki je vključen v razpad fenolnih snovi.

Da bi lahko pojasnili del odprtih vprašanj v povezavi z biokemijskimi procesi obrambe, ki nastane ob interakciji patogena z rastlino, preučili vpliv mehanskih poškodb ter uporabo bikarbonatnih snovi kot sredstev za zatiranje jablanovega škrlupa, smo zastavili štiri ločene poskuse, ki so potekali v več rastnih sezonah.

Poskus 1: Odziv encimske aktivnosti fenilpropanoidne poti in akumulacije fenolnih spojin po okužbi z jablanovim škrlupom

V poskusu spremljanja odziva smo se v prvem delu odločili za monitoring aktivnosti encimov in akumulacije fenolov kožice jabolk v povezavi z okužbo z jablanovim škrlupom. Aktivnost encimov in akumulacijo fenolov smo analizirali v tkivu okuženim z jablanovim škrlupom (pega), tkivu ki obdaja pego (okolica pege) in ju primerjali z zdravim tkivom. V ta poskus sta bili vključeni sorti 'Zlati delišes' in 'Braeburn'. Drugi del poskusa vključuje občutljivo sorto 'Zlati delišes' in odporno sorto 'Florina', kjer smo aktivnost encimov in fenolov glede na različna tkiva tekom razvoja ploda spremljali preko cele rastne sezone. V analizo vsebnosti fenolov in aktivnosti encimov smo vključili kožico ploda. Poskus se je izvajal na lokaciji Laboratorijskega polja Biotehniške fakultete (BF) v Ljubljani, z njim pa smo želeli dobiti sliko aktivnosti encimov in akumulacije fenolov tekom sezone in preveriti, če oziroma koliko je aktivnost encimov in akumulacija fenolov odvisna od oddaljenosti tkiva od mesta okužbe s patogenom.

Poskus 2: Fenolna slika odpornih sort pridelanih na ekološki in integriran način (IP) pridelave

V poskus smo vključili sorte primerne za ekološko pridelavo ('Topaz', 'Florina', 'Carjevič' in 'Šampanska reneta'). Prvo obravnavanje so predstavljala jabolka, ki smo jih pridelovali po navodilih za ekološko pridelavo, drugo pa jabolka pridelana po načelih integrirane pridelave (IP) sadja. V tehnološko zrelih jabolkih smo analizirali fenole, skupne fenole ter antioksidativni potencial tako v kožici kakor tudi mesu plodov. Cilj poskusa je bil ugotoviti, kakšna je razlika v vsebnosti fenolov med različnimi načini pridelave (ekološki oziroma integriran način pridelave) in štirimi sortami pridelave.

Poskus 3: Poskus s natrijevim in kalijevim bikarbonatom

V poskus z natrijevim in kalijevim bikarbonatom smo vključili sorto 'Braeburn'. Poskus smo izvedli na lokaciji Laboratorijskega polja BF v Ljubljani. V poskusu smo tekom rastne sezone spremljali fenolno sestavo v jablanovih listih, v tehnološko zrelih plodovih pa smo spremljali parametre kakovosti, primarne (sladkorji, organske kisline) in sekundarne metabolite (fenoli). Tekom cele sezone smo vizualno ocenjevali stopnjo okužbe. Cilj poskusa je bil ugotoviti, kako se uporaba teh dveh pripravkov obnese v poljskih poskusih in kakšne so razlike v parametrih kakovosti in v vsebnosti primarnih in sekundarnih metabolitov v primerjavi s kontrolo.

Poskus 4: Odziv tkiva na mehansko poškodbo

V ta poskus smo vključili občutljivo sorto 'Zlati delišes' ter odporno sorto 'Florina'. Poskus se je izvajal na lokaciji Laboratorijskega polja BF v Ljubljani. V popolnoma razvitih listih omenjenih sort smo ugotavljali, kakšne spremembe nastanejo v spektru fenolnih spojin, ko oziroma če liste mehansko poškodujemo. Cilj poskusa je bil spremljati, kako se ob mehanski poškodbi rastlina odzove na poškodbo z akumulacijo fenolnih snovi.

Postavili smo naslednje raziskovalne hipoteze:

- Med zdravimi in z glivo *V. inaequalis* okuženimi deli tkiva jabolčne kožice obstajajo razlike v vsebnosti fenolnih snovi ter aktivnosti delovanja encimov, ki so v sintezo fenolnih snovi vključeni.
- Aktivnost delovanja encimov se, skupaj z vsebnostjo fenolnih snovi, med rastno dobo spreminja.
- Med odpornimi in na škrlup občutljivimi sortami jablane obstajajo razlike v aktivnosti encimov, ki sodelujejo pri sintezi fenolnih snovi, kar posledično vodi v višjo vsebnost posameznih fenolnih snovi.
- Akumulacija fenolov po okužbi varira med sortami in se razlikuje tudi glede na oddaljenost od mesta okužbe.
- Način pridelave (integriran/ekološki) vpliva na količino fenolnih snovi.
- Tretiranje dreves z različnimi okolju prijaznejšimi sredstvi (natrijev in kalijev bikarbonat) vpliva na povečano sintezo določenih fenolnih snovi in posledično tudi večjo odpornost rastlin.
- Mehanska poškodba in ne samo okužba s patogenom privede do povečane akumulacije fenolnih snovi.

Rastline se ob okužbi s patogenom odzovejo s povečano akumulacijo fenolov. V naši študiji pa smo poleg same akumulacije poskusili izvedeti tudi, kaj se v tem času dogaja z encimi, ki so vključeni v njihov nastanek. Raziskave so bile opravljene na kožici jabolk, ki je ob listih najbolj izpostavljena okužbi s patogenom, poleg tega pa poškodbe patogena na kožici zmanjšujejo njihovo tržno vrednost. Aktivnost encimov in akumulacijo fenolnih snovi smo spremljali tekom cele rastne sezone, s čimer smo poskusili pridobiti informacijo, kdaj tekom razvoja so ključni trenutki, pomembni za obrambo pred okužbo. Poleg tega smo ugotavljali tudi kakšna je aktivnost encimov in akumulacija fenolov glede na oddaljenost od mesta z okužbo z glivo *V. inaequalis*.

Izbrane iste sorte smo pridelovali na ekološki način in način IP. Ker so rastline pridelane na ekološki način izpostavljene večjim stresom okolja, naj bi se pri njih pojavila višja vsebnost sekundarnih metabolitov (fenolov) tako v plodovih kot tudi v listih.

Natrijev in kalijev bikarbonat smo preizkusili v poljskih poskusih in s tem preverili navedbe nekaterih virov, ki trdijo, da sta ti dve sredstvi uspešni pri zaviranju okužbe z glivo *V. inaequalis*. Plodovi, pridelani z uporabo teh sredstev, pa naj bi imeli zelo dobre parametre kakovosti zunanjega videza in vsebnost primarnih metabolitov ter večjo vsebnost sekundarnih metabolitov.

2 ZNANSTVENI ČLANKI

2.1 ENCIMSKA AKTIVNOST FENILPROPANOIDNE POTI KOT ODGOVOR NA OKUŽBO Z JABLANOVIM ŠKRLUPOM

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Poskus smo izvedli na plodovih sorte 'Zlati delišes', ki smo jih obrali v tehnološki zrelosti (15. september). Želeli smo ugotoviti, kako okužba z jablanovim škrlupom vpliva na aktivnost encimov in vsebnost posameznih fenolov v treh različnih delih kožice jabolk. Kožico plodov smo ločili na pego, tkivo v okolici pege (1-2 mm) in zdravo kožico. Analizirali smo delovanje encimov PAL, CHS/CHI, FHT, FLS in G2'TP. Znano je, da okužba z glivo *Venturia inequalis* spremeni metabolizem nastajanja fenolov v pegi, okolici pege in zdravi kožici. V pegi je bila aktivnost vseh analiziranih encimov večja kot v zdravem tkivu oziroma okolici pege, z izjemo encima G2'TP, pri katerem je bila aktivnost v pegi nižja. V pegi oziroma njeni okolici smo določili večje vsebnosti floridzina kar nakazuje, da se je floretin pretvoril v floridzin. Po analizi vsebnosti fenolov smo ugotovili, da je tkivo pege v primerjavi z zdravim tkivom vsebovalo 3,4-krat več hidroksicimetnih kislin, 1,1-krat več dihidrohalkonov in 1,4-krat več flavan 3-olov. Zdravo tkivo pa je v primerjavi s tkivom pege vsebovalo 1,6-krat več flavonolov. Kljub temu, da je bila aktivnost FLS-a v pegi večja kot v okolici pege oziroma zdravem tkivu, pa je bilo njegovih produktov v pegi najmanj. Zato predvidevamo, da so se produkti transportirali v okolico pege in tam tvorili bariero ali pa se porabili v nadaljnjem encimatskem procesu. Iz dobljenih rezultatov sklepamo, da fenoli sodelujejo v obrambnem mehanizmu jablan proti jablanovem škrlupu. Encimska aktivnost fenilpropanoidne poti je bila v okuženem tkivu bistveno večja kot v zdravem tkivu.

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RESEARCH ARTICLE

Enzyme activity of the phenylpropanoid pathway as a response to apple scab infection

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Dihydrochalcone 2′-*O*-glucosyltransferase; flavonoids; fruits; *Malus domestica*; phenolic compounds; phenylpropanoid pathway; phenylalanine ammonia lyase; *Venturia inaequalis*.

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Abstract

The study was performed on apple trees, 'Golden Delicious' cv., which is a scab-susceptible cultivar. The phenolic content of apple fruit was determined in different parts of the peel. The phenolic compounds were analysed in the scab spot, in the tissue around the spot and in the healthy tissue. We determined the concentration of various phenolic compounds and related enzyme activities. Infection with the *Venturia inaequalis* fungus enhanced the metabolism of phenolic compounds at the scab spot, around the spot and in healthy peel. Compared with the healthy tissue and the tissue around the spot, the scab spot showed higher enzyme activity for all tested enzymes, except for dihydrochalcone 2′-*O*-glucosyltransferase, which had lower activity in the scab spot. In comparison to the healthy peel, the scab spot showed up to 3.4 times more hydroxycinnamic acids, up to 1.1 times more dihydrochalcones and up to 1.4 times more flavan-3-ols. In contrast, the healthy peel showed up to 1.6 times more flavonols than the scab spot.

Introduction

Apple scab, a fruit disease restricted to the *Malus* genus, is caused by the pathogen *Venturia inaequalis* [(Cooke) G. Wint.]. The disease presents a problem of economic significance to growers. It attacks leaves and fruit of apple trees and results in scabby, inferior fruit. Currently existing and some promising potential measures against apple scab include frequent fungicide treatment of scab-susceptible tress, biological control of the disease and the development of scab-resistant apple cultivars by conventional breeding (McHardy, 1996; Köhl *et al.*, 2009).

In plants, phenolic compounds are a chemically diverse and biologically important group of secondary metabolites. It is well known that phenolic compounds are involved in the natural defence reactions of apple trees against various diseases, including apple scab. The phenolic composition of plant tissue can determine the level of susceptibility to/tolerance of fungal infections and pests (Usenik *et al.*, 2004). Many phenolic compounds are directly involved in the plant response to different types of stress. Treutter & Feucht (1990) found that total flavanols content in the skin of scab-resistant varieties was threefold higher than in susceptible varieties. The phenolic derivatives can oxidise and react with proteins, thus causing a loss of enzyme function and restricting the viability of pathogens or they can be deposited inside the cell wall as an important first line in plant defence against infection (Schwalb & Feucht, 1999). A previous study (Mayr *et al.*, 1997; Michalek *et al.*, 1999) revealed that for successive protection, a rapid biosynthesis of flavanols, starting from phenylalanine, was necessary. The inhibition of the enzyme phenylalanine ammonia lyase (PAL) resulted in severe sporulation symptoms.

Phenylalanine ammonia lyase is the key enzyme located at the interface between primary and secondary metabolism. It catalyses the conversion of the amino acid phenylalanine into cinnamic acid, which is an important intermediate in the biosynthesis of flavonoids, lignins, stilbenes and coumarins (Forkmann & Heller, 1999). Fungal invasion triggers the transcription of messenger RNA that codes for PAL, thus increasing the amount of PAL in the plant, which then stimulates the synthesis of

phenolic compounds. In the phenylpropanoid pathway, other enzymes besides PAL are important for the synthesis of different flavonoid classes. Chalcone synthase (CHS) and chalcone isomerase (CHI) lead to the formation of flavanones, which are then further converted by flavanone 3-hydroxylase (FHT) and by dihydroflavonol 4-reductase (DFR) to leucoanthocyanidins (flavanols), which are the precursors of both anthocyanidins and flavan-3-ols (catechins, procyanidins) (Treutter, 2001). Flavonol synthase (FLS) catalyses dehydrogenation of dihydroflavonols to flavonols. Dihydrochalcone 2′-*O*-glucosyltransferase (D2′GT) catalyses the formation of phloridzin.

Peroxidase (POD) is an oxidoreductase that is involved in enzymatic browning reactions, because diphenols may function as reducing substrates in the POD reaction (Burnette, 1977). The role PODs play in the living plant is not completely understood, although they have been associated with cell wall biosynthesis, response to injury, disease resistance and wound repair (Préstano & Manzano, 1993).

The objective of this study was to evaluate the response of apple fruit to infection with *V. inaequalis*. This was investigated at the level of individual phenolic compounds related with selected enzyme activities of the phenylpropanoid pathway. Three different tissues of the same fruit were studied: symptomatic spot, tissue around the spot and healthy tissue. To our knowledge, there is no comparable research approach in the literature. The goal of our experiment was to investigate which parts of the phenylpropanoid pathway increase after infection to shed light on substances responsible for defence against *V. inaequalis*.

Materials and methods

Plant material

The study was performed on apple trees (*Malus domestica* Borkh.), 'Golden Delicious' cv., which is a scab-susceptible cultivar. For the experiment, fruits were taken from 6-year-old trees grafted on the M9 rootstock growing in the University experimental orchard in Ljubljana. In this orchard, the trees were cultivated according to the guidelines for integrated fruit production.

Fruit samples were picked at technological maturity on the 15 September 2008 (125 days after full bloom), which was evaluated using the starch iodine test. The apple skin was peeled as thin as possible (approximately 1-mm thick), and the peel was divided into three parts: scab symptomatic spot (late fruit infection), tissue around the scab spot (1–2 mm) and healthy peel. Three replicates for each part of peel were performed for determination of phenolic compounds and enzyme activity. Samples were immediately shock-frozen in liquid nitrogen and stored at −80°C until analysis of the samples.

Extraction and determination of phenolic compounds

Extraction with some modification was carried out as described by Mikulic Petkovsek *et al.* (2007). The 0.5-g peel samples were extracted with 3 mL of methanol containing 1% 2,6-di-*tert*-butyl-4-methylphenol (BHT) and homogenised with the T-25 Ultra-Turrax (Ika-Labortechnik, Staufen, Germany). BHT was added to the samples to prevent oxidation during the extraction. Samples were placed in a cooled ultrasonic bath (0°C) for 1 h; then they were centrifuged at 15 550 g for 7 min at 10°C and the supernatants were filtered through a Chromafil A0-45/25 polyamide filter produced by Macherey–Nagel (Düren, Germany). The high-performance liquid chromatography (HPLC) analysis was performed on a Surveyor HPLC system with PDA detector (Thermo Finnigan, San Jose, CA, USA). The column was a Gemini C_{18} (150 × 4.6 mm, 3 μm; Phenomenex, CA, USA) operated at 25°C. The elution solvents were 1% formic acid in twice distilled water and 100% acetonitrile. Samples were eluted according to the linear gradient described by Marks *et al.* (2007), with an injection amount of 20 μL and a flow rate of 1 mL min^{-1}. The phenolic compounds were identified by comparing their UV–vis spectra from 220 to 550 nm and their retention times. The hydroxycinnamic acids (chlorogenic, *p*-coumaric, ferulic and caffeic acids) and the monomeric flavan-3-ols (catechin, epicatechin, procyanidin B2) and dihydrochalcones (phloretin) were detected at 280 nm, whereas quercetin-3-*O*-rutinoside, phloridzin, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glucoside and quercetin-3-*O*-galactoside were estimated at 350 nm. The concentrations of phenolic compounds were calculated from the peak areas of samples and the corresponding standards. The concentrations were expressed as mg 100 g^{-1} FW (fresh weight) of peel.

Buffers used

Buffer A (PAL assays): 0.1 M H_3BO_3 + 0.4% Na-ascorbate, pH 8.5. Buffer B (CHS/CHI assays): 0.1 M KPi + 0.4% Na-ascorbate, pH 7.0. Buffer C (FHT assays): 0.1 M Tris/HCl + 0.4% Na-ascorbate, pH 7.25. Buffer D (FLS, D2′GT assays): 0.1 M Tris/HCl + 0.4% Na-ascorbate, pH 7.5. Buffer E (DFR assays): 0.1 M KPi + 0.4% Na-ascorbate, pH 6.8. Buffer F (POD assays): 0.1 M KPi, pH 6.0, 0.1 M H_2O_2, pH 6.5.

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Enzyme preparation

Shock-frozen peel was ground to powder with liquid nitrogen. A total of 0.5 g fine powder, 0.25 g quartz sand, 0.25 g Polyclar AT and 3 mL 0.1 M Tris/HCl (containing 0.4% Na-ascorbate, pH 7.25) was homogenised 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). The protein solution eluted in the excluded volume of the column (crude extract) was used for enzyme assays.

Analyses of enzyme activity

Enzyme assays were performed according to Halbwirth *et al.* (2002), using the assay conditions optimised for apple peel (Table 1). The enzymes assays were incubated for 30 min at 30°C, with the exception of the DFR, which was incubated for 15 min at 30°C. PAL and CHS/CHI assays were stopped with 10 µL of acetic acid, the products were extracted with 200 µL of ethyl acetate and quantified on a scintillation counter. The FHT, DFR and FLS assays were terminated by adding 70 µL of ethyl acetate and 10 µL of acetic acid. To FLS assays, 10 µL of 0.1 M ethylenediaminetetraacetic acid was also added. Organic phases were transferred to precoated cellulose plates (Merck, Darmstadt, Germany) for thin-layer chromatography (TLC) in chloroform/acetic acid/H_2O (10:9:1, v/v/v) for FHT and DFR assays or in 30% acetic acid (FLS). The conversion rates were then determined with a TLC linear analyser (Berthold, Bad Wildbad, Germany). Glucosyltransferase assays were terminated by adding 10 µL of acetic acid and 25 µL of methanol. The mixture was chromatographed on Schleicher and Schüll 2043b paper, using water as solvent system. Zones containing the labelled products were cut out, and radioactivity was quantified on a scintillation counter. All products were identified as described using authentic substances (Fischer *et al.*, 2003).

The protein content was quantified by a modified Lowry procedure (Sandermann & Strominger, 1972) with crystalline BSA as standard.

POD activity was carried out according to the Worthington manual (Worthington Biochemical Corporation, 1972). Briefly, the enzyme preparation was incubated with 0.410 nmol *o*-dianisidine in buffer F (Table 1) and the time-dependent changes in the absorption at 460 nm were determined in comparison with a blank containing only *o*-dianisidine and buffer F.

Enzyme activity was calculated in nkat g^{-1} FW for PAL, CHS/CHI, DFR, D2′GT, FLS and in Δ A min^{-1} for POD.

Statistical analysis

The data were analysed using the Statgraphics Plus 4.0 (Manugistics, Inc., Rockville, MD, USA). Data from the

Table 1 Optimised conditions for assays of main enzymes of the flavonoid pathway in apple peel

Enzyme	Crude extract after gel chromatography (µL)	Substrate(s)	µL	Cofactor and cosubstrate solutions	µL	Buffer	Buffer (µL)	Final volume (µL)
Phenylalanine ammonia lyase	40	(^{14}C)-phenylalanine (0.063 nmol, 548 Bq)	5	—		A	55	100
Chalcone synthase/chalcone isomerase	40	*p*-CuCoA (1 nmol)	5	—		B	50	100
		(^{14}C)-Malonyl-CoA (1.5 nmol, 1300 Bq)	5					
Flavanone 3-hydroxylase	30	(^{14}C)-naringenin (0.036 nmol, 100 Bq)	—	2-Oxoglutarate (1.46 mg mL^{-1})	5	C	60	100
				$FeSO_4 \cdot 7H_2O$ (1.46 mg mL^{-1})	5			
Flavonol synthase	30	(^{14}C)-dihydrokaempferol	—	2-Oxoglutarate (1.46 mg mL^{-1})	5	D	60	100
		(0.036 nmol, 100 Bq)		$FeSO_4 \cdot 7H_2O$ (1.46 mg mL^{-1})	5			
Dihydroflavonol 4-reductase	20	(^{14}C)-dihydroquercetin (0.036 nmol, 100 Bq)	—	NADPH (41.86 mg mL^{-1})	5	E	25	50
Dihydrochalcone 2′-*O*-glucosyltransferase	20	Phloretin (0.3 nmol)	2.5	(^{14}C)-UDPG (0.206 nmol, 2300 Bq)	2.5	D	25	50
Peroxidase	5	*o*-Dianisidine (0.410 nmol)	10			F	1095	1110

chemical analyses were tested for any differences between treatments using one-way analysis of variance (ANOVA). The means and standard errors of the means (mean ± SE) are also reported.

Results and discussion

Analysis of phenols

Analysed phenolics, corresponding enzymes and groups of phenolics are outlined in Fig. 1.

Hydroxycinnamic acids represent the first larger group in the biosynthetic pathways of phenolic compounds. Several types of wounds to apple fruits and leaves induce PAL activity (Michalek *et al.*, 1999), thereby accumulating the level of chlorogenic acid and flavonols (Mayr *et al.*, 1994). In our study, ferulic acid showed the lowest and chlorogenic acid showed the highest concentrations of all hydroxycinnamic acids determined. The tissue at the scab spot showed statistically higher contents of chlorogenic acid and ferulic acid (Table 2) compared with the healthy tissue. The scab spot contained 0.15–0.47 (0.2) mg 100 g^{-1} FW ferulic acid, which is 5–6.7 times more than in healthy peel (Table 2). The spot tissue contained 9.13–11.12 (10.5) mg 100 g^{-1} FW chlorogenic acid, which is 8.5–13.6 times more than in healthy peel (Table 2).

The dihydrochalcone phloridzin, a phloretin 2′*O*-glucoside, is the major phenolic compound in apple extracts (Iwashina, 2000). Phloridzin and aglycone phloretin are often related to resistance to numerous diseases. Studies on apples provide suggestive evidence that the presence of this compound is related to resistance against *V. inaequalis* (Hamauzu, 2006). In our experiment, the healthy peel contained lower values of phloridzin than the spot and the area around it. The concentration in the spot was 154.75–245.58 (174.9) mg 100 g^{-1} FW. The concentration of phloridzin measured in our study in the peel is higher than that reported by Escarpa and Gonzalez (1998); however, we must consider that the reported data were probably gained from healthy peel. The scab spot tissue contained small amounts of phloretin, but for around spot and healthy peel we did not determine any presence of phloretin. Phloridzin and the ratio flavan-3-ol/phloridzin are often discussed with regard to resistance against diseases like apple scab. Upon infection of plants with pathogens (with fungus *V. inaequalis*), they hydrolyse phloridzin to hydroxybenzoic acid, which inhibits the development of fungi (Hamauzu, 2006).

Tissues infected with the *V. inaequalis* fungus show additional accumulation of flavan-3-ols (epicatechin, catechin, procyanidin B2, procyanidin B5 and procyanidin E-B5) (Treutter & Feucht, 1990). In our case, increased accumulation of epicatechin, catechin and procyanidin B2 was noticed in the spot tissue. The tissue around the symptomatic spot and healthy peel did not differ in concentrations of epicatechin, catechin or procyanidin B2. Those concentrations were 1.9–3.8 times lower than

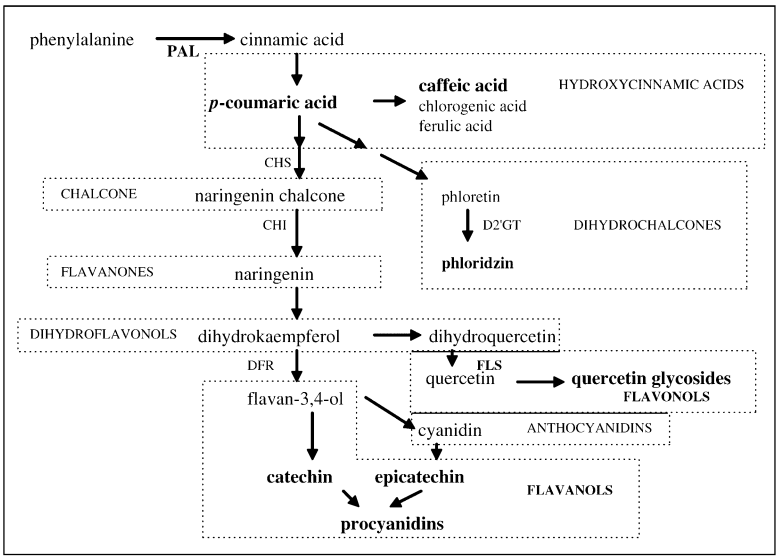

Figure 1 Biosynthetic pathways of phenolic compounds in apple. PAL, phenylalanine ammonia lyase; CHS, chalcone synthase; CHI, chalcone isomerase; DFR, dihydroflavonol 4-reductase; FLS, flavonol synthase; D2′GT, dihydrochalcone 2′-*O*-glucosyltransferase. The individual phenolic and phenolic group mentioned in the text are accentuated in bold lettering.

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Table 2 The content of single phenolic compounds [mean ± SE (mg 100 g^{-1} FW peel)] in apple peel (healthy tissue, scab spot and around scab spot) of 'Golden Delicious' cultivars. Different letters (a, b, c) denote statistically significant differences between different parts of peel

	Around spot	Spot	Healthy	LSD[a] (d.f. = 8)
p-Coumaric acid	1.7 ± 0.5	4.2 ± 1.6	2.0 ± 0.1	3.32
Chlorogenic acid	2.1 ± 0.2b	10.5 ± 0.4c	0.8 ± 0.1a	0.85
Caffeic acid	1.5 ± 0.2	1.7 ± 0.2	1.4 ± 0.1	0.56
Ferulic acid	0.04 ± 0.01a	0.2 ± 0.1b	0.1 ± 0.01a	0.10
Epicatechin	27.1 ± 3.9ab	35.0 ± 0.3b	21.6 ± 0.4a	7.91
Catechin	2.4 ± 0.5a	7.1 ± 1.0b	2.2 ± 0.2a	2.24
Procyanidin B2	36.9 ± 1.7	42.9 ± 1.9	36.5 ± 1.2	5.54
Phloridzin	181.4 ± 57.3	174.9 ± 13.4	168.4 ± 17.0	122.28
Phloretin	n.d.	0.001 ± 0.00002	n.d.	0.0004
Quercetin-3-*O*-rutinoside	0.4 ± 0.2	0.2 ± 0.1	0.4 ± 0.1	0.45
Quercetin-3-*O*-glucoside	24.7 ± 4.0	15.5 ± 0.3	14.5 ± 3.7	10.93
Quercetin-3-*O*-galactoside	42.6 ± 4.4	29.6 ± 3.0	36.1 ± 5.1	14.73
Quercetin-3-*O*-rhamnoside	170.7 ± 29.5b	84.4 ± 6.1a	141.4 ± 14.0ab	66.42
Total quercetin	238.3 ± 38.0b	125.9 ± 7.0a	175.4 ± 31.2ab	112.45

LSD, least significant difference; n.d., not determined.
[a]LSD between means ($P < 0.05$); comparison for different tissues of peel.

in spot tissue (Table 2). The content of procyanidin B2, catechin and epicatechin in different parts of the peel is comparable to results reported by Escarpa and Gonzalez (1998).

Feucht *et al.* (1998) and Mayr & Treutter (1998) reported that, as a response to scab infection, a few cells surrounding the infection site accumulate flavonols during barrier formation. In our experiment, concentration of total flavonols showed the highest values in the tissue around the spot. This indicates that the tissue around the spot acts like a barrier, which should stop further progress of the fungus. Additionally, the concentrations of total flavonols were highest in the healthy peel and lowest in the symptomatic spot. The highest concentration in the flavonol group was that for quercetin-3-*O*-rhamnoside and the lowest that for quercetin-3-*O*-rutinoside (Table 2).

Analysis of enzyme activity

The protein content was significantly higher in scab spots (2.14 μg 10 μL^{-1}) and significantly lower in the healthy peel (0.89 μg 10 μL^{-1}). This could be because of an increased plant protein production as a response to the infection and/or to the presence of fungal proteins in the infected tissues. Therefore, all enzyme activities were expressed per gram fresh tissue rather than per total protein content (specific activity), to keep the impact of potential fungal proteins as low as possible.

PAL is situated at the branch point between primary and secondary metabolism. Therefore, the formation of cinnamic acid is an important regulatory step in the formation of several phenolic compounds. In our analysis, PAL activity could be measured only in the symptomatic spot. In healthy tissue and around the spot, no enzyme activity was detectable (Fig. 2). Saunders & Olechno (1988) reported that differences in mRNA levels produced in plants appear as a response to fungal inoculation. Saunders & O'Neill (2004) reported that alfalfa plants respond to an avirulent fungal inoculation by developing an induced resistance that enhances gene expression, which increases enzymatic activity of PAL in flavonoid metabolism.

The key enzyme for formation of different flavonoid groups is CHS, which catalyses the condensation of three molecules malonyl-CoA with 4-coumaroyl-CoA. The reaction leads to the formation of naringenin chalcone, which is transformed by CHI into naringenin. As CHI does not require any cofactor, it is not possible to measure CHS and CHI activities separately. From all the enzymes measured, the combined CHS/CHI reaction showed highest activity in the tissues (Fig. 2). Compared with the healthy tissue, the spot tissue showed the significantly highest CHS/CHI activity followed by the tissue around the spot. This indicates that infection with *V. inaequalis* causes increased activity leading to formation of different flavonoids.

In the next step, we measured FHT and DFR activities. The activity of DFR was significantly higher in the spot compared with other tissues (Fig. 2). FHT activity, however, could not be detected in any of the analysed tissues. FHT converts flavanones to dihydroflavonols which are subsequently reduced to the corresponding leucoanthocyanidins by DFR. The absence of FHT could provide an explanation for the lack of anthocyanins in cv. Golden Delicious peel, but on the other hand, the

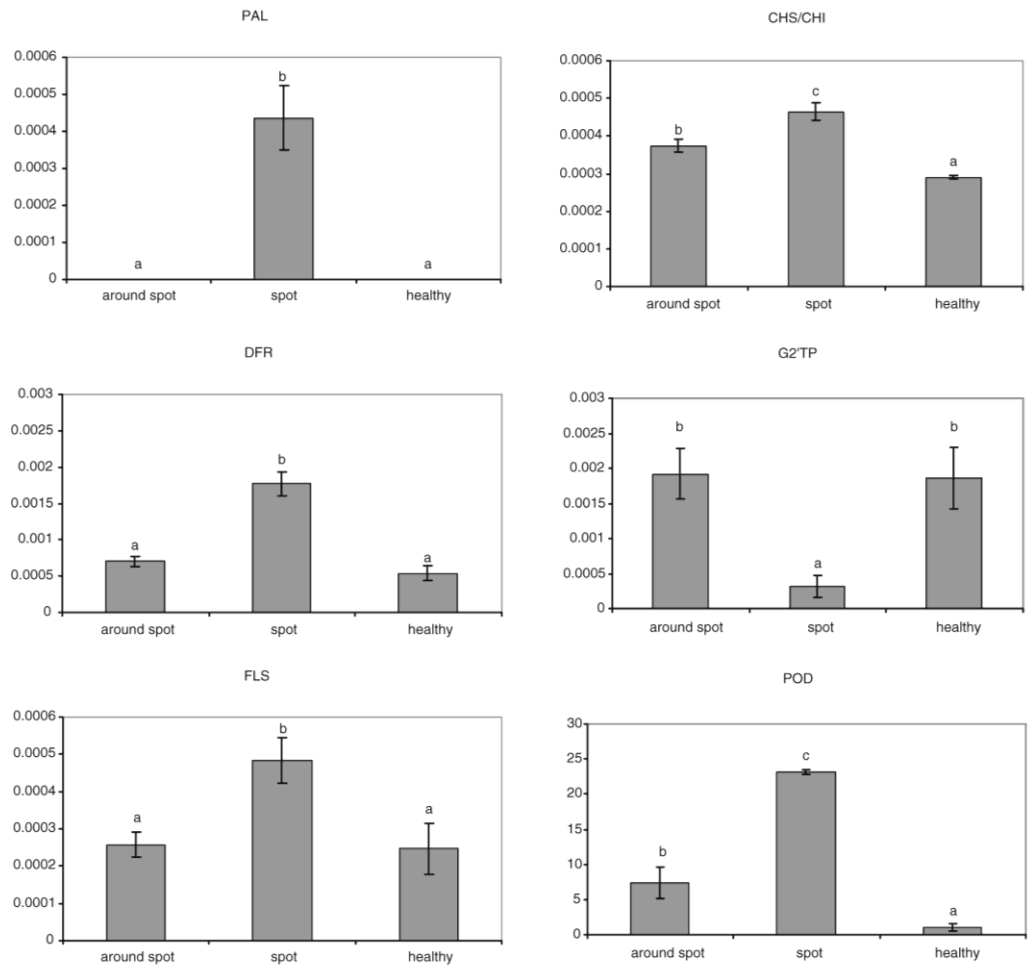

Figure 2 Enzyme activity [mean ± SE (nkat g^{-1} FW) for phenylalanine ammonia lyase (PAL), chalcone synthase/chalcone isomerase (CHS/CHI), dihydroflavonol 4-reductase (DFR), dihydrochalcone 2′-*O*-glucosyltransferase (D2′GT) and in Δ A min^{-1} for peroxidase (POD)]. Least significant difference between means ($P < 0.05$); comparison for different tissues of peel (limits for PAL: 0.0002, CHS/CHI 0.0006, DFR 0.0004, D2′GT 0.001, FLS 0.002, POD 0.104), different letters (a, b, c) denote statistically significant differences between different parts of peel.

accumulation of flavanols must be based on FHT activity because dihydroflavonols are intermediates for flavanol formation. Thus it may be assumed that FHT is active in other developmental stages or that the present FHT activity is below the detection limit.

Flavonol synthase is the key step for the formation of flavonols, such as quercetin derivatives. As with the other flavonoid enzymes, significantly higher activity for FLS was measured in the scab spot compared with the healthy tissue. In contrast, flavonols showed lower concentrations in the spot. Possible explanations could be a transport into the tissue around the infection for barrier formation, increased formation of flavanols at the expense of flavonol formation or up to now unidentified downstream processes. It could also be explained by reduced precursor availability, because FHT activity does not seem to be induced in the tissues.

Dihydrochalcone 2′-*O*-glucosyltransferase is responsible for the conversion of phloretin to phloridzin. In contrast to the other enzymes, the spot showed significantly lower activity compared with the healthy control tissue (Fig. 2). The decrease in the enzyme activity in the infected tissue could be explained by the fact that the aglycone – not the 2′-*O*-glucoside – is considered as active compound against fungus attack. The fact is, as can be seen in Table 2, the aglycone phloretin was only detected

in the scab spot. Also the level of phloridzin in the spot tissue was high, however, it showed no statistically significant difference in the three tissues, but this could be because of the high amounts generally present.

POD activity was significantly higher in the scab spot than in the tissue around the spot and in the healthy tissue (Fig. 2). This is in line with a number of reports on the involvement of POD in plant defence reactions (Sherf & Kolattukudy, 1993; Harrison *et al*., 1995; Zhao *et al*., 2008).

Concluding remarks

Summarising, it could be shown that the polyphenol pathway in apple is dramatically changed after infection with apple scab. This was shown for the first time at the level of flavonoid enzymes and related polyphenolic compounds in three different apple peel tissues. It could be clearly shown that hydroxycinnamic acids and flavanols in scab spots are increased and that the enzymes of the flavonoid pathway are dramatically induced with the exception of D2′GT which decreases in contrast. This might be part of a defence mechanism relying on the enhanced provision of phoretin, the aglycone of phloridzin. Future work will focus on differences of this defence scheme in varieties with different scab susceptibilities.

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2.2 ODGOVOR FENILPROPANOIDNE POTI NA OKUŽBO Z GLIVO *Venturia inaequalis* V DOZOREVAJOČIH JABOLKIH SORTE 'BRAEBURN'

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Response of the phenylpropanoid pathway to *Venturia inaequalis* infection in maturing fruit of 'Braeburn' apple.
Journal of Horticultural Science & Biotechnology, 2010, 85: 465–472.
Prejeto: 17.2.2010, sprejeto: 25.6.2010

V poskusu smo v kožici jabolk okuženih z glivo *Venturia inaequalis* ((Cooke) G.Wint.) preučevali aktivnost ključnih encimov fenilpropanoidne poti in akumulacijo posameznih fenolnih snovi. Zdravo kožico smo primerjali s kožico, na kateri je bila vidna škrlupasta pega, ter tkivom v premeru 1 do 2 mm okoli škrlupaste pege 1 mesec pred polno zrelostjo (140 dni po polnem cvetenju) in v polni zrelosti (175 dni po polnem cvetenju). Izkazalo se je, da okužba z glivo *V. inaequalis* spremeni sintezo nekaterih fenolnih snovi. V primerjavi z zdravo kožico je škrlupasta pega vsebovala 3,1-krat večjo vsebnosti hidroksicimetnih kislin, 1,3-krat večjo vsebnost dihidrohalkonov in 3,9-krat večjo vsebnost flavan 3-olov. Prav tako je škrlupasta pega kazala nekoliko višjo aktivnost encimov PAL, CHS/CHI, FLS in FHT. Vsebnost fenolov je ostajala skoraj nespremenjena med termini vzorčenja, z izjemo epikatehina in kavne kisline, pri katerih je vsebnost z dozorevanjem padala. Na drugi strani se je vsebnost katehina med dozorevanjem povečala. Kar zadeva encimsko aktivnost, smo v obdobju dozorevanja plodov v vseh proučevanih tkivih opazili zmanjšano aktivnost encima CHS/CHI. Študija je pokazala, da se fenilpropanoidna pot v kožici jabolk značilno spremeni po okužbi z jablanovim škrlupom.

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Response of the phenylpropanoid pathway to *Venturia inaequalis* infection in maturing fruit of 'Braeburn' apple

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SUMMARY
This study investigated the activities of key enzymes in the phenylpropanoid pathway and the accumulation of phenolic products in apple peel in response to infection with *Venturia inaequalis* [(Cooke) G. Wint.]. We compared healthy apple peel with apple peel showing symptomatic scab lesions, and with peel tissue from 1 – 2 mm around the scab lesions in fruit 1 month before maturity [140 d after full bloom (DAFB)] and at physiological maturity (175 DAFB). Infection with *V. inaequalis* enhanced the synthesis of some phenolic compounds. Compared to healthy peel, scab lesion tissue had ≤ 3.1-times higher hydroxycinnamic acid content, ≤ 1.3-times higher dihydrochalcone content, and ≤ 3.9-times higher flavan-3-ol content. Scab lesions showed slightly higher phenylalanine ammonia-lyase, chalcone synthase, chalcone isomerase, flavonol synthase, and dihydroflavonol 4-reductase activities. The total amount of phenolics remained relatively stable between the two sampling dates, except for epicatechin and caffeic acid which decreased in amount toward fruit maturity, and levels of catechin which increased in more mature fruit. During fruit maturation, only chalcone synthase and chalcone isomerase activities decreased in all tissues examined. This study showed that the phenylpropanoid pathway in apple fruit peel changed significantly following infection with the apple scab pathogen.

Apple scab, caused by the fungus *Venturia inaequalis* [(Cooke) G. Wint.], is the most important disease present in all apple-growing areas with high Spring and Summer rainfall. Disease control in commercial orchards can require several fungicide treatments per year. An alternative approach is the use of resistant apple cultivars, or treatment with chemical agents which can induce higher resistance to the apple scab pathogen (Brun *et al*., 2008).

Phenolic compounds have been shown to be involved in plant defences against biotic and abiotic stresses (Geibel *et al*., 1994). A large number of different phenolic compounds have been reported to occur in various apple tissues (Treutter, 2001; Bazzi *et al*., 2003). Phenolic compounds in apple, particularly flavonoids, have been claimed to play a role in scab resistance (Treutter and Feucht, 1990). Other phenolics, such as phenolic acids, also contribute to healing through increased lignification of damaged areas. Furthermore, they possess anti-microbial properties, and their concentrations may increase after infection (Bostock *et al*., 1999, Treutter, 2005).

Secondary metabolism is part of the dynamic biochemical processes that are involved in plant growth and differentiation. Interconnections between many different metabolic processes are responsible for the variety of responses of plant tissues to eliciting agents, and highly specialised pathogens are adapted to the specific physiology of the host plant. Research into *Erwinia amylovora* has shown that this bacterium, which causes fire blight disease in apple, is able to decrease accumulation of the natural anti-bacterial compound, phloretin, to below bactericidal levels (Pontais *et al*., 2008).

Previous studies (Mayr *et al*., 1997; Michalek *et al*., 1999a) revealed that the rapid biosynthesis of flavanols from phenylalanine was necessary for protection against pathogens. Inhibition of the enzyme phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) resulted in severe symptoms of sporulation. PAL provides the link between primary plant metabolism and the phenylpropanoid pathway. PAL catalyses the conversion of phenylalanine to *trans*-cinnamic acid, which is a precursor of many different phenolic compounds. The key enzyme in the flavanoid pathway is chalcone synthase (CHS; EC 3.2.1.74). CHS and chalcone isomerase (CHI; EC 3.2.1.14) together lead to the formation of flavanones. Naringenin is converted by flavanone 3-hydroxylase (FHT; EC 1.14.11.9) and dihydroflavonol 4-reductase (DFR; EC 1.1.1.219) to leuco-anthocyanidins (flavanols), which are the precursors of anthocyanidins and flavan-3-ols (e.g., catechins and procyanidins; Treutter, 2001). The latter, in particular, also play an important role in plant disease resistance mechanisms. Flavonol synthase (FLS; EC 1.14.11.23) catalyses the dehydrogenation of dihydroflavonols to flavonols and therefore competes with flavanol-forming enzymes for their common precursors. Dihydrochalcone 2'-*O*-glucosyltransferase (D2'GT; EC 2.4.1.17) catalyses the formation of phloridzin, which is also present in high concentrations in apple peel.

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In addition to its structural and defence-related functions, the phenylpropanoid pathway also produces a variety of phenolics that are closely-associated with fruit quality. However, there are few reports on phenlypropanoid enzyme activities in apple fruit during development, particularly in their responses to infection by pathogens. The objective of this study was to evaluate the biochemical responses of various zones of apple fruit skin to infection with *V. inaequalis*. This was investigated at the level of individual phenolic compounds related to key enzyme activities in the phenylpropanoid pathway. The activities of PAL, CHS/CHI, FHT, DFR, FLS, and D2'GT, and the accumulation of various phenolic compounds (hydroxycinnamic acids, flavan-3-ols, dihydrochalcones, and flavonols) were also investigated. Three different areas of peel tissue on the same fruit (i.e., symptomatic lesions, tissue around lesions, and healthy peel tissue) were analysed at two different stages of fruit maturity (i.e., 1 month before commercial maturity, and at commercial maturity). No comparable research has been reported in the literature.

MATERIALS AND METHODS

Plant material and growth conditions

This study was performed on apple trees (*Malus* × *domestica* Borkh.), cultivar 'Braeburn', which is a scab-susceptible cultivar. Fruits were taken from 9-year-old trees that had been grafted onto M.9 rootstock and were growing at the University Experimental Orchard in Ljubljana. Trees were cultivated according to commercial guidelines for integrated fruit production.

Fruit samples (n = 5) were picked 1 month before commercial maturity, on 15 September 2008 (140 d after full bloom; DAFB), and at commercial maturity on 20 October 2008 (175 DAFB). The apples were peeled using a fruit peeler and the peel was divided into three parts: symptomatic scab lesions, peel tissue 1 – 2 mm around the scab lesions, and healthy fruit peel. All samples were shock-frozen immediately in liquid nitrogen and stored at –80°C until analysis.

Extraction and HPLC determination of phenolic compounds

Extractions were carried out as described by Mikulic Petkovsek *et al.* (2007) with some modifications. Each finely-powdered apple peel sample (0.5 g) was extracted with 3 ml 100% (v/v) methanol containing 1% (w/v) 2,6-di-*tert*-butyl-4-methylphenol (BHT) and homogenised using a T-25 Ultra-Turrax (Ika-Labortechnik, Staufen, Germany). BHT was added to the samples to prevent oxidation during the extraction. All samples were placed in a Sonis 4 (Iskra pio, Ljubljana, Slovenia) ultrasonic bath for 1 h, then centrifuged at 15,550 × *g* for 7 min at 4°C. Each supernatant was filtered through a Chromafil A0-45/25 polyamide filter (Macherey-Nagel, Düren, Germany). HPLC analysis was performed using a Surveyor HPLC system with a PDA detector (Thermo-Finnigan, San Jose, CA, USA). The column was a Gemini C_{18} column (150 mm × 4.6 mm; particle size 3 µm; Phenomenex, Torrance, CA, USA) operated at 25°C. The elution solvents were 1% (v/v) formic acid in double-distilled water (solvent A) and 100% (v/v) acetonitrile (solvent B). Samples were eluted using the linear gradient described by Marks *et al.* (2007), with a sample injection volume of 20 µl and a flow rate of 1.0 ml min^{-1}. Phenolic compounds were identified by comparing their UV-VIS spectra from 220 – 550 nm, and their retention times, with standards. Hydroxycinnamic acids (chlorogenic, *p*-coumaric, ferulic, and caffeic acid), flavan 3-ols (catechin, epicatechin, and procyanidin B2), dihydrochalcone (phloretin), and phloroglucinol were detected at 280 nm; whereas phloridzin, quercetin-3-*O*-rutinoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glucoside, and quercetin-3-*O*-galactoside were detected at 350 nm. The concentration of each phenolic compound was calculated by comparing the peak area of the samples with those of the corresponding standard compound. All concentrations were expressed as mg 100 g^{-1} FW, except in the case of phloretin which was expressed as µg 100 g^{-1} FW.

Enzyme preparation

Each fruit peel sample (0.5 g), plus 0.25 g quartz sand and 0.25 g Polyclar AT was homogenised in 3 ml 0.1 M Tris-HCl pH 7.5 containing 0.4% (w/v) Na-ascorbate in a pre-cooled mortar and pestle. The homogenate was then centrifuged at 10,000 × g for 10 min at 4°C. To remove low molecular compounds, 400 µl of the supernatant was passed through a Sephadex G25 medium chromatography column. The eluate from the column ("crude extract") was used for all enzyme assays. Protein contents were quantified by a modified Lowry procedure (Sandermann *et al.*, 1972) with bovine serum albumin as a standard.

Buffers

The following buffers were used for the various enzyme assays, Buffer A (PAL assays), 0.1 M H_3BO_3 containing 0.4% (w/v) Na-ascorbate, pH 8.5; Buffer B (CHS/CHI assays), 0.1 M potassium phosphate, pH 7.0 containing 0.4% (w/v) Na-ascorbate, pH 7.0; Buffer C (FHT assays), 0.1 M Tris-HCl, pH 7.25 containing 0.4% (w/v) Na-ascorbate; Buffer D (FLS, D2'GT assays), 0.1 M Tris-HCl, pH 7.5 containing 0.4% (w/v) Na-ascorbate, pH 7.5; and Buffer E (DFR assay), 0.1 M potassium phosphate, pH 6.8 containing 0.4% (w/v) Na-ascorbate.

Enzyme assays

PAL assays (100 µl final volume) contained 40 µl "crude extract", 5 µl [^{14}C]-phenylalanine (0.063 nmol) and 55 µl Buffer A. The CHS/CHI assays (100 µl final volume) contained 40 µl "crude extract", 5 µl [^{14}C]-malonyl-CoA (1.5 nmol), 5 µl [^{14}C]-*p*-coumaroyl-CoA (1.0 nmol) and 50 µl Buffer B. The FHT assays (100 µl final volume) contained 8.2 µl [^{14}C]-naringenin (0.036 nmol), 30 µl "crude extract", 5 µl 2-oxoglutarate (1.46 mg ml^{-1}), 5 µl $FeSO_4.7H_2O$ (0.56 mg ml^{-1}) and 60 µl Buffer C. The FLS assays (100 µl final volume) contained 2.9 µl [^{14}C]-dihydrokaempferol (0.036 nmol), 30 µl "crude extract", 5 µl 2-oxoglutarate (1.46 mg ml^{-1}1), 5 µl $FeSO_4.7H_2O$ (0.56 mg ml^{-1}) and 60 µl Buffer D. The DFR assays (50 µl final volume) contained 19 µl (0.036 nmol) [^{14}C]-dihydroquercetin, 20 µl "crude extract", 5 µl NADPH (4.18 mg 100 µl $^{-1}$) and 25 µl Buffer E. The D2'GT assays (50 µl final volume) contained 20 µl "crude extract", 2.5 µl phloretin (300 µM), 2.5 µl [^{14}C]-UDP-glucose and 25 µl Buffer D.

All assays were incubated for 30 min at 30°C, with the exception of the DFR assays which were incubated for 15 min at 30°C. The PAL and CHS/CHI assays were stopped with 200 µl 100% (v/v) ethyl acetate and 10 µl glacial acetic acid. The amounts of the products formed were determined using a WinSpectral 1414 liquid scintillation counter (Wallac, Turku, Finland). The FHT, DFR, and FLS assays were terminated by adding 70 µl 100% (v/v) ethyl acetate and 10 µl glacial acetic acid. Ten µl of 0.1 M EDTA were also added to the FLS assays. The organic phases were transferred to pre-coated cellulose plates (Merck, Darmstadt, Germany) for thin-layer chromatography (TLC) using a 10:9:1 (v/v/v) mix of chloroform/acetic acid/H_2O for the FHT and DFR assays, and 30% (v/v) glacial acetic acid for the FLS assays. Conversion rates were then determined using a TLC linear analyser (Berthold, BadWildbad, Germany). The D2'GT assays were terminated by adding 10 µl glacial acetic acid and 25 µl 100% (v/v) methanol. The mixture was then chromatographed on Schleicher and Schüll 2043b paper using water as solvent. Zones containing the labelled products were cut out, and the radioactivity was quantified using a Winspectral 1414 scintillation counter. All products were identified, as described above, using authentic substances (Fischer *et al.*, 2003). All enzyme activities were expressed as nkat mg^{-1} FW.

Chemicals

The standards used to identify and determine the levels of phenolic compounds in each sample were chlorogenic acid, quercetin-3-*O*-rutinoside, (-)-epicatechin, phloretin, phloroglucinol, and phloridzin from Sigma-Aldrich (Steinheim, Germany), (+)- catechin from Roth (Karlsruhe, Germany), and caffeic acid, *p*-coumaric acid, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-galactoside, procyanidin B2, and ferulic acid from Fluka Chemie GmbH (Buchs, Switzerland). The chemicals used for the mobile phases were HPLC-grade acetonitrile and formic acid from Fluka. Water for the mobile phase was double-distilled and purified using a Milli-Q system (Millipore, Bedford, MA, USA). The following ^{14}C-labelled substrates were used for determining enzyme activities L-[U-^{14}C] phenylalanine and [2-^{14}C]-malonyl-coenzyme A, (Amersham International, Freiburg, Germany). [^{14}C]-Naringenin, [^{14}C]-dihydrokaempferol, and [^{14}C]-dihydroquercetin were prepared as described (Fischer *et al.*, 2003).

Statistical analysis

The results were analysed using Statgraphics Plus Version 4.0 (Manugistics Inc., Rockville, MD, USA). Data from all chemical analyses were tested for spatial or temporal differences between the samples using one-way analysis of variance (ANOVA). Differences between apple peel tissues were analysed independently for each time point using Duncan's multiple range test with a significance level of $P \leq 0.05$. A one-way analysis of variance (ANOVA) was also carried out to determine differences in the contents of phenolic compounds between the different sampling dates followed by the LSD test with a significance level of $P \leq 0.05$. Means and standard errors of the means (means + SE) are reported.

RESULTS AND DISCUSSION

Analysis of phenolic compounds

Hydroxycinnamic acids represented the largest group of products in the biosynthetic pathways for phenolic compounds. Chlorogenic acid contents were higher than those of the other hydroxycinnamic acids analysed and were comparable on both sampling dates. On both sampling dates, tissues infected with the apple scab fungus contained statistically higher quantities of chlorogenic acid compared with tissues around the lesions or healthy apple peel (Figure 1C). The chlorogenic acid contents of healthy peel varied from 0.04 – 1.24 mg 100 g^{-1} FW. Ferulic acid contents were at least 17-times lower, but showed a similar distribution (Figure 1A). The amount of caffeic acid was statistically lower at 175 DAFB than at 140 DAFB (Table I). Peel tissue surrounding the scab lesions accumulated *p*-coumaric and caffeic acid acid (Figure 1B, D). Compared to healthy peel, apple scab lesions contained 1.4- to 1.7-times higher concentrations of caffeic acid, and 1.6- to 3.1-times more *p*-coumaric acid. This agreed with previous reports on the accumulation of hydroxycinnamic acids in the response of apple leaves to fungus infection. Mikulic Petkovsek *et al.* (2008) and Williams and Kuç (1969) found that hydroxycinnamic acids inhibited the growth and sporulation of *V. inaequalis.*

Dihydrochalcone phloridzin, a phloretin 2' *O*-glucoside, was the major phenolic compound in apple fruit extracts (Iwashina, 2000). Phloridzin and its corresponding aglycone, phloretin, have often been linked to resistance to numerous diseases. Studies on apple have shown that resistance to *V. inaequalis* was related to the presence of this compound (Hamauzu, 2006). The fungus degrades phloridzin to its toxic aglycone, phloretin. In our experiments, healthy apple peel and tissues around the symptomatic lesions contained significantly lower levels of phloridzin than the scab lesion itself. Phloridzin concentrations in scab lesions ranged from 114.7 – 238.8 mg 100 g^{-1} FW (Figure 1F), which was 6.5-times higher than in healthy peel. It was therefore evident that phloridzin accumulated when apple peel tissue was infected with *V. inaequalis*. This effect was also reported by Mikulic Petkovsek *et al.* (2008) and by Leser and Treutter (2005). The aglycone, phloretin, was only found in small amounts in scab lesion tissues, while surrounding

TABLE I

Differences in the levels of selected phenolic compounds in the peel of Malus × domestica *'Braeburn' between two sampling dates (140 DAFB and 175 DAFB) by one-way ANOVA*

Compound	Concentration (mg 100 g^{-1} FW) 140 DAFB	175 DAFB	ANOVA[‡]
Total quercetin	81.56 ± 7.78[†]	63.15 ± 8.60	NS
Epicatechin	24.11 ± 1.26	20.02 ± 1.33	*
Catechin	2.16 ± 0.32	4.64 ± 0.37	***
Ferulic acid	0.13 ± 0.03	0.16 ± 0.02	NS
Phloridzin	52.83 ± 5.8	66.05 ± 8.82	NS
Phloroglucinol	112.64 ± 11.6	139.22 ± 13.2	NS
Caffeic acid	0.95 ± 0.09	0.65 ± 0.07	**
Chlorogenic acid	1.82 ± 0.42	2.74 ± 0.53	NS
p-Coumaric acid	1.55 ± 0.13	1.16 ± 0.16	NS
Procyanidin B2	28.89 ± 2.05	26.54 ± 2.15	NS
Phloretin[†]	0.70 ± 0.08	0.77 ± 0.08	NS

[†]Values are means ± SD (n=5).
[‡]NS,*, **, ***; non-significant, significant at $P < 0.05$, $P < 0.01$, or $P < 0.001$ respectively.
[†]Phloroglucinol in µg 100 g^{-1} FW.

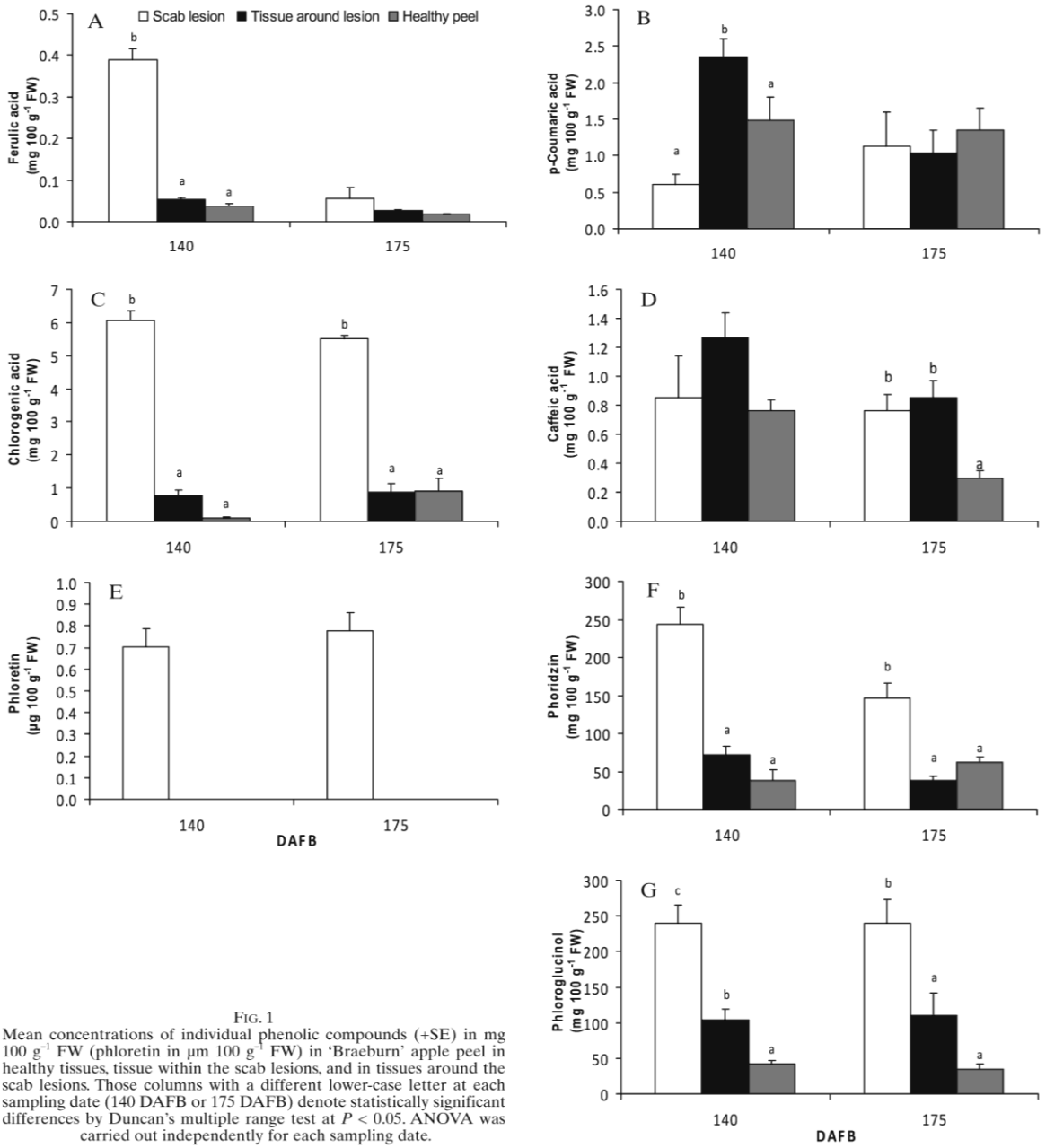

FIG. 1
Mean concentrations of individual phenolic compounds (+SE) in mg 100 g^{-1} FW (phloretin in µm 100 g^{-1} FW) in 'Braeburn' apple peel in healthy tissues, tissue within the scab lesions, and in tissues around the scab lesions. Those columns with a different lower-case letter at each sampling date (140 DAFB or 175 DAFB) denote statistically significant differences by Duncan's multiple range test at $P < 0.05$. ANOVA was carried out independently for each sampling date.

tissues and healthy apple peel contained none (Figure 1E). However, phloretin rather than phloridzin has been claimed to be the key agent in defence reactions, related to formation of the highly reactive *o*-quinone (Elstner *et al.*, 1996). In addition, phloretin can be degraded to form phloroglucinol, which inhibited development of the fungus (Hamauzu, 2006). In our experiments, apple scab-infected tissues contained statistically greater quantities of phloroglucinol on both sampling dates, compared to tissues around the lesions or healthy peel. Phloroglucinol concentrations in the lesions ranged from 196.2 – 292.0 mg 100 g^{-1} FW (Figure 1G), which was 6.9-times higher than in healthy apple peel.

Flavan-3-ols are capable of precipitating proteins, which may explain the presence of catechins in the defence mechanisms of plants (Treutter, 1989). Mayr *et al.* (1997) related the rapid accumulation of catechins and oligomeric pro-anthocyanidins at the sites of infection to the restriction of fungal spread. Feucht *et al.* (1998) reported that epicatechin was the main flavanol synthesised during fruit infection by *V. inaequalis*. In our study, the highest levels of flavanols were found for procyanidin B2, compared to epicatechin and catechin.

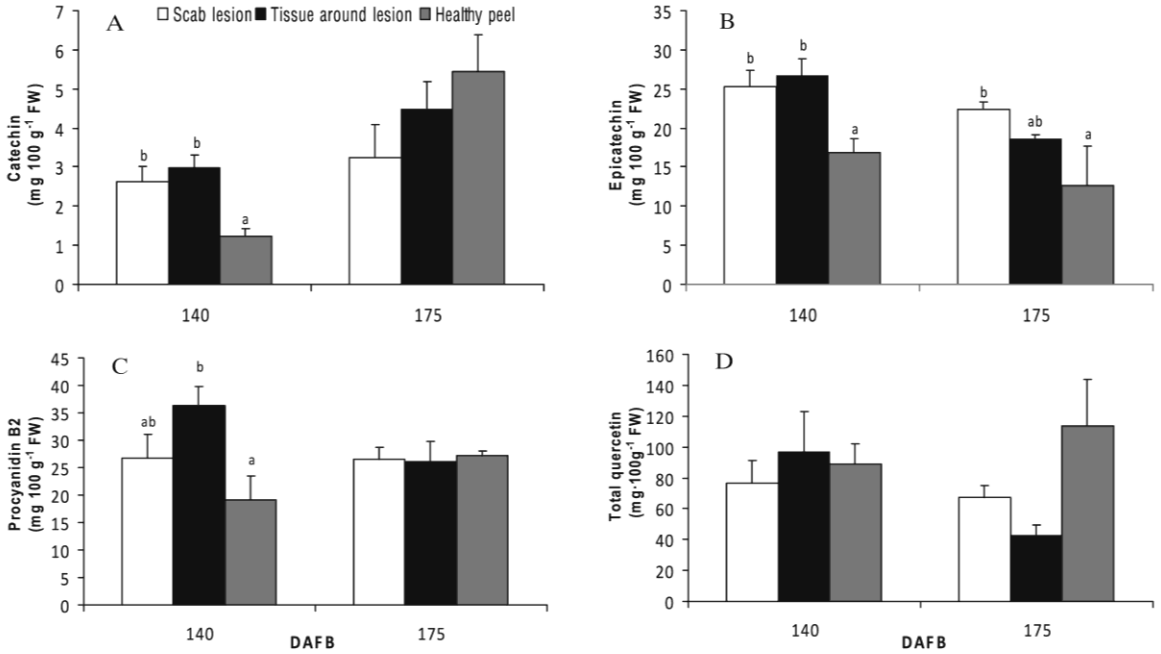

FIG. 2
Mean concentrations of flavanols and flavonols (+SE) in mg 100 g^{-1} FW in 'Braeburn' apple peel in healthy tissues, tissue within the scab lesions, and in tissues around the scab lesions. Those columns with a different lower-case letter at each sampling date (140 DAFB or 175 DAFB) denote statistically significant differences by Duncan's multiple range test at $P < 0.05$. ANOVA was carried out independently for each sampling date.

The amounts of epicatechin differed significantly between treatments (Table I) and appeared lower at 175 DAFB (Figure 2B). This was also noted in healthy fruit during maturation by Mayr *et al.* (1994). Tissues around the scab lesions contained the highest amounts of epicatechin compared to lesion tissue or healthy peel (Figure 2B). Treutter and Feucht (1990) and Feucht *et al.* (1994) also observed a dramatic increase in the levels of catechins and their polymers in the boundary zones around the sites of infection by *V. inaequalis* in apple leaves. In our case, statistically higher concentrations of catechins were observed at 175 DAFB, regardless of the tissue sample (Figure 2A; Table I).

Mikulic *et al.* (2009) reported the highest concentrations of flavonols in the zone between scab lesions and healthy skin tissue. In our experiments, the concentrations of individual phenolic compounds (data not shown) and total quercetin-*O*-glucosides (i.e., the

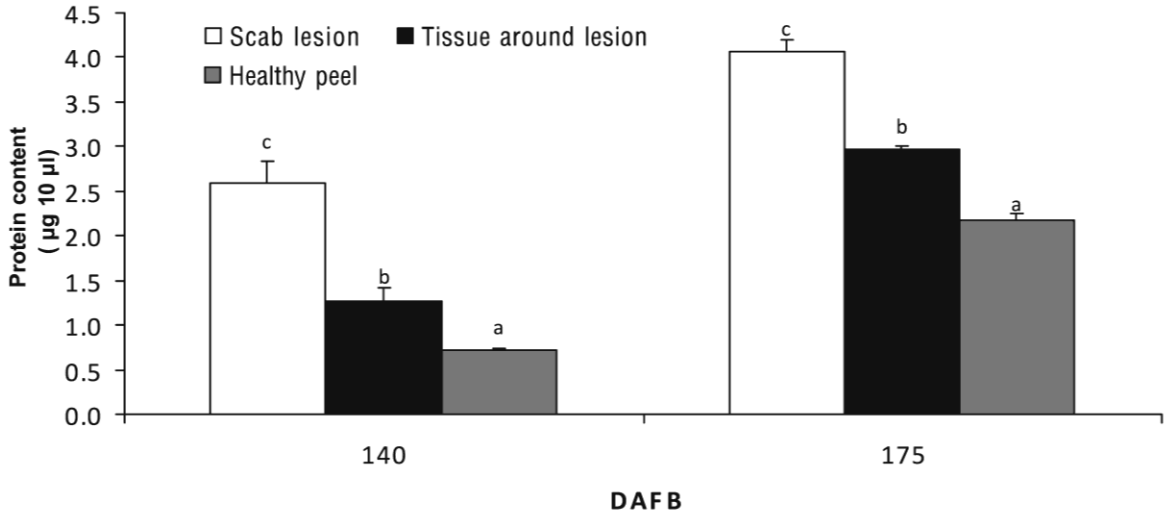

FIG. 3
Mean concentrations of total proteins (+SE) in µg 10 μl^{-1} "crude tissue" extract from 'Braeburn' apple peel in healthy tissues, tissues within the scab lesions, or in tissues around the scab lesions. Columns with a different lower-case letter at each sampling date (140 DAFB or 175 DAFB) denote statistically significant differences by Duncan's multiple range test at $P < 0.05$. ANOVA was carried out independently for each sampling date.

sum of quercetin-3-*O*-rutinoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glucoside, and quercetin-3-*O*-galactoside) showed no statistically significant differences between the different tissues (Figure 2D) on either fruit maturity date (140 DAFB and 175 DAFB). This also agreed with the results of Picinelli *et al.* (1995), who found no relationship between the level of flavonols and scab resistance in apple leaves.

Analyses of enzyme activities

Protein contents were highest in scab lesion tissue and lowest in healthy apple peel (Figure 3). This could be due to increased protein production in response to fungal infection and/or to the presence of added fungal proteins in infected tissues. All enzyme activities were therefore expressed as mg^{-1} FW, rather than per total protein content (specific activity), in order to reduce the impact of additional fungal proteins. Figure 4 shows a comparison of the activities of the five key enzymes examined in the different apple peel samples on the two sampling dates.

PAL, which catalyses deamination of the amino acid phenylalanine, is a key enzyme and represents the interface between primary and secondary metabolism. The resulting product, cinnamic acid, is a precursor for the biosynthesis of flavonoids, lignin, and alkaloids. In our analysis, PAL activity could only be measured in symptomatic scab lesions and in the tissues around the lesions on both sampling dates. By 175 DAFB, PAL activities had increased, particularly around the symptomatic lesions, but remained almost unchanged within the scab lesions (Figure 4A). Previous studies (Mayr *et al.*, 1997; Michalek *et al.*, 1999b) revealed that the rapid biosynthesis of flavanols from phenylalanine was necessary for successful plant protection. Inhibition of PAL activity resulted in severe symptoms of fungal sporulation in a resistant cultivar. Saunders and Olechno (1988) reported that differences in PAL mRNA levels in plants occurred as a response to fungal inoculation. Saunders and O'Neill (2004) reported that alfalfa plants responded to inoculation with an avirulent fungus by developing induced resistance that enhanced *PAL* gene expression, which increased the activity of PAL for flavonoid metabolism.

CHS is the key enzyme for the formation of flavonoids because it produces the first C_{15}-structure which is an intermediate for the formation of all further classes of flavonoids. The CHS reaction leads to the formation of naringenin chalcone, which is converted by CHI into naringenin, the first true flavonoid structure. At 175 DAFB, statistically higher CHS/CHI activities were detected in symptomatic lesion tissues (Figure 4B) than

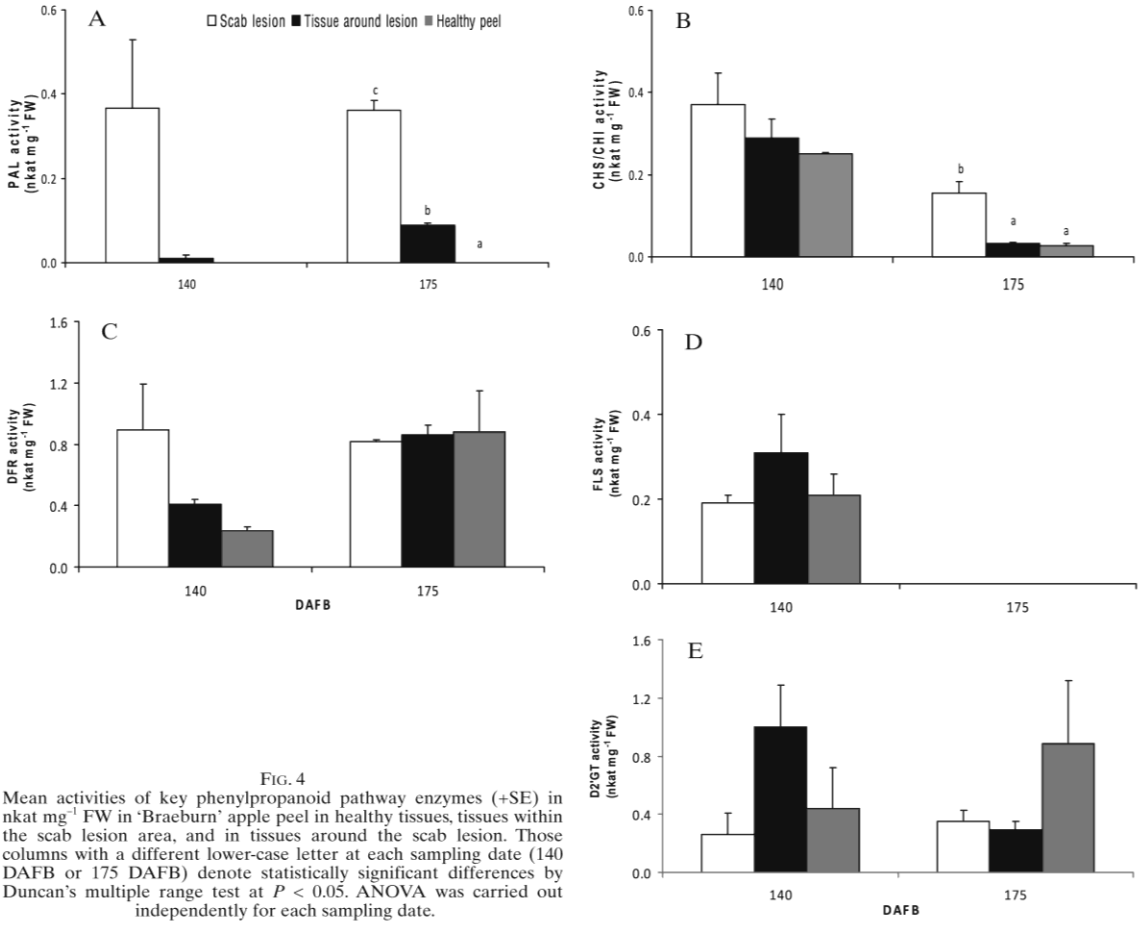

FIG. 4
Mean activities of key phenylpropanoid pathway enzymes (+SE) in nkat mg^{-1} FW in 'Braeburn' apple peel in healthy tissues, tissues within the scab lesion area, and in tissues around the scab lesion. Those columns with a different lower-case letter at each sampling date (140 DAFB or 175 DAFB) denote statistically significant differences by Duncan's multiple range test at $P < 0.05$. ANOVA was carried out independently for each sampling date.

in healthy apple peel tissue, or in tissues surrounding the scab lesions, which showed no differences in activity. Between 140 DAFB and 175 DAFB, CHS/CHI activities decreased in all tissues, indicating that the processes of fruit maturation lowered the levels of enzyme activity (Figure 4B).

DFR is an NADPH-dependent oxido-reductase which reduces the oxo-group at position-4 to provide flavan-3,4-diols; precursors for the formation of catechins, anthocyanidins, and epicatechins (Forkmann and Heller, 1999). DFR activities showed no statistically significant differences between any of the peel zones studied. By 175 DAFB, DFR activities had increased around the scab lesions and in healthy apple peel, but had decreased in lesion tissues (Figure 4C).

FHT and FLS are di-oxygenases which require 2-oxoglutarate, Fe^{2+}, and ascorbate as co-factors, and catalyse the formation of flavanones and flavonols. FHT activity was undetectable on either sampling date. FLS activity could only be measured at 140 DAFB (Figure 4D). However, those flavonols that are the products of this enzyme showed higher concentrations in scab-infected tissues and in surrounding tissues than in healthy apple peel.

D2'GT converts phloretin to phloridzin. In contrast to the other enzymes examined, symptomatic lesions showed lower D2'GT activities than healthy peel tissue on both sampling dates. The decrease in D2'GT enzyme activity in infected peel tissues could be explained by the fact that the aglycone, not the 2'-*O*-glucoside, is considered to be the active compound against fungal attack. In fact, as can be seen in Figure 4E, the aglycone (phloretin) was only detected in scab lesions. The levels of phloridzin in lesion tissues were also statistically higher. At 175 DAFB, D2'GT activities were higher in lesions and in healthy peel tissue than in immature fruit at 140 DAFB. D2'GT activities around the lesions decreased between the two sampling dates (Figure 4E).

CONCLUSIONS

Knowledge of the phenolic compounds and enzyme activities present in fruit tissues is an indispensable pre-condition for future studies on complex host-pathogen interactions and polyphenol-mediated disease resistance. We have shown that phenolic compounds accumulate in apple peel in response to infection by *V. inaequalis*. This results from the higher activities of key phenylpropanoid pathway enzymes in scab lesions and in surrounding tissues. All key enzyme activities, except DFR and G2'TP, were lower at 175 DAFB which indicates that these enzyme activities decreased with fruit maturity. Nevertheless, only decreases in caffeic acid and epicatechin levels were noticed, while the levels of all other phenolic compounds remained constant. Our results represent an important contribution to increase our knowledge of the activity of the phenlypropanoid pathway in apple fruit. Future work should focus on differences in this defense scheme in apple varieties having different levels of susceptibility to scab, and on the responses to infection at the early stages of fruit development which are crucial for successful resistance to the apple scab pathogen.

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2.3 POLIFENOLNI METABOLIZEM V JABOLČNI KOŽICI RAZVIJAJOČIH SE PLODOV PRI NA JABLANOV ŠKRLUP ODPORNI IN OBČUTLJIVI SORTI

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Polyphenol metabolism of developing apple skin of a scab resistant and a susceptible apple cultivar.
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Tekom razvoja jabolk (*Malus domestica* Borkh.) smo v kožici spremljali vsebnost glavnih fenolnih snovi (flavonoli, flavanoli, dihidrohalkoni, hidroksicimetne kisline, antociani) in aktivnost encimov PAL, CHS/CHI, FLS, FHT, DFR in POD, ki so z njihovo vsebnostjo povezani. Skozi sezono smo vzorčili v petih terminih, v poskus pa sta bili vključeni dve sorti jabolk, in sicer na škrlup občutljiva sorta 'Zlati delišes' in na škrlup odporna sorta 'Florina'. Vzorčenja so pri občutljivi sorti vključevala zdravo kožico, kožico, na kateri je bila vidna škrlupasta pega, in tkivo oziroma del kožice v okolici škrlupaste pege, medtem ko smo pri sorti 'Florina' v vzorčenjih analizirali samo zdravo kožico plodov. Izmed vseh analiziranih encimov, je pri obeh preučevanih sortah encim CHS/CHI kazal največjo aktivnost, PAL pa najnižjo. Zdrava kožica na jablanov škrlup občutljive in na jablanov škrlup odporne sorte ni kazala razlik v glavnih fenolnih skupinah, zastopanih v kožici. Vseeno pa smo pri odporni sorti 'Florina' v zadnjih fazah razvoja ploda opazili povečano delovanje fenolnih encimov, za kar je po našem mnenju odgovorno predvsem nastajanje antocianov v dozorevajočih plodovih. Značilne razlike v fenolnem metabolizmu je bilo zaznati v vseh treh tkivih občutljive sorte 'Zlati delišes'. Povečano vsebnost hidroksicimetnih kislin, dihidrohalkonov in flavan 3-olov je bilo zaznati v škrlupasti pegi in tkivu okoli škrlupaste pege. Encimi PAL, DFR, FHT in POD so kazali višjo aktivnost v škrlupasti pegi v primerjavi z ostalimi analiziranimi tkivi, medtem ko je bila aktivnost encimov CHS/CHI in FLS pri vseh obravnavanjih precej podobna. V začetnih fazah okužbe z jablanovim škrlupom (začetku sezone) je prišlo do večje akumulacije fenolnih snovi, medtem ko je encimska aktivnost začela naraščati v poznih fazah razvoja ploda.

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ORIGINAL PAPER

Polyphenol metabolism of developing apple skin of a scab resistant and a susceptible apple cultivar

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Abstract During fruit development, the concentration of main polyphenols (flavonols, flavanols, dihydrochalcones, hydroxycinnamic acids, anthocyanins) and the activities of related enzymes (phenylalanine ammonia lyase, chalcone synthase/chalcone isomerase, flavanone 3-hydroxylase, dihydroflavonol 4-reductase, flavonol synthase, peroxidase) were monitored in apple (*Malus domestica* Borkh.). The seasonal survey was performed at five different sampling dates and included the healthy peel of the resistant cultivar 'Florina' and healthy peel, scab symptomatic spot and the tissue around the infected spot of the susceptible cultivar 'Golden Delicious'. From all enzymes tested, chalcone synthase/chalcone isomerase had the highest activity in both cultivars, while phenylalanine ammonia lyase had the lowest. The healthy peels of the susceptible and the resistant cultivar did not show differences in the accumulation of the main polyphenol groups present in the apple skin. However, in the resistant cultivar 'Florina', an increase of polyphenol enzyme activities could be observed in late stages of fruit development, which seems to be related to the anthocyanin accumulation in ripe fruits. Significant differences in the polyphenol metabolism were observed in the three different tissues of the susceptible cultivar 'Golden Delicious'. Increased concentrations of hydroxycinnamic acids, dihydrochalcones and flavan-3-ols were found in the scab symptomatic spots and surrounding tissues. Phenylalanine ammonia-lyase, dihydroflavonol 4-reductase, flavanone 3-hydroxylase and peroxidase showed higher activities in the scab symptomatic spot compared to other analysed tissues, whereas the activities of other enzymes remained unchanged. Highest induction of polyphenol accumulation after scab infection was observed in early developmental stages, whereas enzyme activities were increased in later stages.

Keywords Apple scab · Dihydroflavonol 4-reductase · Flavanol · Hydroxycinnamic acid · *Malus domestica* · Phenylalanine ammonia-lyase

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Introduction

Apple scab, also known as black spot, is caused by the fungus *Venturia inaequalis* (Cooke) G. Wint. It is one of the most serious diseases of apple that occurs in almost all apple-producing countries and causes huge economic losses (MacHardy 1996). Previous work on resistance mechanisms demonstrated the significance of flavonoids, especially flavan 3-ols in leaf (Bazzi et al. 2003; Leser and Treutter 2005; Mikulic Petkovsek et al. 2009; Treutter 2005) and fruit (Mikulic Petkovsek et al. 2007, 2009). Differences in phenolic compounds between susceptible and resistant apple cultivars are especially evident in the content of flavanols, phloridzin and hydroxycinnamic acids. Similar response to *Venturia inaequalis* (Cooke) G. Wint. was reported in apple leaves and fruit (Treutter and Feucht 1990a; Picinelli et al. 1995; Michalek et al. 1999; Mikulic Petkovsek et al. 2007; Gosch et al. 2010).

However, relatively little is known about the defence reactions in the apple skin as a response to scab infection. Recently we have shown that apple scab provokes dramatic, but locally restricted changes in the polyphenol metabolism of the apple peel (Slatnar et al. 2010a, b). Thus, the activities of many enzymes of the polyphenol pathway were distinctly increased in the scab spot compared to the healthy tissue and the area around the spot. Likewise, the concentrations of hydroxycinnamic acids, flavanols and dihydrochalcones were increased in the scab spot and surrounding areas (Slatnar et al. 2010a).

The polyphenol composition is primarily genetically determined but can be also influenced by various factors (Treutter 2010). As a rule, the polyphenol spectrum changes during fruit ripening (Mayr et al. 1995; Halbwirth et al. 2006). In early fruit stages, apple skin contains high levels of hydroxycinnamic acids, flavonols and proanthocyanidins, which are assumed to play an important role in pathogen defence, herbivory deterrence and UV protection. During ripening, the concentrations of these compounds decrease and in some apple cultivars, anthocyanins accumulate (Lister et al. 1994). An understanding of the biosynthesis of polyphenols is important as they influence fruit quality, plant development and plant health (Cheng and Breen 1991).

The dihydrochalcones are the predominant polyphenols in *Malus domestica* Borkh. leafs. It was suggested that a presence of specific highly active dehydrogenase, which catalyses the NADPH dependent formation of 4-hydroxy-dihydrocinnamoyl-CoA from *p*-coumaroyl-CoA, is responsible for formation of high concentration of dihydrochalcones (Gosch et al. 2009). In addition, different flavonoid classes are present in apple fruit: flavanones, dihydroflavonols, flavonols, leucoanthocyanidins, flavan 3-ols and anthocyanidins (Lata et al. 2009; Treutter 2001). Figure 1 provides an overview of the biosynthesis of polyphenols and related enzymes involved in the formation of the main flavonoid classes. Peroxidase was included because it converts phenolic structures into quinoid structures, which might be part of the defence reaction.

The main aim of the present study was to follow the changes in enzyme activities and accumulation of phenolic compounds during the growing season in the skin of a susceptible and a resistant apple cultivar. Our previous studies have shown that there was a change in enzyme activities and phenolic accumulation after scab infection in the ripe fruit; however, the analysis was only conducted at one harvesting date (Slatnar et al. 2010a, b). With a seasonal monitoring of polyphenol accumulation and the related enzyme activity, the involvement of the polyphenol metabolism in the defence mechanisms against *Venturia inaequalis* ((Cooke) G. Wint.) is investigated in more detail.

Materials and methods

Chemicals

For the quantification of phenolic compounds, chlorogenic acid (5-caffeoylquinic acid), cyanidin-3-galactoside and rutin (quercetin-3-*O*-rutinoside) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), (−)-epicatechin, quercetin (quercetin-3-*O*-rhamnoside), quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, *p*-coumaric acid, procyanidin B2, caffeic acid from Fluka Chemie GmBH (Buchs, Switzerland), quercetin-3-*O*-arabinofuranoside and quercetin-3-*O*-xyloside from Apin Chemicals (Abingdon, UK) and (+)-catechin from Roth (Karlsruhe, Germany). Methanol for extraction of phenolic compounds was acquired from Sigma. The chemicals for mobile phases were HPLC grade acetonitrile and formic acid from Fluka. Water for the mobile phase was bidistilled and purified with the Milli-Q system (Millipore, Bedford, MA, USA). L-[U-^{14}C] Phenylalanine and [2-^{14}C]-malonyl-coenzyme A, were obtained from Amersham International (Freiburg, Germany). [^{14}C]-Naringenin, [^{14}C]- dihydrokaempferol and [^{14}C]-dihydroquercetin were prepared as recently described (Fischer et al. 2003).

Plant material and experimental design

The study was performed on apple trees (*Malus domestica* Borkh.), cv. 'Florina', which is a scab-resistant cultivar, and cv. 'Golden Delicious', which is a scab-susceptible cultivar. Fruits were taken from 9-year-old trees grafted on the M9 rootstock growing in the University experimental orchard in Ljubljana. The samples were picked five times during the growing season: 30th June 2009 (60 days after full bloom, DAFB); 14th July 2009 (74 DAFB); 28th July 2009 (88 DAFB); 11th August 2009 (102 DAFB) and 15th September 2009 (137 DAFB). The apple skin was peeled as thin as possible (approximately 1 mm thick), and the peel of the susceptible cultivar was divided into three parts: scab symptomatic spot (late fruit infection), tissue around the scab spot (1–2 mm) and healthy peel. In the scab-resistant cultivar 'Florina', only the healthy peel was analysed because no symptomatic spot occurred. Five repetitions of apple peel per cultivar were prepared for all the analyses, each consisting of ten different fruits. The samples were separated in two parts; one was taken for the analysis of the enzyme activity and one for phenolic quantification. Samples were immediately shock-frozen in liquid nitrogen and stored at −80°C until use.

Extraction and analysis of phenolic compounds

Extraction was carried out as described by Mikulic Petkovsek et al. (2007) with a few modifications. The apple

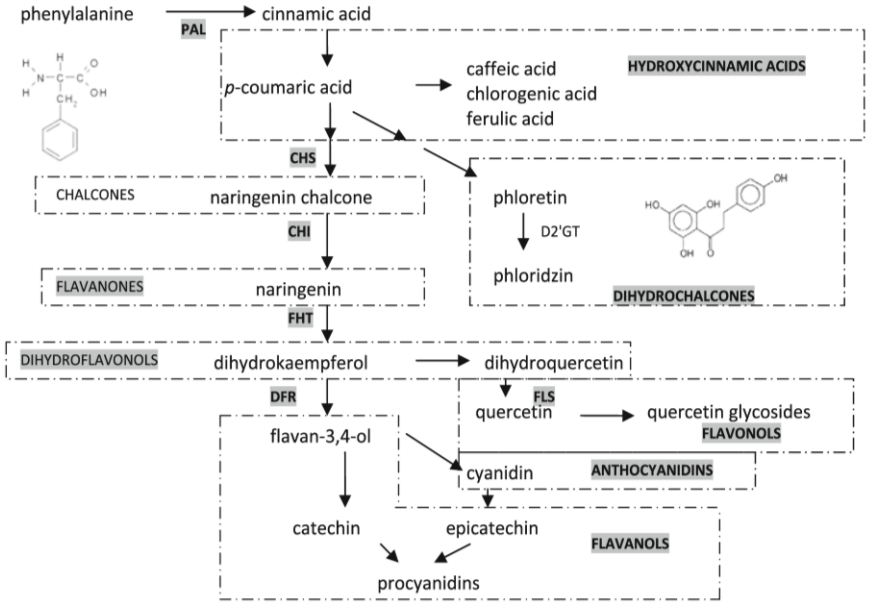

Fig. 1 Biosynthetic pathways of phenolic compounds in apple. *PAL* phenylalanine ammonia lyase, *CHS* chalcone synthase, *CHI* chalcone isomerase, *FHT* flavanone 3-hydroxylase, *DFR* dihydroflavonol 4-reductase, *FLS* flavonol synthase, *D2′GT* dihydrochalcone 2′-*O*-glucosyltransferase. The individual phenolics and phenolic groups mentioned in the text are accentuated in *bold lettering*. The enzymes and polyphenols analysed are highlighted by *grey shading*

peel was ground to a fine powder with liquid nitrogen and 0.5 g of the powder were extracted with 3 ml methanol containing 3% (v/v) HCOOH and 1% (w/v) 2,6-di-tert-butyl-4-methylphenol (BHT) in an ultrasonic bath for 1 h. The treated samples were centrifuged for 7 min at 10,000 rpm. The supernatant was filtered through a Chromafil AO-45/25 polyamide filter (Macherey–Nagel, Düren, Germany) and transferred to a vial prior to injection into a HPLC system. Samples were analysed using a Thermo Finnigan Surveyor HPLC system (Thermo Scientific, San Jose, CA, USA) with a diode array detector at 280 nm (hydroxycinnamic acids, dihydrochalcones and flavan-3-ols), 350 nm (flavonols) and 530 nm (anthocyanins). A Phenomenex (Torrance, CA, USA) HPLC column C18 (150 × 4.6 mm, Gemini 3 µm) protected with a Phenomenex security guard column operated at 25°C was used. The injection volume 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; and finally washing and reconditioning of the column. Identification of compounds was achieved by comparing retention times and their UV–VIS spectra from 200 to 600 nm, as well as by the addition of an external standard. Compounds were identified and quantified using a mass spectrometer (Thermo Scientific, LCQ Deca XP MAX) with an electrospray interface (ESI) operating in negative/positive ion mode. The analyses were carried out using full-scan data dependent MS^n scanning from *m/z* 115 to 2,000. Quantification was achieved according to concentrations of a corresponding external standard.

The concentrations of phenolic compounds were calculated from the peak areas of samples and the corresponding standards. An unknown procyanidin dimer was quantified and expressed in procyanidin B2 equivalents. In the peak of procyanidin B2, an additional unknown procyanidin trimer was detected and the whole peak was expressed as procyanidin B2. 4′-*O*-*p*-coumaroylquinic acid was quantified and expressed as *p*-coumaric acid, phloretin-2′-*O*-xylosylglucoside was quantified as phloridzin and quercetin-3-*O*-arabinopyranoside was quantified as quercetin-3-*O*-arabinofuranoside. Concentrations were expressed as mg/100 g fresh weight of peel (mg/100 g FW).

Extraction and assay of enzyme

Buffers used Buffer A (phenylalanine ammonia lyase—PAL assays; EC 4.3.1.5): 0.1 M H_3BO_3 + 0.4% Na-ascorbate, pH 8.5. Buffer B (chalcone synthase/chalcone isomerise—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 (flavanone 3-hydroxylase—FHT assays; FHT; EC 1.14.11.9): 0.1 M Tris/HCl + 0.4% Na-ascorbate, pH 7.25. Buffer D (flavonol

synthase—FLS assays; FLS; EC 1.14.11.23): 0.1 M Tris/HCl + 0.4% Na-ascorbate, pH 7.5. Buffer E (dihydroflavonol 4-reductase—DFR assays; DFR; EC 1.1.1.219): 0.1 M KPi + 0.4% Na-ascorbate, pH 6.8. Buffer F (peroxidase—POX assays): 0.1 M KPi, pH 6.0, 0.1 M H_2O_2, pH 6.5.

Enzyme preparation Shock-frozen peel was ground to powder with liquid nitrogen. A total of 0.5 g fine peel powder was homogenised with 0.25 g quartz sand, 0.25 g Polyclar AT, and 3 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). The protein solution eluted in the excluded volume of the column (crude extract) was used for enzyme assays.

Analyses of enzyme activity Enzyme assays were performed as previously described (Slatnar et al. 2010a, b), using the assay conditions optimised for apple peel (Table 1). The assays were incubated for 30 min at 30°C. PAL and CHS/CHI assays were stopped with 10 µl of acetic acid and the products were extracted with 200 µl of ethyl acetate, whilst substrates remained in the aqueous phase. The amount of product formed was quantified by measuring the radioactivity of 100 µl of the organic phase on a scintillation counter. The FHT, DFR, and FLS assays were terminated by adding 70 µl of ethyl acetate and 10 µl of acetic acid. To FLS assays 10 µl of 0.1 M EDTA were also added. Organic phases were transferred to precoated cellulose plates (Merck, Germany) for thin-layer chromatography (TLC) in chloroform/acetic acid/H_2O (10:9:1, v/v/v) for FHT and DFR assays or in 30% acetic acid for FLS. The conversion rates were then determined with a TLC linear analyzer (Berthold, Germany). By using authentic substances, all products were identified as described (Fischer et al. 2003).

The protein content was quantified by a modified Lowry procedure (Sandermann and Strominger 1972) with crystalline BSA as standard.

POX activity was carried out according to the Worthington manual (Worthington Biochemical Corporation 1972). Briefly, the enzyme preparation was incubated with 0.410 nmol *O*-dianisidine in buffer F (Table 1) and the time-dependent changes in the absorption at 460 nm were determined in comparison with a blank containing only *o*-dianisidine and buffer F.

Enzyme activity was calculated in nkat/g FW for PAL, CHS/CHI, DFR, FHT, FLS and in Δ A/min for POX (Figs. 4, 5).

Statistical analysis

The data was analysed using the Statgraphics Plus 4.0 (Manugistics, Inc., Rockville, MD, USA). Data from all chemical analyses was tested for differences among treatments using one-way analysis of variance (ANOVA). Differences among tissues were carried out independently for each sampling date using Duncan's multiple range test with a significance level of 0.05. The means and standard errors of means (mean ± SE) are also reported.

Results

In the apple skin, five main polyphenol classes could be identified: hydroxycinnamic acids, dihydrochalcones, flavan 3-ols, flavonols and anthocyanins. The total amounts of the respective classes are shown in Fig. 2, because the individual members followed a similar seasonal course. During fruit development, the hydroxycinnamic content decreased slightly but steadily. The other flavonoid classes showed an increase from sampling date 1 to sampling date 2 and slight decreases thereafter, with the exception of the flavonol content in cv. 'Golden Delicious', which increased towards ripening.

Flavonols were the prevalent group and typically showed a concentration between 200 and 300 mg/100 g FW (Fig. 2d). Seven different quercetin-glycosides were identified (quercetin-3-*O*-rutinoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-arabinofuranoside and quercetin-3-*O*-arabinopyranoside). The most abundant flavonol in apple peel was quercetin-3-*O*-galactoside, followed by quercetin-3-*O*-arabinofuranoside and quercetin-3-*O*-rhamnoside (data not shown). Soluble flavan 3-ols were the second largest group showing a concentration of 100–200 mg/100 g FW at all sampling dates. The monomeric flavanols catechin and epicatechin, the flavanol dimer procyanidin B2 and two further unknown procyanidins (a dimer and a trimer) were found in the apple peel. Epicatechin was the dominant soluble flavan 3-ol, whereas the unknown procyanidin dimer was only present in traces (ca. 1%). In the healthy apple peel, the dihydrochalcone content was typically around 100 mg/100 g FW and thus, slightly lower than the flavan 3-ol content (Fig. 2b). In the group of dihydrochalcones, no statistical differences were observed between resistant and susceptible healthy peel, as reported by Mikulic Petkovsek et al. (2007). Phloridzin (phloretin 2′-*O*-*β*-D-glucoside) was the prevalent dihydrochalcone, but phloretin 2-*O*-xylosylglucoside was also present. The dihydrochalcone content ranged from 50 to 100 mg/100 g FW (Fig. 2b). In the group of hydroxycinnamic acids, chlorogenic acid showed the highest

Table 1 Optimised conditions for assays of main enzymes of the flavonoid pathway in apple peel

Enzyme	Crude extract after gel chromatography (µl)	Substrate(s)	µl	Cofactor and cosubstrate solutions	µl	Buffer	Buffer (µl)	Final volume (µl)
PAL	40	(^{14}C)-phenylalanine (0.063 nmol, 548 Bq)	5	–		A	55	100
CHS/CHI	40	*p*-CuCoA (1 nmol)	5	–		B	50	100
		(^{14}C)-malonyl-CoA (1.5 nmol, 1300 Bq)	5					
FHT	60	(^{14}C)- naringenin	–	2-oxoglutarate (1.46 mg ml^{-1})	5	C	30	100
		(0.036 nmol, 100 Bq)		$FeSO_4 \times 7\ H_2O$ (1.46 mg ml^{-1})	5			
FLS	40	(^{14}C)-dihydrokaempferol	–	2-oxoglutarate (1.46 mg ml^{-1})	5	D	50	100
		(0.036 nmol, 100 Bq)		$FeSO_4 \times 7\ H_2O$ (1.46 mg ml^{-1})	5			
DFR	20	(^{14}C)-dihydroquercetin (0.036 nmol, 100 Bq)	–	NADPH (41.86 mg ml^{-1})	5	E	25	50
POX	5	*O*-dianisidin (0.410 nmol)	10			F	1,095	1,110

concentration followed by 4-*O*-*p*-coumaroylquinic acid. Caffeic acid was only present in low amounts (data not shown). The total hydroxycinnamic content was below 50 mg/100 g FW (Fig. 2a).

No statistically significant differences in the contents of hydroxycinnamic acids, dihydrochalcones, flavan 3-ols, and flavonols were found in the apple peel of the susceptible and the resistant cultivar, with the exception of sampling date 2, where the flavonol and flavanol content in the peel of the resistant cultivar was higher than in the peel of the susceptible cultivar (Fig. 2c, d).

The most striking difference in the polyphenol composition of the two cultivars was the increasing anthocyanin content in ripe 'Florina' fruits. Cyanidin-3-*O*-galactoside was the major anthocyanin present in apple skin. Even in ripe fruits, the anthocyanin content in the skin remained below 10 mg/100 g FW and was therefore quite low compared to other polyphenol classes. Although the red colour occurs at late developmental stages in the apple skin, anthocyanins were found also in the early stages of 'Florina' (Fig. 2e).

The protein content was highest at the latest developing stages and lowest in young fruitlets. On none of the sampling dates, statistically significant differences in the protein content were observed between the healthy skin of the resistant and the susceptible cultivars (Fig. 3). PAL activity was undetectable at the first sampling date in all analysed tissues. In healthy peel of the susceptible and the resistant variety, there was no significant difference in the enzyme activities during the experimental period (Fig. 4a). The highest activity was observed in the healthy peel of the resistant cultivar on the second sampling date and decreased on the fourth and fifth sampling dates in comparison to susceptible cultivar's healthy peel. CHS/CHI activity had comparable values in the healthy peel of both cultivars (Fig. 4b). A statistically higher FHT activity was detected on the fifth sampling date in healthy peel of the resistant apple cultivar, (Fig. 4c). At this sampling date, DFR activity increased 16.5-fold in the healthy peel of 'Florina' compared to the susceptible cultivar. On the third and fifth sampling dates, the activity increased in the resistant healthy peel by 0.5-fold to 0.7-fold in comparison to susceptible healthy peel.

Changes caused by the infection with *Venturia inaequalis*

At all sampling dates, the highest content of hydroxycinnamic acids was measured in the scab symptomatic spot, followed by the tissue around the spot. The effect was highest at the earliest sampling dates and decreased continuously from 3.2-fold to 2.1-fold concentrations (Fig. 2a). A statistically higher content of dihydrochalcones was observed in the scab symptomatic spot and in the surrounding tissue compared to the healthy peel (Fig. 2b). The scab symptomatic spot accumulated 2.3-fold (in ripe fruits) to 5-fold (in immature fruits) higher concentrations compared to the healthy peel. The seasonal monitoring revealed statistically highest amounts of flavan-3-ols in the scab symptomatic spot tissue in the susceptible cultivar followed by the tissue around the scab symptomatic spot (Fig. 2c). In comparison to the healthy peel, the

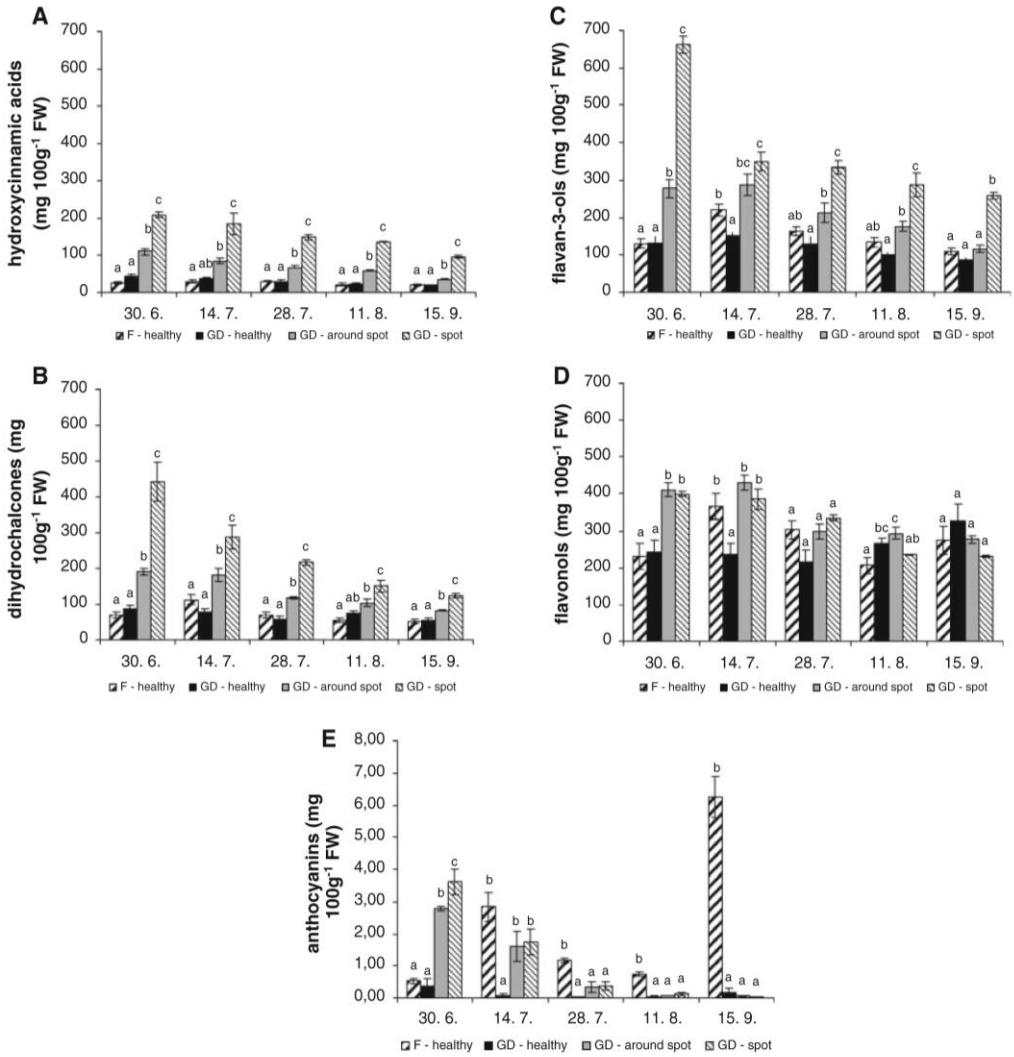

Fig. 2 Changes in phenolic compounds [means ± SE (mg 100 g^{-1} FW)] among different tissue of 'Golden Delicious' (*GD*) and 'Florina' (*F*) cultivar apple skin during fruit development. *Different letters* (*a–c*) denote statistically significant differences among different parts of peel on the same sampling time by Duncan's multiple range test at $P < 0.05$

scab symptomatic spot and the tissue around the spot contained statistically higher amounts of flavonols on the first and second sampling date. On all other sampling dates there were no statistically significant differences between different parts of apple peel (Fig. 2d). The scab symptomatic spot contained more than twofold higher contents of total flavonols compared to the healthy peel. On the first and second sampling dates there was a statistically higher amount of anthocyanins in the spot tissue and tissue around the spot compared to the healthy peel of 'Golden Delicious'. This was also visible as increased red colouration around the infection site. Later on the presence of anthocyanins decreased and in ripe fruit it was almost below detection. As expected, highest amounts of anthocyanins were detected in the red coloured apples of 'Florina' from the second sampling date onwards (Fig. 2e).

The scab symptomatic spot contained statistically highest amounts of protein. However, it remains open whether this is a result of increased plant protein formation at the infection site or due to the presence of fungal proteins. In order to keep the potential impact of fungal proteins as low as possible, all enzyme activities were expressed as per gramme of fresh tissue rather than per total protein content (specific activity).

Scab infection caused a clear increase of POX, PAL, DFR, and FHT activity on all sampling dates, whereas

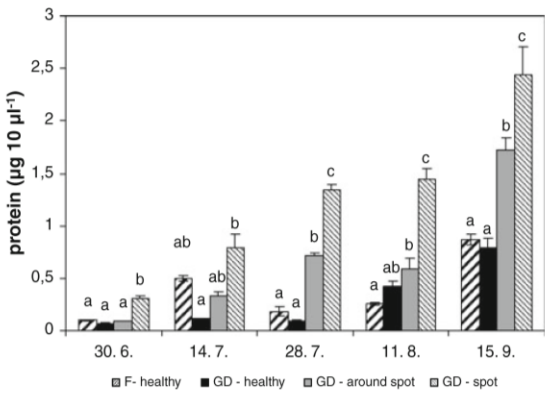

Fig. 3 Protein content [mean ± SE (µg 10 µl^{-1})] among different tissue of 'Golden Delicious' (*GD*) and 'Florina' (*F*) cultivar apple skin during fruit development. *Different letters* (*a–c*) denote statistically significant differences among different parts of peel in same sampling time by Duncan's multiple range test at $P < 0.05$

CHS and FLS activity remained more or less unchanged (Figs. 4, 5). POX activity was statistically increased in scab symptomatic spot on all monitoring dates (Fig. 5). The increase ranged from 8.0-fold to 69.0-fold in the scab symptomatic spot and from 2.1-fold to 10.0-fold in the tissue around scab symptomatic spot. From the flavonoid enzymes, the highest impact was found on the PAL activity, which was increased by 2.6-fold on the second sampling date to 21.4-fold on the fifth sampling date in the scab symptomatic spot. In the tissue around the spot, an increase by 1.2-fold on the second sampling date to 4.5-fold on the fifth sampling date was observed in comparison to the healthy peel (Fig. 4a). However, later on statistically higher activities were observed in the scab symptomatic spot. In contrast, no statistical differences (Fig. 2e) were detected in the tissue around the spot compared to the healthy peel. DFR activity increased in the scab symptomatic spot by 6.7-fold to 44.2-fold. The increase of FHT activity after scab infection was not so pronounced as for PAL and DFR, but on the first sampling date a 2.8 times higher activity was observed.

Discussion

The apple skin is the first barrier against external influences such as biotic and abiotic stress for the protection of the developing seeds in the fruits. The armamentarium is based on combined mechanical and chemical measures. Secondary metabolites contribute to both protection types. The phenylpropanoid pathway provides a wide variety of compounds including precursors of lignin, sunprotective agents and antimicrobial compounds (Dixon and Paiva 1995). These compounds may be constitutively present; others are only synthesised as direct response to stress exposure. Flavonoids are an important part of the game particularly in the defence against UV light and pathogens (Harborne and Williams 2000). The flavonoid composition in developing fruits and their modulation by external factors have been extensively reported (Treutter 2001, 2010). Although the contribution of polyphenols to apple scab resistance is largely documented (Treutter and Feucht 1990a; Picinelli et al. 1995; Michalek et al. 1999; Mikulic Petkovsek et al. 2007), the mechanism is still poorly understood. Therefore, we studied the polyphenol metabolism in the apple skin at the level of enzymes and metabolite accumulation. In the susceptible apple cultivar, healthy peel, tissue around the scab symptomatic spot and a scab symptomatic spot were investigated. The resistant cultivar did not develop scab spots and therefore only healthy peel was available.

Five polyphenol classes (Fig. 2), six related enzymes (Figs. 4, 5) and total protein (Fig. 3) content were included in the studies. PAL provides the link between the primary plant metabolism and the phenylpropanoid pathway. PAL catalyses the conversion of phenylalanine to *trans*-cinnamic acid, which is a precursor of many different phenolic compounds. The first step in the flavonoid pathway is catalysed by chalcone synthase. Three molecules of acetate-derived malonyl-CoA and one molecule of *p*-coumaroyl-CoA are condensed to generate a tetrahyhdroxychalcone as precursor for all flavonoid classes. The formation of the first real flavonoid structure is catalysed by chalcone isomerase, which is responsible for a stereospecific ring closure isomerisation step to form the 2*S*-flavanones and naringenin. The next step in the flavonoid pathway is the formation of dihydroflavonols by the stereospecific 3-hydroxylation of flavanones. The enzyme responsible, flavanone 3-hydroxylase, is a Fe^{2+}, 2-oxoglutarate-dependent dioxygenase. DFR is an NADPH-dependent oxidoreductase, which reduces the oxo-group at position 4 to provide flavan-3,4-diols, the precursors for the formation of catechins, anthocyanidins, and epicatechins (Forkmann and Heller 1999). DFR competes with FLS for the common substrates. FLS is a 2-oxoglutarate-dependent dioxygenase, introducing a double bond between the C-2 and C-3 of dihydroflavonols. POX is the key enzyme in the lignin biosynthesis (Bruce and West 1989) and it has been implicated in a number of physiological functions that may contribute to resistance including cross-linking of extension monomers and lignification (Walter 1992). They are also associated with the deposition of phenolic compounds into plant cell walls during resistance interaction (Graham and Graham 1991). In addition, they convert phenolic compounds into quinoid structures, which typically interact with proteins. Particularly, in damaged cells after pathogen attack, the loss of compartmentation results in the contact between cytosolic

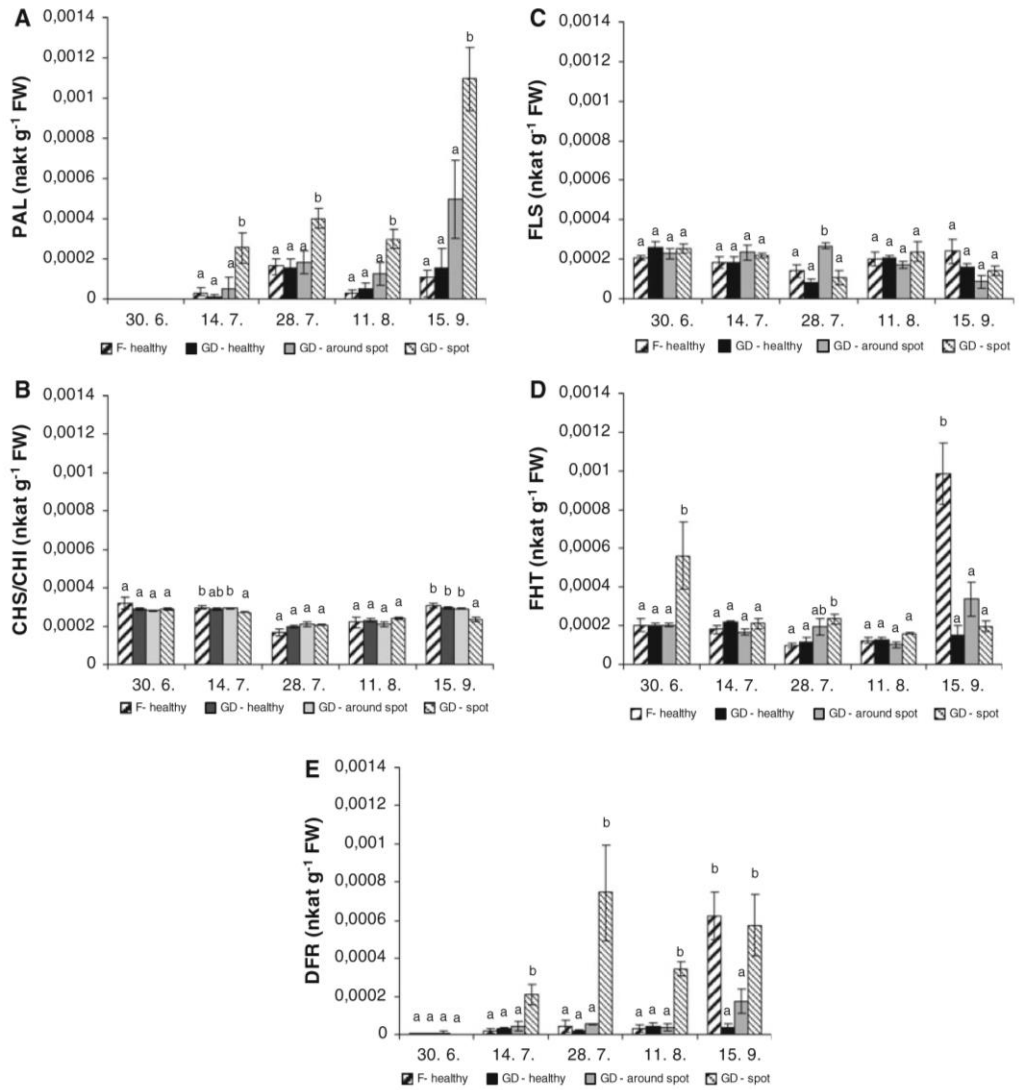

Fig. 4 Changes in enzyme activity [means ± SE (nkat g^{-1} FW)] among different tissue of 'Golden Delicious' (*GD*) and 'Florina' (*F*) cultivar apple skin during fruit development. *Different letters* (*a–b*) denote statistically significant differences among different parts of peel on the same sampling date by Duncan's multiple range test at $P < 0.05$

peroxidases and polyphenols previously stored in vacuoles. The conversion into quinoid structures may increase the defence potential of constitutively present phenolic compounds.

Polyphenol metabolism in a resistant and a susceptible apple cultivar during fruit development

A main and obvious difference of the two cultivars is the ability of red blush formation of cultivar 'Florina' in contrast to the typically yellow coloured, 'Golden Delicious'. Thus, the most striking difference in the polyphenol composition of the two cultivars was the increasing anthocyanin content in ripe 'Florina' fruits. However, it is unlikely that this is decisive for scab resistance. For apple, it was previously reported that the anthocyanin concentration shows two peaks: the first occurring in young fruitlets during cell division and the second in fully developed apples during maturity (Saure 1990) which corresponds to our results. Although the red colour occurs at late developmental stages in the apple skin, anthocyanins were found also in the early stages of 'Florina'. This could be related to the formation of epicatechin in the early stages, which requires the anthocyanidin cyanidin as

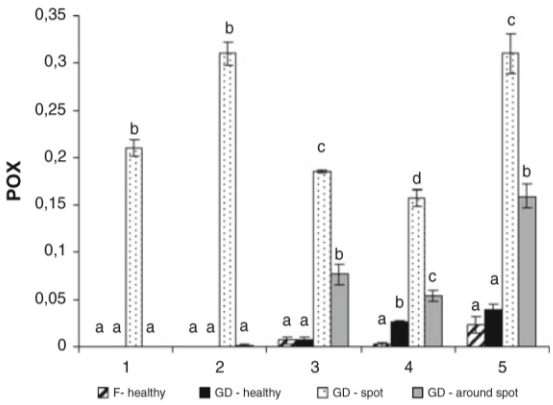

Fig. 5 Changes in POX activity [means ± SE (ΔA min^{-1})] among different tissues of 'Golden Delicious' (*GD*) and 'Florina' (*F*) cultivar apple skin during fruit development. *Different letters* (*a–d*) denote statistically significant differences between different parts of peel on the same sampling time by Duncan's multiple range test at $P < 0.05$

intermediate (Dixon et al. 2004). However, it remains then unclear why it is present as the galactoside also in the early stages and why the yellow variety 'Golden Delicious' shows significantly lower amounts although the epicatechin formation is comparable in the early stages. It could be speculated that red apple skin is dependent on the presence of a glycosyltransferase, which stabilises the anthocyanins by sugar addition in the late stages as described for grapevine (Boss et al. 1996).

The protein content was highest at the latest developing stages and lowest in young fruitlets as reported for other fruits such as guava (Bashir et al. 2003). However, the protein content measured depends strongly on the protein preparation method used and may be influenced by the presence of interfering compounds frequently present in unripe fruits (Halbwirth et al. 2009).

The statistically higher FHT activity in healthy peel of the resistant apple cultivar can be explained with higher synthesis of anthocyanins, clearly quantified on the last sampling date. The increase in the DFR activity may primarily the consequence of the anthocyanin formation in the last stages of ripening (Fig. 4e) although it can be also linked to the formation of flavan 3-ols. DFR is an NADPH-dependent oxidoreductase which reduces the oxo-group at position 4 to provide flavan-3,4-diols, the precursors for the formation of catechins, anthocyanidins, and epicatechins (Forkmann and Heller 1999).

Changes caused by the infection with *Venturia inaequalis*

The scab infection caused dramatic changes in the polyphenol metabolism at both the level of enzyme activities and local polyphenol accumulation. The effect was visible throughout the whole season. However, with the exception of flavonols, the rise in polyphenol accumulation was more pronounced in the early developmental stages. The changes in the enzyme activities were more divergent and showed higher increases—if any—in the late stages.

At all sampling dates, the highest content of hydroxycinnamic acids was measured in the scab symptomatic spot, followed by the tissue around the spot. This is probably a result of stress conditions in the plant, which are caused by the presence of *Venturia inaequalis*. Increased amounts of hydroxycinnamic acids were also reported by Mikulic Petkovsek et al. (2009) because of stress conditions.

An involvement of dihydrochalcones in pathogen defence is frequently disputed because all apple varieties generally contain high amounts of phloridzin. However, differences in disease resistance could be determined by divergent local accumulation or by differences in the speed of the oxidation cascade (hypersensitive reaction) after pathogen attack rather than the actual amount of phloridzin present in the plant (Gosch et al. 2010). MacHardy (1996) and Hrazdina et al. (1997) reported that phloridzin produced by apple in response to *Venturia inaequalis*, accumulated around the infection sites and assumed that the corresponding aglyone phloretin, which is generated by the fungus or plant glucosidases after pathogen attack, negatively affects the pathogen growth. The hypothesis that the aglycone rather than the 2′-*O*-glucoside is involved in the pathogen defence is strengthened by Slatnar et al. (2010a) who found a decrease of UDP-glucose: phloretin 2′-*O*-glycosyltransferase activity in the scab symptomatic spot compared to healthy tissue, which contrasted with the observed increase of the other flavonoid enzymes.

The role of catechin in the pathogen defence of plants might be their interaction with proteins and inhibition of the enzymes secreted by pathogenic fungi (Bors and Michel 2002). It is suggested that a rapid accumulation of catechin and oligomeric proanthocyanidins at the infection site restricting the fungal spread (Treutter and Feucht 1990b; Mayr et al. 1997).

The accumulation of high flavonol amounts in the apple skin is probably related to the physiological function of UV protection but may be also related to pathogen defence (Dixon and Paiva 1995). Mikulic Petkovsek et al. (2007) measured higher values of flavonols in scab resistant varieties compared to the susceptible ones which was also confirmed by our studies. Pathogenic invasion may explain higher quercetin levels in infected tissues, because it is known that flavonols are synthesised by plants in response to pathogen attack (Dixon and Paiva 1995). In contrast, Picinelli et al. (1995) found no relation between flavanol levels and scab resistance.

These results confirmed our first reports on ripe apple fruit (Slatnar et al. 2010a, b). Increased PAL activity was also observed during plant pathogen interaction (Fuenfgelder et al. 1994; Vidhyasekaran 1997; Anand et al. 2009). DFR activity was also strongly induced after scab infection, although it was undetectable on the first sampling date. This corresponds to our previous study (Slatnar et al. 2010a, b) and to Mayr et al. (1997), who suggested that a rapid accumulation of catechin and oligomeric proanthocyanidins at the infection site might have restricted the fungal spread. For rapid accumulation of phenolic compounds, higher enzyme activity might be significant. The increase in POX activity could be related to an increased lignification process as a response against the infection with *Venturia inaequalis*.

Although the scab infection caused clear changes in the polyphenol metabolism at the level of enzymes and polyphenol accumulation, there was a clear temporal discrepancy. The increased PAL and DFR activities in the late developmental stages correspond well to increased concentrations of hydroxycinnamic acids and flavan 3-ols and to decreased flavonol accumulation. However, the dramatic increase of the contents of hydroxycinnamic acids, dihydrochalcones, flavan 3-ols and anthocyanins at the early sampling dates is not reflected by the enzyme activity patterns observed. This could be because very young fruits are highly susceptible to apple scab and the infection occurred probably very early after blooming. The symptoms on the fruits developed much later; therefore, the accumulation of the phenolics at our first sampling date was probably high, meanwhile the enzyme activity was already decreasing. At this stage, it also cannot be completely excluded that discrepancy between high phenolic content and low corresponding enzyme activity is due to the presence of disturbing metabolites in the young fruits, although the gel chromatography step performed during the enzyme preparation should largely remove such molecules. Alternatively, the possibility of a polyphenol transport from other tissues to infection sites should be considered and investigated in future. In further studies, this should be investigated more in detail.

Conclusion

Many different studies reported higher amounts of phenolic compounds in resistant cultivars, but in this study, the polyphenol accumulation and activities of related enzymes were comparable. It could be clearly shown that the polyphenol metabolism is strongly activated in the apple skin of the cultivar 'Golden Delicious' and that this effect is specifically restricted to the infection site. In addition, it could be shown for the first time that the polyphenol accumulation is much higher at the beginning of the season. However, it is obvious that the strong defence reaction is not enough to overcome the disease. The scab resistance of cv. 'Florina' is based on the presence of the V_f gene, which results in gene-for-gene interactions involving a plant receptor protein that recognises a pathogen elicitor (Gessler and Pertot 2011). It is possible that this induces a spectrum of defence mechanisms, which also includes polyphenol-based defence. The availability of cisgenic Gala trees (Gessler and Pertot 2011), which differ from their parent plant only in the presence of the V_f gene, will allow studying as to what extent the polyphenol metabolism is involved in this type of scab resistance.

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2.4 VPLIV EKOLOŠKE/INTEGRIRANE PRIDELAVE NA VSEBNOST FENOLNIH SNOVI V LISTIH IN PLODOVIH ŠTIRIH RAZLIČNIH SORT JABLANE V OBDOBJU 2 LET

MIKULIČ-PETKOVŠEK Maja, SLATNAR Ana, ŠTAMPAR Franci, VEBERIČ Robert
The influence of organic/integrated production on the content of phenolic compounds in apple leaves and fruits in four different varieties over a 2-year period.
Journal of the Science of Food and Agriculture, 2010; 90: 2366–2378
Prejeto: 30.4.2010, sprejeto: 20.6.2010

Na vsebnost fenolov v sadju lahko vpliva sadna vrsta, rastni in klimatski dejavniki, rastna sezona, skladiščni pogoji in industrijski postopki. Namen študije je bil primerjati vsebnost posameznih fenolov, skupnih fenolov in antioksidativnega potenciala pri štirih različnih sortah, ki smo jih tekom dveh let pridelovali na ekološki oziroma integriran način pridelave. Določili smo številne fenolne snovi, ki pripadajo skupinam hidroksicimetnih kislin, flavanolov, dihidrohalkonov in antocianov. Vsebnost vseh analiziranih skupin je bila v ekološko pridelanih listih in plodovih večja (ne vedno statistično) kot v listih in plodovih iz IP. Listi iz ekološke pridelave so imeli 10-20 % višjo vsebnost skupnih fenolov, kot listi iz IP. V obeh letih je imela kožica ekološko pridelanih jabolk večji antioksidativni potencial kot jabolka iz IP, višja pa je bila tudi vsebnost skupnih fenolov. Raziskava je med drugim pokazala tudi to, da ima sorta velik vpliv na vsebnost fenolnih snovi in antioksidativni potencial jabolk.

Research Article

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The influence of organic/integrated production on the content of phenolic compounds in apple leaves and fruits in four different varieties over a 2-year period

Maja Mikulic Petkovsek,* Ana Slatnar, Franci Stampar and Robert Veberic

Abstract

BACKGROUND: Phenolics in fruit may be influenced by the fruit type, cultivation conditions of a plant, climatic conditions, growing season, storage conditions, and industrial processing. The aim of this study was to compare single phenolic compounds, total phenolics and antioxidant capacity of four varieties of apple grown under organic and integrated conditions over a 2-year period.

RESULTS: Several different phenolic compounds belonging to the groups of hydroxycinnamic acids, flavanols, dihydrochalcones, flavonols and anthocyanins were quantified. The levels of all analysed groups of phenolics were higher (not always significantly) in the organically grown apple leaves and fruit than in the leaves or apples from integrated production. Leaves from organic trees had 10–20% higher content levels of total phenols than the leaves from trees in the integrated growing system. In both years, organic production affected the increase in the antioxidant activity of apple peel. Organic apples have higher total phenolic content than the integrated grown ones.

CONCLUSION: Cultivation type had an important influence on the polyphenolic level and antioxidant capacity of the apple fruits and leaves. Apples from organic production showed a higher content of hydroxycinnamic acids, flavanols, dihydrochalcones, quercetins and total phenolics than apples from integrated cultivation.
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Keywords: *Malus domestica* (Borkh.); hydroxycinnamic acids; dihydrochalcones; flavonoids; antioxidant capacity; agricultural practice

INTRODUCTION

Apple is one of the most extensively produced and consumed fruits worldwide. It is available on the market for the whole year and, owing to its polyphenol content and dietary databases, represents a major source of intake for these compounds in the European diet.[1] Epidemiological investigations have revealed an inverse correlation between apple consumption and many chronic human diseases (cardiovascular disease, asthma and pulmonary dysfunction, diabetes and cancer).[2]

The main structural classes of apple phenolic compounds include hydroxycinnamic acids, dihydrochalcones, flavonols, catechins and oligomeric procyanidins, as well as anthocyanins in red apples.[3] Phenolics in fruit may be influenced by the fruit type (species and variety), cultivation conditions of a plant (environmental and cultivation techniques), climatic conditions, growing seasons, storage conditions, and industrial processing.[4]

In theory, differences between organic and integrated cultivation may lead to enhanced content levels of phenolics in organically produced food. Many studies have shown the importance of fertilization for the phenolic content.[5,6] Thus differences in fertilization practices may affect the phenolic content. In organic farming, nutrients are supplied through compost, manure and plant-derived byproducts. Organic nitrogen is transformed into the inorganic form by soil microflora, so the level of nutrient availability to plants may be difficult to control. Lower nitrogen availability leads to increased synthesis of phenylpropanoids.[7] It is known that injuries in plant tissue influence the metabolism of pre-formed antifungal agents and increase the activity of some enzymatic systems. Phenolic compounds are involved in plant defence against biotic stress factors[8] and increased disease pressure due to the lack of pesticide use, which could elevate the phenolic content of organically produced fruit.[3]

Consumer awareness of the relationship between food and health, together with environmental concerns, has led to an increased demand for organically produced food. Consumer studies have shown multiple reasons for organic preferences, including environmental and socioeconomic concerns, taste preferences, and the belief that organic foods are healthier.[9] The last decade has seen a significantly increased interest in organic food production, since conventional methods have caused various environmental problems.[10]

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The available data on the effect of cultivation system on phenolic content of fruit and vegetables give controversial results.[9] These are difficult to interpret, because growing conditions varied widely and different methods of sampling and analysis were used. Recent studies compared secondary metabolites with total antioxidant capacity using various methods, but the results showed large differences in values, probably reflecting different extraction methods, apple cultivars, growing regions, soil type and different durations of light exposure. There are limited numbers of studies comparing organically and conventionally grown fruit (or integrated practices with controlled fertilizer and pesticide use). These results are also difficult to compare, because of several methodological problems. The majority of these studies avoided assessing levels of secondary plant metabolites because their role in human health was not yet appreciated.[11]

The aim of this study was to compare the single phenolic compounds, total phenolics and antioxidant capacity of four varieties of apple grown under organic and integrated conditions over a 2-year period. A limited number of papers[12,13] address this issue only for selected phenolics in apple fruit or for one cultivar only, or for differing growing locations, while our research includes all major phenolic compounds as total phenolics present in apple leaves and fruit in the same orchard (the same soil, climate conditions and length of insolation). This was done on fruit at their technological maturity in the scab-resistant cultivars 'Florina' and 'Topaz', as well as on the scab-susceptible cultivars 'Crown Prince Rudolf' and 'Reinette de Champagne', which can be grown using both cultivation techniques. During the two growing seasons, the phenolic profile in apple leaves was also monitored in both growing practices. Based on prior studies, we hypothesized that cultivation technique would have an influence on the synthesis of some phenolic compounds and alter the physiological response of the plant.

MATERIALS AND METHODS

Plant material and growing conditions

The experiment was carried out during the growing seasons of 2008 and 2009. For the experiment, leaves and fruit were taken from 9-year-old apple trees grafted on to M9 rootstock, growing at the Ljubljana location (latitude 46° 2′ N, longitude 14° 28′ E). Ten trees of each scab-resistant cultivar 'Florina' and 'Topaz', and 10 trees of the scab susceptible cultivars 'Crown Prince Rudolf' and 'Reinette de Champagne' were grown either organically or in integrated production. The resistant cultivars carry the dominant *Vf* gene for resistance to apple scab. The orchard had no irrigation system. Organic and integrated orchards were located close to each other, so that the effects of microclimate and soil type could be reduced.

Measures in the integrated orchard were taken in accordance with the integrated pest management guidelines.[14] Organic production was managed according to the European Union Council Regulation (EEC) No. 2092/91.[15]

Leaf sampling was performed six times at 21-day intervals between 30 May and 12 September. Ten fully developed, healthy leaves were collected from each tree. For each cultivar, the leaves were sampled from 10 trees on different dates. The leaves were frozen in liquid nitrogen and stored at −20 °C until the preparation of samples.

Fruit sampling was performed at technological maturity, which was evaluated using the starch iodine test. Picking dates were 20 September for 'Crown Prince Rudolf' and 'Reinette de Champagne' (143 days after full bloom (DAFB)), 26 September for 'Topaz' (149 DAFB) and 10 October for 'Florina' (163 DAFB). Immediately after harvest, the fruit (peel and pulp separately) were frozen in liquid nitrogen and stored at −20 °C until preparation of the samples.

Chemicals

The following standards were used for the quantification of phenolic compounds: chlorogenic acid (5-caffeoylquinic acid), phloretin and rutin (quercetin-3-*O*-rutinoside) from Sigma-Aldrich Chemie (Steinheim, Germany), ferulic acid, (−)-epicatechin, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, *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 extraction of phenolics was acquired from Sigma. Chemicals for the mobile phases were 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 total phenolic content, Folin–Ciocalteu phenol reagent (Fluka Chemie GmbH), sodium carbonate (Merck, Darmstadt, Germany), gallic acid and ethanol (Sigma) were used. For the antioxidant capacity 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid and methanol were purchased from Sigma.

Extraction and determination of individual phenolic compounds

The frozen leaves were lyophilized and ground to a fine powder in a mortar chilled with liquid nitrogen. Extraction with some modification was done as described by Mikulic Petkovšek *et al.*[16] The fine powder (40 mg) was extracted with methanol (3 mL) containing 1% 2.6-di-*tert*-butyl-4-methylphenol (BHT) for 30 min in a cooled water bath using sonification. BHT was added to the samples to prevent oxidation during extraction. There was no interference with the extracted phenols during the subsequent HPLC analysis, because it was eluted at the end of the gradient or in the equilibration delay between the two analyses.

The apples were peeled with a mechanical peeler, and the peel was separated from the pulp. The extraction of fruit samples (peel and pulp separately) was done as described by Mikulic Petkovsek *et al.*,[4] with some modification. Fresh apple samples were ground to a fine powder in a mortar chilled with liquid nitrogen. The samples of 10 g pulp or 5 g peel were extracted with 10 mL methanol containing 1% (v/v) formic acid and 1% (w/v) BHT in a cooled ultrasonic bath for 1 h.

After extraction, the leaf or fruit extracts were centrifuged for 10 min at 10 000 rpm. The supernatant was filtered through the Chromafil AO-45/25 polyamide filter produced by Macherey-Nagel and transferred to a vial prior into injection into the HPLC system.

The polyphenolics were analysed on a Thermo Finnigan Surveyor HPLC system with a diode array detector, at 280, 350 and 530 nm. The hydroxycinnamic acids (chlorogenic, caffeic and 4-*O*-*p*-coumaroylquinic acids), the monomeric flavan 3-ols (catechin, epicatechin, procyanidin B1, B2) and dihydrohalcones (phloretin, phloretin-2-*O*-xylosylglucoside) were detected at 280 nm, whereas phloridzin, quercetin-3-*O*-rutinoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-arabinofuranoside, quercetin-3-*O*-arabinpyranoside and quercetin hexoside were estimated at 350 nm and anthocyanins (cyanidin galactoside, cyanidin-3-arabinoside, peonidin-3-galactoside, cyanidin-7-arabinoside, cyanidin-3-xyloside) were analysed at 530 nm. Spectra of the compounds were recorded between 200 and 600 nm. The column was

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a Gemini C_{18} (150 × 4.6 mm, 3 µm; Phenomenex, USA) operated at 25 °C. 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.*,[17] with an injection amount of 20 µL and a flow rate of 1 mL min^{-1}.

The identification of compounds was achieved by comparing retention times and spectra as well as by adding the standard solution to the sample. All phenolic compounds were also confirmed using a mass spectrometer (Thermo Scientific, LCQ Deca XP MAX) with an electrospray interface (ESI) operating in negative ion mode. Analysis was carried out using MS/MS scanning from *m/z* 115 to 1000.

Concentrations of phenolic compounds were calculated from the peak areas of the sample and the corresponding standards. The concentrations were expressed as mg g^{-1} dry weight (DW) for leaves and as mg kg^{-1} fresh weight (FW) for fruit peel or pulp. For compounds lacking standards, quantification was carried out using similar compounds as standards. Thus 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 (peonidin-3-galactoside, cyanidin-7-arabinoside, cyanidin-3-xyloside) were quantified in equivalents of cyanidin-3-galactoside. Procyanidins, which in our extracts were found to be represented by dimers and trimers, were quantified in equivalents of procyanidin B2.

Determination of total phenolic content

The extraction of fruit or leaf samples for the determination of total phenolics was made according to the same protocol as for phenolics, with the difference that no BHT was added. The total phenolic content of extracts was assessed using the Folin–Ciocalteu phenol reagent method.[18] To 100 µL of the sample extracts, 6 mL bidistilled water and 500 µL Folin–Ciocalteu reagent were added; after resting between 8 s and 8 min at room temperature, 1.5 mL sodium carbonate (20% w/v) was added. The extracts were mixed and allowed to stand for 30 min at 40 °C before measuring the absorbance on a spectrophotometer (Perkin Elmer, UV/visible Lambada 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 g^{-1} DW of leaf tissue and in mg kg^{-1} FW of fruit peel or pulp. Absorption was measured in three replications.

Determination of antioxidant activity with the DPPH radical scavenging method

The extraction of fruit samples for the determination of antioxidant activity was made according to the same protocol as for total phenolics. The free radical scavenging activity of apple extracts was measured according to the DPPH method reported by Brand-Williams *et al.*,[19] with some modifications. A methanolic solution (50 µL) of extract was placed in 96-well microplates, and 200 µL of a 0.1 mmol L^{-1} methanolic solution of DPPH was added and allowed to react in the dark at room temperature. The decrease in absorbance of DPPH at 520 nm was measured at 5 min intervals by spectrophotometer (MRX Dynex Technologies), until absorbance stabilized (30 min). Methanol was used as blank solution, and a DPPH solution without test samples served as the control. All sample analyses were performed in triplicate. The DPPH radical scavenging activity of apple methanolic extracts was expressed as milligrams of ascorbic acid equivalents per kilogram of fresh peel or pulp (AEAC = ascorbic acid equivalent antioxidant capacity) in 30 min of reaction time. Identification of the antioxidant capacities of the samples at various concentrations was made using the standard curves of ascorbic acid.

Statistical analysis

The data were analysed using the Statgraphics Plus 4.0 program (Manugistics Inc., Rockville, MD, USA). Data from the chemical analyses were tested for any differences between organic and integrated production using one-way analysis of variance (ANOVA). Significant differences between means were determined by Student's *t*-test, with a significance level of 0.05. Multivariate statistical analysis (hierarchical cluster analysis, discriminate analysis and classification) was conducted in order to interpret the differences in phenolic compounds amongst apple cultivars and cultivation techniques. Ward's method based on square Euclidian distance was used to interpret the differences in total phenolic content and antioxidant capacity in peel.

RESULTS AND DISCUSSION

In the four apple cultivars we have identified 19 phenolics belonging to various groups (Tables 1–4 and Figs 1 and 2). Tables 1–4 present the content levels of various individual phenolics, total phenolics and antioxidant capacity in apple pulp and peel in two types of production.

The total content of hydroxycinnamic acids in apple leaves is presented in Fig. 1(A, B). Chlorogenic acid represents the greatest amount of hydroxycinnamic acids in apple leaves (~55–65%). Mayr *et al.*[20] also observed that the content of chlorogenic acid increased during seasonal development in the leaves of 'Golden Delicious'; it reached its peak in older leaves, and then content levels slowly decreased. From Fig. 1(A,B) it can be seen that organic production led to higher content levels of hydroxycinnamic acid in apple leaves. Such a trend was particularly noticed in 2009 when the leaves from organic production on the majority of dates had statistically higher content levels of hydroxycinnamic acids in comparison to the leaves from integrated production. The difference in their content between both production types increased over the growth period: the September leaves from organic production had levels of hydroxycinnamic acids three times higher than the leaves from integrated production. The increased content of hydroxycinnamic acids in 2009 compared to the previous year may be attributed to a higher number of insolation hours and lower quantity of precipitation, which probably caused stress in the plants. It is known that hydroxycinnamic acids increase under stress conditions.[8]

In the fruit pulp and peel, we found the following hydroxycinnamic acids: chlorogenic, 4-*O*-*p*-coumaroylquinic and caffeic acids. In flesh, chlorogenic acid was found at the highest mean concentrations (24–180 mg kg^{-1} FW) in almost all the samples included in the study and represented 17–42% of the total HPLC polyphenolics. The results show that in 2008 the fruit (skin and pulp) of the 'Florina' and 'Reinette de Champagne' cultivars from organic production had significantly higher content levels of total hydroxycinnamic acids compared to the fruit from integrated production. Organic production caused an increase in the pulp by 1.1–2.6 times and in the skin by 1.2–3.2 times in comparison to integrated production. It is interesting that there was no response from the 'Crown Prince Rudolf' cultivar in any year to two different

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Table 1. Content of individual phenolic compounds (mean ± SE, mg kg^{-1} FW), total phenolic content (mean ± SE, mg GAE kg^{-1} FW) and antioxidant capacity (mean ± SE, mg ascorbic acid kg^{-1} FW) in pulp of four apple cultivars in 2008

	Florina			Topaz			Reinette de Champagne			Crown Prince Rudolf		
Compound	I	O	Sig.	I	O	Sig.	I	O	Sig.	I	O	Sig.
Chlorogenic acid	33 ± 2.2	109 ± 11	***	51 ± 0.9	54 ± 0.6	NS	151 ± 3.6	176 ± 4.7	**	37 ± 3.6	43 ± 4.1	NS
Caffeic acid	18 ± 1.8	28 ± 2.3	*	0.7 ± 0.02	1.3 ± 0.07	***	4.0 ± 0.13	5.6 ± 0.57	*	3.9 ± 0.25	3.8 ± 0.18	NS
4-*p*-Coumaroylquinic acid	1.9 ± 0.2	4.2 ± 0.4	**	1.6 ± 0.10	2.2 ± 0.08	**	9.6 ± 0.55	13.2 ± 1.53	NS	4.8 ± 0.18	3.7 ± 0.16	*
Total hydroxycinnamic acids	**53 ± 3.7**	**138 ± 15.3**	**	**53 ± 0.9**	**57 ± 0.4**	*	**165 ± 4.0**	**192 ± 8.3**	*	**46 ± 4.0**	**51 ± 4.6**	**NS**
Catechin	15 ± 2.6	45 ± 2.3	***	7.6 ± 0.11	9.4 ± 0.34	**	19.2 ± 0.5	18.5 ± 2.6	NS	21.8 ± 1.5	17.8 ± 0.4	NS
Epicatechin	39 ± 4.7	17 ± 2.8	**	32 ± 0.4	32 ± 0.4	NS	18.9 ± 0.9	27.3 ± 3.9	NS	25.6 ± 1.7	27.3 ± 1.9	NS
Procyanidin B1	10 ± 1.9	15 ± 3.5	NS	5.8 ± 0.17	8.1 ± 0.51	*	5.6 ± 0.42	6.1 ± 1.21	NS	6.8 ± 0.95	7.5 ± 1.46	NS
Procyanidin B2	39 ± 2.2	26 ± 3.7	*	46 ± 0.6	47 ± 0.7	NS	30.7 ± 2.1	42.6 ± 4.3	*	30.2 ± 1.2	34.8 ± 2.2	NS
Procyanidin Bx	3.9 ± 0.12	2.6 ± 0.53	NS	2.8 ± 0.24	4.3 ± 0.14	*	3.4 ± 0.85	6.8 ± 0.76	*	5.0 ± 0.23	6.0 ± 0.16	NS
Procyanidin trimer	12 ± 1.1	13 ± 2.5	NS	13 ± 1.0	16 ± 0.2	NS	11.8 ± 1.9	19.8 ± 1.8	*	15.2 ± 1.1	14.4 ± 1.0	NS
Total flavanols	**124 ± 7.2**	**132 ± 12.7**	**NS**	**106 ± 2.2**	**119 ± 2.6**	*	**88 ± 5.3**	**130 ± 16**	*	**111 ± 2.7**	**94 ± 8.1**	**NS**
Phloretin 2-xylosylglucoside	2.6 ± 0.12	2.7 ± 0.32	NS	1.7 ± 0.08	2.1 ± 0.04	*	8.0 ± 0.37	9.2 ± 0.80	NS	16.2 ± 1.1	16.4 ± 1.2	NS
Phloridzin	8.7 ± 0.62	7.8 ± 0.66	NS	4.5 ± 0.21	5.4 ± 0.21	*	12.7 ± 0.49	19.1 ± 1.48	**	6.3 ± 0.15	5.8 ± 0.34	NS
Total dihydrochalcones	**11.4 ± 0.6**	**10.6 ± 0.9**	**NS**	**6.3 ± 0.19**	**7.5 ± 0.22**	*	**20.7 ± 2.1**	**28.3 ± 2.0**	**	**23.1 ± 0.8**	**22.3 ± 1.5**	**NS**
Quercetin 3-galactoside	0.11 ± 0.03	0.08 ± 0.02	NS	0.12 ± 0.008	0.15 ± 0.01	NS	0.05 ± 0.004	0.06 ± 0.01	NS	0.31 ± 0.07	0.38 ± 0.15	NS
Quercetin 3-glucoside	0.17 ± 0.02	0.17 ± 0.02	NS	0.09 ± 0.01	0.11 ± 0.02	NS	0.37 ± 0.03	0.39 ± 0.06	NS	0.41 ± 0.05	0.26 ± 0.07	NS
Quercetin 3-xyloside	0.70 ± 0.07	0.37 ± 0.06	*	0.14 ± 0.01	0.16 ± 0.06	NS	0.52 ± 0.04	0.91 ± 0.17	NS	0.27 ± 0.03	0.25 ± 0.03	NS
Quercetin 3-arabinofuranoside	0.33 ± 0.01	0.33 ± 0.009	NS	0.21 ± 0.007	0.28 ± 0.02	NS	0.34 ± 0.03	0.48 ± 0.05	NS	0.12 ± 0.01	0.12 ± 0.03	NS
Quercetin 3-rhamnoside	2.7 ± 0.35	1.7 ± 0.17	NS	0.71 ± 0.02	0.93 ± 0.02	***	1.0 ± 0.04	1.6 ± 0.20	*	0.90 ± 0.06	0.88 ± 0.08	NS
Total flavonols	**3.9 ± 0.52**	**2.7 ± 0.22**	**NS**	**1.3 ± 0.05**	**1.7 ± 0.05**	**	**2.2 ± 0.20**	**3.7 ± 0.64**	*	**1.9 ± 0.22**	**1.8 ± 0.36**	**NS**
Total phenolic content[a]	**277 ± 6.6**	**343 ± 6.9**	***	**226 ± 7.7**	**241 ± 9.7**	**NS**	**259 ± 13**	**346 ± 22**	*	**227 ± 13**	**328 ± 13**	**
Antioxidant capacity[b]	362 ± 2.2	365 ± 1.2	NS	362 ± 0.6	364 ± 0.8	NS	364 ± 0.2	364 ± 0.1	NS	349 ± 1.6	355 ± 1.2	NS

[a] TPC, total phenolic content determined by Folin–Ciocalteu method (mg gallic acid kg^{-1} FW).
[b] AEAC, ascorbic acid equivalent antioxidant capacity (mg ascorbic acid kg^{-1} FW).
NS, not significant; Sig., significance.
Asterisks indicate statistically significant differences at: * *P*-value below 0.05; ** *P*-value below 0.01; *** *P*-value below 0.001.

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Table 2. Content of individual phenolic compounds (mean ± SE, mg kg^{-1} FW), total phenolic content (mean ± SE, mg GAE kg^{-1} FW) and antioxidant capacity (mean ± SE, mg ascorbic acid kg^{-1} FW) in pulp of four apple cultivars in 2009

	Florina			Topaz			Reinette de Champagne			Crown Prince Rudolf		
Compound	I	O	Sig.	I	O	Sig.	I	O	Sig.	I	O	Sig.
Chlorogenic acid	24.3 ± 2.4	27.4 ± 4.0	NS	63 ± 1.5	45 ± 2.6	**	180 ± 2.7	179 ± 4.4	NS	26.0 ± 1.3	26.4 ± 1.9	NS
Caffeic acid	23.4 ± 1.7	21.1 ± 1.0	NS	1.6 ± 0.02	1.1 ± 0.18	NS	5.4 ± 0.10	4.2 ± 0.25	**	3.4 ± 0.14	3.3 ± 0.22	NS
4-*p*-Coumaroylquinic acid	1.3 ± 0.13	2.0 ± 0.09	*	2.9 ± 0.14	1.4 ± 0.22	NS	11.4 ± 0.66	11.9 ± 0.10	NS	3.6 ± 0.73	4.0 ± 0.21	NS
Total hydroxycinnamic acids	**52 ± 3.8**	**50 ± 5.0**	**NS**	**67 ± 1.8**	**48 ± 3.0**	******	**201 ± 4.7**	**196 ± 4.1**	**NS**	**33.6 ± 0.4**	**33.9 ± 2.2**	**NS**
Catechin	22.8 ± 0.6	21.0 ± 0.5	NS	5.7 ± 0.43	3.7 ± 0.50	*	7.6 ± 0.32	10.3 ± 0.7	*	3.9 ± 0.96	2.9 ± 0.34	NS
Epicatechin	32.1 ± 1.4	28.7 ± 0.7	NS	31.2 ± 0.9	38.2 ± 1.6	**	26.9 ± 1.4	32.7 ± 2.4	NS	22.7 ± 1.5	27.5 ± 0.1	*
Procyanidin B1	9.9 ± 0.41	9.6 ± 0.27	NS	7.9 ± 0.17	7.2 ± 0.41	NS	12.1 ± 0.9	12.6 ± 1.4	NS	5.9 ± 0.32	6.6 ± 0.24	NS
Procyanidin B2	33.1 ± 1.3	29.2 ± 2.8	NS	52.1 ± 1.1	54.0 ± 2.0	NS	33.7 ± 1.9	33.1 ± 2.6	NS	21.4 ± 1.2	33.0 ± 0.3	**
Procyanidin Bx	2.3 ± 0.31	2.8 ± 0.55	NS	7.5 ± 1.26	3.7 ± 0.82	NS	4.4 ± 0.25	3.1 ± 3.4	*	2.2 ± 0.29	3.1 ± 0.09	*
Procyanidin trimer	9.3 ± 1.0	9.2 ± 1.89	NS	19.7 ± 1.7	13.7 ± 2.1	NS	14.9 ± 0.7	15.2 ± 1.2	NS	8.2 ± 1.21	9.7 ± 1.35	NS
Total flavanols	**110.4 ± 2.6**	**102 ± 4.7**	**NS**	**119 ± 8.9**	**112 ± 8.1**	**NS**	**99.6 ± 3.9**	**89.3 ± 8.3**	**NS**	**65.0 ± 3.8**	**84.1 ± 2.9**	*****
Phloretin 2-xylosylglucoside	2.4 ± 0.13	2.1 ± 0.17	NS	1.7 ± 0.06	1.5 ± 0.09	NS	9.2 ± 0.42	10.6 ± 0.36	NS	6.3 ± 0.88	10.0 ± 1.16	*
Phloridzin	7.7 ± 0.31	6.8 ± 0.68	NS	4.7 ± 0.25	3.8 ± 0.23	NS	16.4 ± 0.88	18.9 ± 1.57	NS	3.1 ± 0.38	3.9 ± 0.07	NS
Total dihydrochalcones	**10.1 ± 0.38**	**9.0 ± 0.84**	**NS**	**6.6 ± 0.32**	**5.5 ± 0.32**	**NS**	**26.4 ± 1.4**	**28.2 ± 1.8**	**NS**	**7.7 ± 0.23**	**13.9 ± 1.2**	******
Quercetin 3-galactoside	0.12 ± 0.011	0.10 ± 0.14	NS	0.10 ± 0.023	0.03 ± 0.004	*	0.05 ± 0.011	0.13 ± 0.010	**	0.1 ± 0.036	0.02 ± 0.012	NS
Quercetin 3-glucoside	0.28 ± 0.025	0.22 ± 0.037	NS	0.02 ± 0.003	0.02 ± 0.003	NS	0.49 ± 0.045	0.76 ± 0.018	**	0.09 ± 0.038	0.06 ± 0.026	NS
Quercetin 3-xyloside	0.05 ± 0.020	0.08 ± 0.024	NS	0.16 ± 0.008	0.22 ± 0.021	NS	0.64 ± 0.055	1.08 ± 0.055	**	0.48 ± 0.152	0.15 ± 0.019	**
Quercetin 3-arabinofuranoside	0.40 ± 0.035	0.26 ± 0.027	*	0.15 ± 0.082	0.26 ± 0.042	NS	0.03 ± 0.036	0.53 ± 0.048	*	0.10 ± 0.01	0.11 ± 0.01	NS
Quercetin 3-rhamnoside	3.0 ± 0.14	2.2 ± 0.17	*	0.02 ± 0.003	0.02 ± 0.003	NS	1.4 ± 0.09	1.7 ± 0.09	NS	0.69 ± 0.066	0.74 ± 0.023	NS
Total flavonols	**4.0 ± 0.17**	**2.8 ± 0.23**	*****	**1.1 ± 0.09**	**1.1 ± 0.07**	**NS**	**3.1 ± 0.13**	**4.2 ± 0.15**	******	**1.4 ± 0.05**	**1.1 ± 0.05**	*****
Total phenolic content[a]	**216 ± 6.5**	**208 ± 23**	**NS**	**262 ± 20**	**519 ± 24**	*******	**362 ± 3**	**380 ± 15**	**NS**	**174 ± 9**	**494 ± 3**	*******
Antioxidant capacity[b]	365 ± 0.1	366 ± 0.5	NS	335 ± 16.7	309 ± 11.2	NS	364 ± 0.4	365 ± 0.4	NS	351 ± 5.3	288 ± 10.4	**

Different letters between organic (O) and integrated (I) production within one cultivar denote significant differences (Student's *t*-test, $P < 0.05$).
[a] TPC, total phenolic content determined by Folin–Ciocalteu method (mg gallic acid kg^{-1} FW).
[b] AEAC, ascorbic acid equivalent antioxidant capacity (mg ascorbic acid kg^{-1} FW).
NS, not significant; Sig. significance.
Asterisks indicate statistically significant differences at: * *P*-value below 0.05; ** *P*-value below 0.01; *** *P*-value below 0.001.

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Table 3. Content of individual phenolic compounds (mean ± SE, mg kg^{-1} FW), total phenolic content (mean ± SE, mg GAE kg^{-1} FW) and antioxidant capacity (mean ± SE, mg ascorbic acid kg^{-1} FW) in peel of four apple cultivars in 2008

	Florina			Topaz			Reinette de Champagne			Crown Prince Rudolf		
Compound	I	O	Sig.	I	O	Sig.	I	O	Sig.	I	O	Sig.
Chlorogenic acid	165 ± 19	576 ± 38	***	144 ± 17	129 ± 8	NS	218 ± 10	279 ± 9.9	**	119 ± 13	124 ± 21	NS
Caffeic acid	18 ± 0.7	31 ± 2.2	**	13 ± 0.9	14 ± 0.9	NS	14 ± 1.0	18 ± 0.9	*	14 ± 1.3	13 ± 1.5	NS
4-*p*-Coumaroylquinic acid	15 ± 0.2	23 ± 0.6	***	10 ± 0.7	12 ± 0.6	NS	12 ± 1.5	16 ± 1.7	NS	13 ± 1.6	16 ± 0.1	NS
Total hydroxycinnamic acids	**198 ± 19**	**631 ± 36**	***	**181 ± 16**	**144 ± 14**	**NS**	**246 ± 13**	**317 ± 13**	**	**146 ± 15**	**157 ± 23**	**NS**
Catechin	222 ± 13	556 ± 32	***	156 ± 18	187 ± 5.6	NS	69 ± 3.8	96 ± 1.4	***	182 ± 15	154 ± 26	NS
Epicatechin	270 ± 11	205 ± 04	**	152 ± 6.2	178 ± 13	NS	95 ± 10	102 ± 7.0	NS	208 ± 7.5	170 ± 9.6	*
Procyanidin B1	38 ± 2	138 ± 11	***	41 ± 1.4	47 ± 2.3	NS	28 ± 2.6	37 ± 2.0	*	52 ± 6.1	89 ± 4.8	**
Procyanidin B2	266 ± 6	141 ± 28	**	198 ± 15	276 ± 17	*	163 ± 19	146 ± 16	NS	222 ± 8.2	200 ± 6.8	NS
Total flavanols	**796 ± 25**	**1079 ± 39**	***	**588 ± 48**	**659 ± 42**	**NS**	**337 ± 33**	**380 ± 24**	**NS**	**684 ± 31**	**592 ± 52**	**NS**
Phloretin 2-xylosylglucoside	100 ± 4.6	94 ± 3.6	NS	55 ± 1.0	65 ± 4.2	*	129 ± 5.8	155 ± 4.7	*	290 ± 8.7	276 ± 19	NS
Phloridzin	137 ± 4.4	104 ± 2.8	**	62 ± 2.8	79 ± 3.2	**	66 ± 7.8	72 ± 4.0	NS	153 ± 15	222 ± 23	NS
Total dihydrochalcones	**237 ± 4.4**	**197 ± 4.0**	***	**117 ± 2.4**	**139 ± 8.3**	*	**195 ± 9.5**	**226 ± 2.9**	*	**429 ± 30**	**499 ± 41**	**NS**
Quercetin 3-rutinoside	3.3 ± 0.7	29 ± 2.7	***	17 ± 3.3	19 ± 2.8	NS	44 ± 2.9	37 ± 1.5	NS	91 ± 18	110 ± 28	NS
Quercetin 3-galactoside	85 ± 10.5	197 ± 16	**	198 ± 11	179 ± 5.1	NS	139 ± 6.8	118 ± 11	NS	258 ± 17	215 ± 19	NS
Quercetin 3-glucoside	9.6 ± 1.4	81 ± 3.4	***	39 ± 5.4	40 ± 3.8	NS	57 ± 1.9	54 ± 3.2	NS	129 ± 14	120 ± 18	NS
Quercetin 3-xyloside	56 ± 5.1	98 ± 4.5	**	68 ± 6.5	75 ± 2.6	NS	67 ± 2.4	74 ± 2.1	NS	112 ± 7.3	112 ± 16	NS
Quercetin 3-arabinopyranoside	2.9 ± 0.1	2.5 ± 0.1	NS	1.6 ± 0.04	1.9 ± 0.1	NS	3.9 ± 0.15	4.8 ± 0.19	*	10.3 ± 1.0	13.1 ± 1.6	NS
Quercetin 3-arabinofuranoside	74 ± 7.3	157 ± 11	***	113 ± 8	102 ± 3.2	NS	92 ± 2.5	100 ± 4.2	NS	168 ± 7.6	144 ± 14	NS
Quercetin 3-rhamnoside	93 ± 6.5	135 ± 8.8	**	126 ± 4.5	127 ± 6.8	NS	42 ± 2.1	55 ± 0.4	**	65 ± 3.1	63 ± 12	NS
Total flavonols	**297 ± 18**	**668 ± 26**	***	**598 ± 36**	**562 ± 11**	**NS**	**431 ± 8.8**	**424 ± 30**	**NS**	**876 ± 47**	**778 ± 107**	**NS**
Cyanidin 3-galactoside	80 ± 10	342 ± 18	***	68 ± 9.2	58 ± 6.2	NS	6.4 ± 1.1	2.0 ± 0.6	*	58 ± 6.9	60.8 ± 9.9	NS
Other anthocyanins	7.6 ± 0.9	45 ± 3.6	***	4.0 ± 0.27	5.3 ± 2.95	NS	0.3 ± 0.11	0.2 ± 0.03	NS	5.8 ± 0.76	6.1 ± 1.02	NS
Total anthocyanins	**88 ± 11**	**387 ± 21**	***	**80 ± 10**	**69 ± 4.1**	**NS**	**6.7 ± 1.2**	**2.1 ± 0.6**	*	**65 ± 7.6**	**67 ± 11**	**NS**
Total phenolic content [a]	**1511 ± 24**	**1780 ± 10**	***	**1238 ± 45**	**1362 ± 41**	**NS**	**1259 ± 15**	**1601 ± 72**	**	**1719 ± 16**	**2027 ± 30**	**NS**
Antioxidant capacity [b]	1234 ± 7.7	1321 ± 1.7	***	1324 ± 1.5	1335 ± 2.2	*	1294 ± 4.2	1332 ± 2.9	***	1284 ± 40	1455 ± 37	*

Different letters between organic (O) and integrated (I) production within one cultivar denote significant differences (Student's *t*-test, $P < 0.05$).
[a] TPC, total phenolic content determined by Folin–Ciocalteu method (mg gallic acid kg^{-1} FW).
[b] AEAC, ascorbic acid equivalent antioxidant capacity (mg ascorbic acid kg^{-1} FW).
NS, not significant; Sig., significance.
Asterisks indicate statistically significant differences at: * *P*-value below 0.05; ** *P*-value below 0.01; *** *P*-value below 0.001.

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Table 4. Content of individual phenolic compounds (mean ± SE, mg kg^{-1} FW), total phenolic content (mean ± SE, mg GAE kg^{-1} FW) and antioxidant capacity (mean ± SE, mg ascorbic acid kg^{-1} FW) in peel of four apple cultivars in 2009

	Florina			Topaz			Reinette de Champagne			Crown Prince Rudolf		
Compound	I	O	Sig.	I	O	Sig.	I	O	Sig.	I	O	Sig.
Chlorogenic	235 ± 18	291 ± 16	NS	53 ± 2	157 ± 23	**	294 ± 15	287 ± 4.4	NS	31 ± 3.1	33 ± 3.5	NS
Caffeic	23 ± 0.6	34 ± 1.7	***	14 ± 1.4	57 ± 2.7	***	22 ± 1.0	22 ± 0.8	NS	25 ± 0.5	19 ± 4.1	*
4-*p*-Coumaroylquinic	45 ± 2.1	70 ± 5.5	**	35 ± 0.9	42 ± 2	*	52 ± 1.0	42 ± 0.5	**	47 ± 1.7	54 ± 1.1	*
Total hydroxycinnamic acids	**325 ± 6.7**	**387 ± 14**	*	**102 ± 4**	**252 ± 27**	**	**366 ± 14**	**355 ± 4.7**	**NS**	**109 ± 3.7**	**105 ± 4.4**	**NS**
Catechin	175 ± 5	214 ± 25	NS	69 ± 7	64 ± 3.5	NS	89 ± 18	86 ± 27	NS	95 ± 19	109 ± 38	NS
Epicatechin	266 ± 2.2	302 ± 8.4	*	114 ± 2.2	175 ± 6	**	130 ± 4.0	137 ± 12	NS	148 ± 12	163 ± 4.9	NS
Procyanidin B1	38 ± 3.9	64 ± 4.5	*	48 ± 0.8	53 ± 8	NS	67 ± 0.8	11 ± 0.9	NS	36 ± 1.6	58 ± 3.7	**
Procyanidin B2	184 ± 15	313 ± 19	**	188 ± 4.4	197 ± 9	NS	193 ± 2.0	188 ± 7.8	NS	206 ± 19	268 ± 5.4	*
Total flavanols	**683 ± 45**	**952 ± 69**	*	**456 ± 8**	**504 ± 14**	**	**476 ± 17**	**467 ± 59**	**NS**	**511 ± 41**	**591 ± 33**	*
Phloretin 2-xylosylglucoside	58 ± 2.9	66 ± 8.1	NS	34 ± 2.1	31 ± 1.7	NS	166 ± 4.7	180 ± 2.8	NS	256 ± 7.2	289 ± 3.9	*
Phloridzin	124 ± 7	162 ± 11	*	56 ± 3.8	59 ± 4.9	NS	88 ± 2.7	98 ± 2.1	*	99 ± 2.8	125 ± 6.7	*
Total dihydrochalcones	**192 ± 4.1**	**222 ± 6.8**	*	**89 ± 5.5**	**89 ± 6.3**	**NS**	**266 ± 9.3**	**276 ± 1.6**	**NS**	**366 ± 13**	**409 ± 6.5**	*
Quercetin 3-rutinoside	6.8 ± 0.7	8.2 ± 2.4	NS	12 ± 2.6	18 ± 2.9	NS	139 ± 9.6	73 ± 5.7	**	51 ± 2.5	103 ± 14	*
Quercetin 3-galactoside	143 ± 1.1	202 ± 26	NS	179 ± 14	203 ± 18	NS	265 ± 3.0	246 ± 14	NS	198 ± 34	264 ± 26	NS
Quercetin 3-glucoside	18 ± 1.2	17 ± 3.6	NS	38 ± 5.7	47 ± 5.7	NS	130 ± 1.8	101 ± 11	NS	88 ± 21	139 ± 15	NS
Quercetin 3-xyloside	62 ± 3.5	103 ± 7.3	**	65 ± 1.7	68 ± 7.8	NS	114 ± 2.1	136 ± 10	NS	58 ± 7.9	92 ± 5.0	*
Quercetin 3-arabinopyranoside	14 ± 1.2	22 ± 3.3	*	9 ± 1.2	9.2 ± 1.1	NS	14 ± 2.0	15 ± 2.9	NS	9.3 ± 0.4	16 ± 1.1	**
Quercetin 3-arabinofuranoside	102 ± 4.8	142 ± 13	*	92 ± 1.9	96 ± 8.9	NS	162 ± 6.7	169 ± 10	NS	80 ± 10	129 ± 6.1	*
Quercetin 3-rhamnoside	173 ± 4.3	200 ± 11	NS	186 ± 1.7	188 ± 8.7	NS	81 ± 3.9	106 ± 7.1	*	56 ± 5.6	80 ± 4.2	*
Total flavonols	507 ± 18	708 ± 68	*	588 ± 28	597 ± 54	NS	916 ± 11	941 ± 87	NS	470 ± 103	869 ± 51	*
Cyanidin 3-galactoside	134 ± 8.2	154 ± 5.4	NS	33 ± 3	89 ± 8.7	***	2.7 ± 0.5	1.7 ± 0.1	NS	17 ± 3.8	499 ± 6.9	**
Other anthocyanins	21 ± 3.9	29 ± 3.5	NS	3.4 ± 0.31	5.4 ± 1.05	NS	0.16 ± 0.02	0.37 ± 0.25	NS	2.0 ± 0.5	5.7 ± 0.4	**
Total anthocyanins	**158 ± 12**	**181 ± 3.4**	**NS**	**37 ± 3.3**	**96 ± 9.5**	**	**1.9 ± 0.4**	**1.5 ± 0.5**	**NS**	**19 ± 4.3**	**56 ± 7.4**	**
Total phenolic content [a]	**1412 ± 57**	**1567 ± 44**	**NS**	**1440 ± 37**	**1607 ± 46**	*	**1555 ± 62**	**1631 ± 84**	**NS**	**1302 ± 48**	**1618 ± 54**	**
Antioxidant capacity [b]	1397 ± 1.4	1406 ± 2	*	1406 ± 1.4	1420 ± 1.1	***	1424 ± 1.2	1423 ± 1.1	NS	1416 ± 2.5	1595 ± 7.4	**

[a] TPC, total phenolic content determined by Folin–Ciocalteu method (mg gallic acid kg^{-1} FW).
[b] AEAC, ascorbic acid equivalent antioxidant capacity (mg ascorbic acid kg^{-1} FW).
NS, not significant; Sig., significance.
Asterisks indicate statistically significant differences at: * *P*-value below 0.05; ** *P*-value below 0.01; *** *P*-value below 0.001.

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production types, since the content levels of hydroxycinnamic acids in these fruit did not differ. Similarly, in yellow plums and strawberry, individual phenolic acids were found to be systematically higher in organically grown fruit than in conventionally grown ones.[21,22] In contrast to this, in apples of the 'Golden Delicious' cultivar, higher concentrations of hydroxycinnamic acids were found in integrated than in organic production.[12] It became evident in 2008 that the 'Reinette de Champagne' cultivar was outstanding in having the highest content of hydroxycinnamic acids in fruit.

The monomeric flavanols catechin and epicatechin, and the polymeric flavanol procyanidin B2, were determined in apple leaves. The leaves from organic production had content levels of total flavanols higher by 10–38% in both years in comparison to the leaves from integrated production (Fig. 1(C,D)). This could be attributed to the fact that leaves from organic production were infected with different pathogens, which caused accumulation of flavan-3-ols in the leaves. Treutter and Feucht[23] also reported content levels of extracted monomeric flavan-3-ols in pear leaves infected with *Gymnosporangium sabinae* that were six times higher in comparison with healthy leaves. Other researchers also reported higher synthesis of flavanols when the leaves were attacked by the fungus *Venturia inaequalis*.[8,24,25]

The following flavanols were analysed in fruit: catechin, epicatechin, procyanidin B1, B2 and, additionally, in the pulp the still unknown procyanidin dimer and the unknown procyanidin trimer. As reported by several authors,[12,26] polyphenols in the flesh were almost completely represented (88–96%) by flavanols and hydroxycinnamics. Fruit of cultivar 'Reinette de Champagne' in 2008 and 'Crown Prince Rudolf' in 2009 produced organically had a statistically significant higher content of total flavanols than fruit grown in integrated production. The same effect was evident in the skin of the 'Florina' cultivar in both years, in the pulp of the 'Topaz' cultivar in 2008 and in the next year in its skin. The reason for the presence of catechins in defence mechanisms of plants might be found in the fact that flavan-3-ols may interact with proteins and inhibit the enzymes secreted by diverse pathogenic fungi.[23] Mayr *et al.*[27] reported that a rapid accumulation of catechins and oligomeric proanthocyanidins at the infection site restricted the fungal spread. Feucht *et al.*[28] indicate that epicatechin is the main flavanol synthesized during damage of fruit by the *V. inaequalis* fungus. In contrast to our results, Chinnici *et al.*[12] report higher flavanol content in integrated than in organic production.

Among dihydrochalcones in leaves, we analysed phloridzin and phloretin. The dihydrochalcones and, within this group, phloridzin (phloretin 2′-*O*-β-*D*-glucoside) are the main polyphenols in leaves, representing more than 90% of the extractable phenolics.[29] Technologically ripe fruit contains only ∼5–10% of phloridzin from the total phenols analysed. Over the 2008 growing period, the difference in content of total dihydrohalcones in leaves between integrated and organic production was very narrow (Fig. 1(E)). One year later, at the first three dates of sampling, the leaves from organic production had significantly higher levels of total analysed dihydrohalcones, about 7–12% more than leaves from integrated production, while later in the growing period the difference between the two production types was not significant (Fig. 1(F)). Higher levels of dihydrohalcones in leaves are often connected with scab infection. It was reported that infection with *Venturia inaequalis* fungus increased higher phloridzin synthesis.[8] This effect was also confirmed by other researchers.[30,31] Although in our case no visual symptoms were noticed on sampled leaves, some infection in organically produced apples could already have been present.

Besides phloridzin, in apples we also analysed dihydrochalcone phloretin 2-xylosylglucoside. Its content in pulp ranged from 1.5 to 16.4 mg kg^{-1} FW and in peel from 31 to 290 mg kg^{-1} FW, thus attaining the rank of other authors' results.[12,17] The apple peel possessed 15–30 times more total dihydrochalcones than the pulp. The 'Crown Prince Rudolf' cultivar was outstanding in both years for the highest values of total dihydrochalcones in fruit skin. In 2008, apples of the 'Topaz' and 'Reinette de Champagne' cultivars from organic production had significantly higher content levels of total dihydrochalcones than apples from integrated production. The same value was obtained in the 'Crown Prince Rudolf' cultivar the following year. Pathogenic pressure (apple scab particularly) and moderate nitrogen fertilization have been reported to directly influence the metabolism of phloridzin in apple leaves,[8,30] which could explain the higher content of dihydrochalcones in organic apples. Similarly, Chinnici *et al.*[12] found that the phloridzin content in apple peel was higher in organic fruit production than in the integrated one.

With regard to quantity, quercetins are the second major phenol group after phloridzin in apple leaves and the major group in apple peel. Their share of the content levels in leaves represents only one-fifth of the quantity of phloridzin. In apple leaves and fruit, several quercetin glycosides were detected: quercetin-3-*O*-rutinoside (not detected in pulp), quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-arabinopyranoside (not detected in pulp), quercetin-3-*O*-arabinofuranoside, quercetin-3-*O*-rhamnoside and quercetin-3-*O*-xyloside.

Organic production contributed to a 10–30% increase in quercetins in apple leaves in comparison to integrated production (Fig. 2(A,B)). A statistically significant difference was found only on one date in 2008 and on three dates in 2009. The scab infection on leaves probably caused an increased synthesis of flavonols. Feucht[32] came to the same conclusion: i.e., that leaves infected with the *Venturia inaequalis* fungus accumulated flavonols.

The main source of quercetin glycosides is the peel. In the pulp, five quercetin glycosides were found in very low amounts. Among quercetin glycosides, the most abundant individual compound in peel was quercetin-3-*O*-galactoside, followed by quercetin-3-*O*-arabinofuranoside and quercetin-3-*O*-rhamnoside. The peel of 'Florina' apples and the pulp of 'Topaz' and 'Reinette de Champagne' cultivars from organic production had significantly higher levels of total quercetins in both years than those from integrated production. Pathogenic pressures may explain the higher quercetin level in organic samples, since it is known that flavonols such as quercetins are antimicrobial compounds synthesized by plants in response to pathogen attack.[33] The opposite was reflected in the pulp of 'Florina' and 'Crown Prince Rudolf' cultivars in 2009, when integrated production had significantly higher content levels of total flavonols in comparison with organic production. Similarly, Chinnici *et al.*[12] report a higher content of total quercetins in integrated production in comparison with organic production in the 'Golden Delicious' cultivar. Also, in yellow plums from conventional production, higher levels of quercetins in comparison with organically grown plums were found.[21]

The qualitative composition of peel polyphenols was almost analogous to that observed for flesh, with the exception of the cyanidin glycosides, which were present only in red apple peel. It became evident that the peel of the 'Florina' cultivar from organic production in 2008 had significantly higher content of

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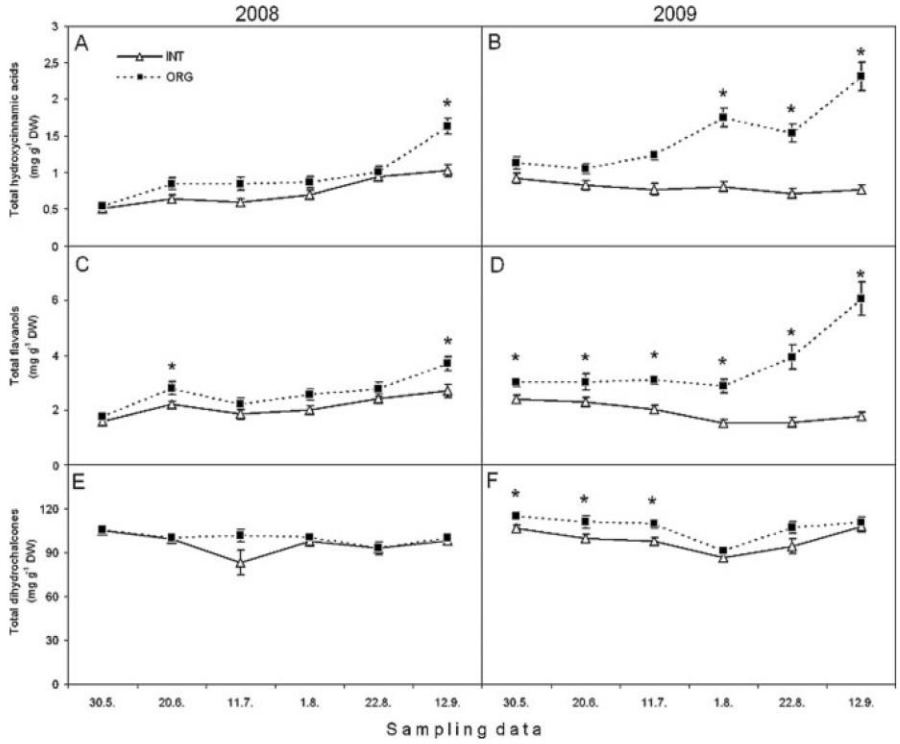

Figure 1. Content of total hydroxycinnamic acids, total flavanols and total dihydrochalcones (mg g^{-1} DW) in apple leaves from organic (org) and integrated (int) fruit production on sampling dates in the 2008 and 2009 growing seasons (mean values and standard error bars). *Statistically significant differences between organic and integrated fruit production.

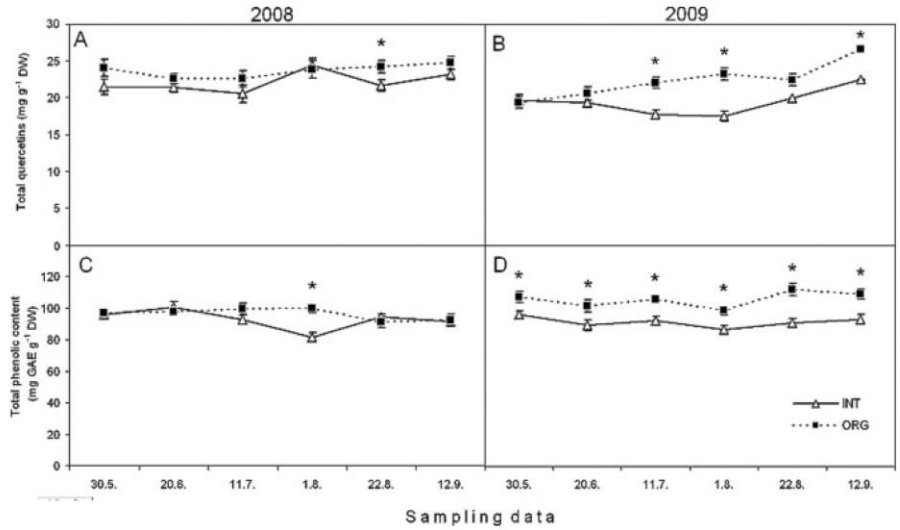

Figure 2. Content of total quercetins (mg g^{-1} DW) and total phenolic content (mg GAE g^{-1} DW) in apple leaves from organic (org) and integrated (int) fruit production on sampling dates in the 2008 and 2009 growing seasons (mean values and standard error bars). *Statistically significant differences between organic and integrated fruit production.

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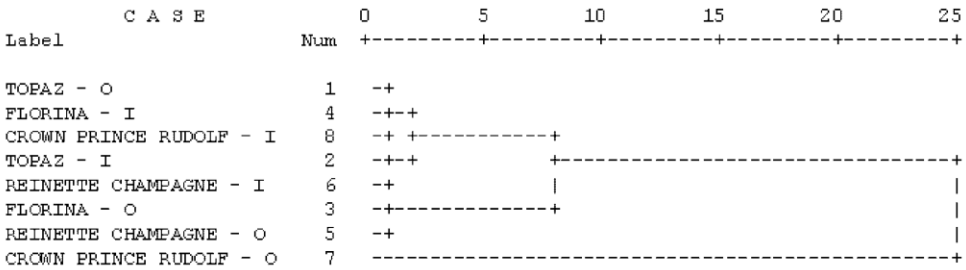

Figure 3. Dendrogram for a 2-year mean value of antioxidant potential and total phenolic content of different apple cultivars in two growing systems (O, organic; I, integrated production), using Ward's method based on square Euclidian distance from total phenolic content and antioxidant capacity.

total anthocyanins than that from integrated production. The same goes for the 'Topaz' and 'Crown Prince Rudolf' cultivars from organic production in 2009. Higher levels of anthocyanins in organic production may be attributed to probably less vigorous growth of shoots and, therefore, to better insolation of trees. Olsson *et al.*[22] also report higher levels of anthocyanins in organically grown strawberries in comparison with those from conventional production. Contrary to that, in grapes, anthocyanin concentrations were observed to be higher in the peel from conventional production.[34]

The impact of the organic type of apple production is reflected in the content of total phenols. In 2009, on all sampling dates, the leaves from organic production had 10–20% higher levels of total phenols than those from integrated production (Fig. 2(C,D)). In 2008, the ranking of the content of total phenols in leaves was almost the same in both types of production.

As reported by several authors,[3,4,12,35] peel was richer than pulp in single phenolic compounds as well as in total polyphenolics. Apple peel has a total phenolic content 3–7.5 times higher than pulp. As in leaves, the effect of organic production became evident in fruit, too. In 2008, apples of the 'Florina' and 'Reinette de Champagne' cultivars as well as apples of the 'Topaz' and 'Crown Prince Rudolf' cultivars in 2009, all from organic production, had a significantly higher content of total phenols than apples from integrated production (Tables 1–4). In a similar study, organically grown peaches, pears,[36] strawberries[22] and marionberries[37] also had a higher total phenolic content when compared with the conventionally grown ones. Lamperi *et al.*[13] reported that organic apple peel showed on average about 25% higher total polyphenolics than integrated peel. Contrary to that, higher total phenolic content was found in conventionally grown yellow plums than in the organically grown ones.[21] Also, Chinnici *et al.*[12] adduced proof that integrated apple peel and pulp were richer in polyphenols and had higher antioxidant capacities than organic apples. Some studies reported that there were no differences in levels of total phenolics between the conventional and organic fruit.[5,38,39]

In our experiments, the antioxidant capacity of apple extracts was expressed as ascorbic acid equivalents. The AEAC values of peels are about 3.4- to 4.5-fold higher than the respective pulps. Owing to their high radical scavenging activities and high concentrations, quercetin glycosides and flavanols proved to be major contributors to the antioxidant activity in peels.[12] In both years, organic production affected the statistical increase of antioxidant activity in apple peel for the 'Florina', 'Topaz' and 'Crown Prince Rudolf' cultivars. However, the type of production had no influence on changing the antioxidant potential of apple flesh, except for the 'Crown Prince Rudolf' cultivar in 2009 (Table 2). Chinnici *et al.* also reported that for apple pulp no statistical differences in the total antioxidant capacity were found between organic and integrated extracts.[12] The effect of organic production is probably more immediately evident in fruit peel than in the flesh, which is connected with the physiological role of skin in providing a physical and chemical barrier against pathogen attack. Integrated fruit is protected by synthetic pesticides and need not rely so heavily on its own defence mechanisms, which include synthesis of phenolic compounds.

Figure 3 shows significant differences among different cultivars in mean values of total phenolics and antioxidative potential, with the exception of 'Reinette de Champagne'. Mean values are higher in apples grown according to organic practice. According to many studies, describing health beneficial properties of organic fruit, this is yet another confirmation that consumers should not be focused solely on external quality of apples (visual parameters) but also consider high phenolic content and antioxidative potential of such fruit.

For individual phenolic compounds, total phenolics and antioxidant capacity in apple peel and pulp, the influence of year, cultivar and their interaction was also statistically evaluated and is presented in Tables 5 and 6. For peel, the interaction between year and cultivar was significant for both cultivation techniques in the case of all phenolics, except for phloretin 2-xylosylglucoside and the antioxidant capacity. For apple pulp, the interaction between year and cultivar was significant for integrated production in almost all phenolics, while for organic production it was not significant for the majority of phenolics identified. Since cultivar had a greater influence than year on the amount of phenolics, we assume that the interaction is mainly the consequence of this factor. As shown in Tables 1–4, there are great differences in the content of phenolics between individual cultivars. The reason for that can probably be found in the great genetic differences between the apple cultivars analysed. That cultivars can also have an influence on the amount of phenolics has been reported by many papers.[3,4,13,26,35] In the case of leaves, year and cultivar were also taken into account, as well as dates, since we obtained data from six different sampling dates (Table 7). Also in this case, the interaction of all three factors was significant for the levels of almost all phenolics under integrated production, while for organic production the interaction for the majority of studied phenolics was not statistically significant. All three factors contributed to the different outcome. We concluded from these results that the accumulation of phenolic compounds in apple tissues is, apart from cultivation techniques, also influenced by growing season as well as variety and sampling data.

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Table 5. Two-way ANOVA for single phenolic compounds, total phenolics (TPC) and antioxidant capacity (AC) in apple peel of 2 years, four cultivars and the interaction year × cultivar (Y × C)

	Year		Cultivar		Y × C	
Phenolic compound	Integr.	Organic	Integr.	Organic	Integr.	Organic
Chlorogenic acid	***	***	***	***	***	***
Caffeic acid	*	*	***	***	***	***
4-*p*-Coumaroylquinic	***	***	***	***	***	***
Catechin	***	***	***	***	***	***
Epicatechin	NS	NS	***	***	**	**
Proc. B1	***	***	***	***	***	***
Proc. B2	NS	NS	NS	NS	NS	NS
Phloretin 2-xylglucoside	NS	NS	***	***	**	**
Phloridzin	NS	NS	***	***	***	***
Q rutinoside	NS	NS	***	***	**	**
Q galactoside	NS	NS	NS	NS	***	**
Q glucoside	*	*	***	***	***	***
Q xyloside	**	**	NS	NS	***	***
Q arabinopyranoside	***	***	**	**	***	***
Q arabinofuranoside	**	**	**	**	***	***
Q rhamnoside	***	***	***	***	***	**
Cyanidin galactoside	***	***	***	***	***	***
Other anthocyanins	***	***	***	***	***	***
TPC	*	*	NS	NS	**	**
AC	***	***	*	*	NS	NS

NS, not significant; Integr., integrated production; Organic, organic production.
Asterisks indicate statistically significant differences at: * *P*-value below 0.05; ** *P*-value below 0.01; *** *P*-value below 0.001.

Table 6. Two-way ANOVA for single phenolic compounds, total phenolics (TPC) and antioxidant capacity (AC) in apple pulp of 2 years, four cultivars and the interaction year × cultivar (Y × C)

	Year		Cultivar		Y × C	
Phenolic compound	Integr.	Organic	Integr.	Organic	Integr.	Organic
Chlorogenic acid	***	NS	***	***	***	NS
Caffeic acid	NS	NS	***	***	NS	NS
4-*p*-Coumaroylquinic	NS	NS	***	***	***	NS
Catechin	***	***	***	***	*	***
Epicatechin	***	NS	***	NS	***	NS
Proc. B1	NS	NS	***	NS	**	NS
Proc. By	NS	*	NS	NS	**	NS
Proc. B2	NS	NS	***	***	*	NS
Proc. trimer	NS	NS	*	NS	*	NS
Phloretin 2-xylglucoside	***	*	***	***	***	***
Phloridzin	NS	NS	***	***	***	NS
Q galactoside	NS	*	**	NS	*	*
Q glucoside	NS	NS	***	***	***	*
Q xyloside	NS	NS	***	***	**	***
Q arabinofuranoside	NS	NS	*	**	NS	NS
Q rhamnoside	***	NS	***	***	***	NS
TPC	NS	***	**	***	**	***
AC	NS	***	***	***	NS	***

NS, not significant; Integr., integrated production; Organic, organic production.
Asterisks indicate statistically significant differences at: * *P*-value below 0.05; ** *P*-value below 0.01; *** *P*-value below 0.001.

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Table 7. Three-way ANOVA for single phenolic compounds and total phenolics (TPC) in apple leaves of 2 years, four cultivars, six sampling dates and the interaction year × cultivar × date (Y × C × D)

	Year		Cultivar		Dates		Y × C × D	
Phenolic compound	Org.	Integr.	Org.	Integr.	Org.	Integr.	Org.	Integr.
Chlorogenic acid	***	***	***	***	***	**	***	*
4-*p*-Coumaroylquinic	***	***	***	***	***	NS	***	NS
Catechin	***	**	***	***	***	***	***	NS
Epicatechin	***	***	***	***	***	***	***	NS
Proc. B2	***	***	***	***	***	***	***	*
phloretin	NS	***	***	***	***	***	*	***
phloridzin	***	NS	***	***	***	***	***	*
Q rutinoside	***	***	***	***	***	***	*	NS
Q galactoside	**	***	NS	***	***	***	***	NS
Q glucoside	**	***	***	***	***	***	**	NS
Q xyloside	NS	***	*	***	***	***	***	NS
Q arabinopyranoside	**	***	***	***	**	***	NS	NS
Q arabinofuranoside	NS	***	***	***	***	***	***	NS
Q rhamnoside	NS	***	***	***	NS	***	***	NS
Q hexoside	***	***	***	***	***	***	*	*
TPC	***	NS	**	NS	NS	NS	*	NS

NS, not significant; Integr., integrated production; Organic, organic production.
Asterisks indicate statistically significant differences at: * *P*-value below 0.05; ** *P*-value below 0.01; *** *P*-value below 0.001.

CONCLUSIONS

Our results showed higher levels of phenolic compounds and antioxidant capacity in organic apples and leaves when compared with those from integrated production. Apples from organic production showed a higher content of hydroxycinnamic acids, flavanols, dihydrochalcones, quercetins and total phenolics than apples from integrated cultivation. The reason for higher phenolic levels in organically grown apples lies in the fact that the trees are exposed to various stress factors, like diseases, pests, lack of mineral nutrients, etc., which induce the accumulation of phenolic compounds. The idea is based on the fact that phenylpropanoids act as defence compounds in plants.[4,33] From the nutritional point of view, since most phenolics were located in the apple peel, the consumption of unpeeled organic apples is recommended to maximize the dietary intake of antioxidant compounds. Organic production is also friendlier to the environment and wildlife, because it avoids the use of man-made fertilizers and pesticides.

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2.5 VPLIV UPORABE BIKARBONATNIH SOLI, SREDSTEV ZA ZATIRANJE JABLANOVEGA ŠKRLUPA, NA METABOLITE V PLODOVIH IN LISTIH JABLANE

SLATNAR Ana, ŠTAMPAR Franci, VEBERIČ Robert
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V poljskem poskusu smo preučevali učinkovitost in fitotoksičnost neorganskih fungicidov kalijevega bikarbonata (KBK) in natrijevega bikarbonata (NaBK) pri zatiranju jablanovega škrlupa. Njuno učinkovitost smo primerjali s kontrolo, pri kateri smo uporabili vodo ter fungicidnim tretiranjem; klasično metodo za varstvo pred jablanovim škrlupom na sorti ´Braeburn´. Da bi preučili vse možne vplive teh dveh sredstev, smo na plodovih spremljali stopnjo okužbe, zunanje parametre kakovosti plodov (maso, trdoto, barvo) ter vsebnost primarnih in sekundarnih metabolitov. Na listih pa smo analizirali in beležili stopnjo okužbe ter vsebnost sekundarnih metabolitov in kalija. Rezultati so pokazali, da sta sredstvi učinkoviti pri varstvu pred jablanovim škrlupom in pri dani aplikaciji ne povzročata fitotoksičnih učinkov. Zunanji parametri kakovosti ploda so bili primerljivi s tistimi, pridelanimi na drevesih s fungicidnim obravnavanjem. Tretiranje s KBK je izzvalo višje vsebnosti sladkorjev in ekoloških kislin, ki so bile primerljive s tistimi, ki smo jih izmerili v plodovih fungicidnega obravnavanja. V raziskavi nismo zaznali statističnih razlik v vsebnosti analiziranih fenolov med fungicidnim obravnavanjem in tretiranjem s KBK oz. NaBK. Vsebnost fenolnih snovi v listih tretiranih s KBK oz. NaBK so bile primerljive s tistimi iz fungicidnega obravnavanja, kar nakazuje aktivnost obrambnega mehanizma v listih jablane. Uporaba KBK je pozitivno vplivala na vsebnost kalija v listih skozi celotno rastno sezono. Glede na znano dejstvo, da produkti neekoloških fungicidov niso toksični za zdravje človeka, bi v ekološki pridelavi KBK oz. NaBK potencialno lahko bili perspektivni sredstvi za varstvo nasadov pred jablanovim škrlupom.

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Influence of bicarbonate salts, used against apple scab, on selected primary and secondary metabolites in apple fruit and leaves

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ABSTRACT

In a field experiment, the effectiveness and phytotoxicity of inorganic fungicides such as potassium bicarbonate (PBC) and sodium bicarbonate (SBC) were compared with water applications for the control and fungicide treatments as a classical method for controlling scab infections in cultivar 'Braeburn'. To examine all possible effects of this inorganic fungicide disease severity, external quality parameters (mass, firmness, colour), and the content of primary and secondary metabolites were measured and recorded on fruit; and disease severity, the content of secondary metabolites and accumulation of potassium were monitored in apple leaves. The results indicate that both inorganic fungicides are effective against apple scab and do not cause any phytotoxicity at given application doses. External fruit quality parameters were comparable with fruit produced with fungicide treatments. Similarly, the PBC treatments exhibited a positive effect on higher content of sugars and organic acids in comparison to fungicide treatments. No significant differences between fungicide and PBC or SBC treatments were observed in the content of analysed phenolic compounds. The content of phenolic compounds in leaves, were comparable to those in fungicide treated trees. However, this only indicates the activity of defence mechanisms in apple leaves. The use of PBC also positively affected the potassium accumulation in leaves through the growing season. Given that these products are not toxic to human health, PBC and SBC can potentially present a perspective protection in the apple orchards especially for the control of apple scab in organic fruit growing.

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1. Introduction

Apple scab, also known as black spot, caused by *Venturia inaequalis* ((Cook.) Wint.) is one of the most serious diseases of apple reported in almost all apple producing regions causing huge economic losses (up to 70% reduction in apple production). Scab infection leads to deformation in shape and size of the fruit, premature leaf and fruit drop, and enhanced susceptibility of tree to chilling and freezing injuries (McHardy, 1996).

Apples are prone to infection by pathogenic microorganisms during and after the growing season. Fungal pathogens are the major cause of economic losses in apple production and a challenge to crop protection strategies. The awareness and concern about possible health risks and the environmental impact of agrochemicals intensively used in the recent decades in conventional farming (Gessler and Pertot, 2012; Reganold et al., 2001; Tilman, 1999) increased the consumer's demand for healthier fruit produced in a more environmentally friendly manner. For these reasons, integrated and organic farming have become more widespread in apple production (Peck et al., 2005).

Chemical control of apple scab caused by *V. inaequalis* presents a considerable part of the pest control measures necessary for apple orchard protection when it is planted with one or several cultivars susceptible to the disease. However, these intensive treatments have a negative impact on the beneficial fauna and can lead to the outbreak of certain pests (Cuthbertson and Murchie, 2003), as well as to problems of *V. inaequalis* resistance to the active ingredient used (Parisi et al., 1994; Steinfeld et al., 2001).

In order to control apple scab in organic fruit growing, copper or sulphur products are widely used (Jamar et al., 2010; Mitre et al., 2009, 2010). These have several disadvantages, such as copper accumulation in soil, reduced efficiency at low temperatures, low fruit quality in terms of commercially important appearance etc. Therefore, a replacement for copper and sulphur products must be considered for more environmentally friendly and economical apple production (Kelderer et al., 2008).

In recent years, a broad range of products based on potassium bicarbonate (PBC) have been used as an alternative to products based on copper and sulphur (Jamar et al., 2007, 2008, 2010). Bicarbonate is considered harmless from an ecotoxicological and toxicological point of view (Environmental Protection Agency–EPA,

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1999) and this product has already been introduced in Annex II of European Regulation EEC 2091/92 list of active substances, which may be used as plant protection products in organic farming (Kelderer et al., 2005, 2006). Ilhan et al. (2006) reported that sodium bicarbonate (SBC) effectively inhibited spore germination and germ tube elongation of *V. inaequalis* in vitro. Favourable conditions for scab infection are still rather undefined in the orchard and also, the course of infection is not fully researched. However, combinations of sulphur and PBC offer increased effectiveness during distribution and development of the spore population in the orchard. Applied at the initial time of the infection or soon afterwards, sulphur is effective on the spores that are still in their germination phase, and the bicarbonates stop the spores that are already penetrating into the leaf.

The practical experience on apple scab control with bicarbonates is still limited, but laboratory and field trials indicate that the highest efficacy is shortly after the moment the infection has started. The objectives of this study were: (1) to evaluate the effectiveness of inorganic fungicides such as SBC and PBC for scab control (effects on fruit weight, firmness, disease severity on fruit and leaves, visible discolourations of the skin); (2) to evaluate the influence of this compounds on primary and secondary metabolites; and (3) to evaluate the effect of PBC on the content of potassium in apple leaves in apple orchard system. We hypothesised that both bicarbonate salts could influence plant secondary metabolism, which in return could induce higher plant resistance to apple scab.

2. Materials and methods

2.1. Chemicals

The following standards were used for the quantification of sugars and organic acids: sucrose, glucose and fructose; and citric and malic acids from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). The following standards were used for the quantification of phenolic compounds: chlorogenic acid (3-caffeoylquinic acid), phloretin and rutin (quercetin-3-*O*-rutinoside) from Sigma–Aldrich, ferulic acid, (−)-epicatechin, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, *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–Aldrich. The chemicals for the mobile phases were HPLC–MS grade acetonitrile and formic acid from Fluka. Water for mobile phase was twice distilled and purified with the Milli-Q system (Millipore, Bedford, MA). For the total phenolic content, Folin–Ciocalteu phenol reagent (Fluka), sodium carbonate (Merck, Darmstadt, Germany), gallic acid and methanol (Sigma–Aldrich) were used.

2.2. Experimental site, design, and treatment

The study was performed on apple trees (*Malus domestica* Borkh.), cv. 'Braeburn', a scab-susceptible cultivar, in the growing season 2010. Fruits were harvested from 9-year-old trees grafted on the M9 rootstock growing in the University experimental orchard in Ljubljana (latitude: 46°2′, longitude: 14°28′).

The experimental treatments and the application protocol for the treatments were as followed: (1) water-treated control, (2) fungicide treated in accordance with the integrated pest management guidelines (Rules on integrated production of fruit), (3) 1% SBC (Merck) and (4) 1% PBC (Merck). The first application was on April 19th. The treatments were repeated every 10 days until harvest and 14 applications were completed in the growing period. Spray solutions were applied to the trees with a hand gun sprayer and each tree received 1 L of water/fungicide/SBC/PBC solution per application.

For the leaf phytotoxicity severity assessment, 10 shoots per tree were monitored during the whole growing season and 10 fully developed leaves per shoot were surveyed. The following leaf phytotoxicity levels were recorded: 0 = no damage; 1 = 0–2% damage; 2 = 2–5% damage; 3 = 5–20% damage; and 4 = >20% of the leaf surface damaged. The leaf damage of apple scab severity was evaluated using the grading scale described by Parisi et al. (1993): score 0 = no visible symptoms; score 1 = 0% < percentage of scabbed leaf surface (sls) ≤ 1%; score 2 = 1% < sls ≤ 5%; score 3 = 5% < sls ≤ 10%; score 4 = 10% < sls ≤ 25%; score 5 = 25% < sls ≤ 50%; score 6 = 50% < sls ≤ 75%; score 7 = sls > 75%.

Samples for the potassium content analysis were taken three times during the growing period (20 May, 20 June, and 20 September). A combined sample of 10 mature leaves per tree from different types of one-year shoots were analysed in five repetitions for each treatment. Plant samples were dried at 40 °C till 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 are removed by precipitation and filtration. The filtrate was diluted to the desired volume (100 mL) with twice distilled water and the concentration of potassium was determined by atomic absorption spectrometry (Analyst 800, Perkin Elmer). The absorbance of potassium in the sample solution was determined by comparison with the absorbance of calibration solutions and was proportional to the concentration of this element in the solution. The ionisation and chemical interferences were controlled by the addition of caesium and lanthanum buffer solutions to standards and samples.

Fruit sampling was performed at technological maturity, which was determined by the starch iodine test. The harvesting date was October 10th. Five fruit (five repetitions for each treatment) were peeled as thin as possible and peel and pulp (separately) were immediately shock-frozen in liquid nitrogen and stored at −20 °C until analysis of the samples. Fruit disease assessment was monitored at harvest and disease severity was quantified using a scale developed by Tomerlin and Jones (1983). The scale consists of five categories; (0) no visible lesion; (1) <10% fruit surface infected; (2) 10–25% fruit surface infected; (3) 25–50% fruit surface infected; (4) >50% fruit surface infected.

2.3. External fruit characteristics

Fruit firmness, colour and mass were evaluated at technological maturity in order to determine the effect of different treatments on the external quality parameters. The measurements were done on 25 randomly selected fruits per each treatment. Fruit mass was measured for each fruit separately on electronic scales. Firmness was measured on four peeled sides of each fruit with a penetrometer (tr Italy, TR Turoni, Forli, Italy) equipped with a 11 mm tip.

Peel colour was measured by a portable colorimeter (CR-10 Chroma; Minolta, Osaka; Japan) with C illuminant. The colorimeter was calibrated with a white standard calibration plate before use. In 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). The a^* and b^* values extend from −60 to 60: a^* negative is for green and a^* positive is for red and b^* negative is for blue and positive for yellow. The hue angle ($h°$) is expressed in degrees from 0 to 360 (0° = red, 90° = yellow, 180° = green and 270° = blue). Colour was measured in the middle of each fruit (two repetition per fruit on different sides) to ensure equal measurement conditions.

2.4. Analysis of individual sugars and organic acids

The samples were analysed for their content levels of sugars (sucrose, glucose and fructose) and organic acids (malic and citric acid). Whole apples were halved, cut into small pieces and 10 g of fresh mass was immersed into 50 mL of twice distilled water and homogenized with a T-25 Ultra-Turrax (Ika-Labortechnik, Staufen, Germany). The samples were left for extraction for half an hour at room temperature with frequent stirring, and the extracted samples were centrifuged at 10 000 rpm for 7 min at 10 °C (Eppendorf Centrifuge 5810R, Hamburg, Germany). The supernatants were filtered through a 0.45 μm cellulose ester filter (Macherey-Nagel, Düren, Germany), transferred to a vial and analysed according to the method described by Slatnar et al. (2011) using high performance liquid chromatography (HPLC; Thermo Scientific, Finnigan Spectra System, Waltham, MA, USA). 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).

2.5. Extraction and determination of individual phenolic compounds

The frozen leaves were lyophilized and ground to a fine powder in a mortar chilled with liquid nitrogen. The fine powder (40 mg) was extracted with methanol (3 mL) containing 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 during the extraction. The apples were peeled with a peeler, and the peel was separated from the pulp. The extraction of fruit samples (peel and pulp separately) was done as described by Slatnar et al. (2011). Fresh apple samples were ground to a fine powder in a mortar chilled with liquid nitrogen. The samples of 10 g pulp or 5 g peel were extracted with 10 mL methanol containing 1% (w/v) BHT in BHT and 3% (v/v) formic acid in a cooled ultrasonic bath (0 °C) for 1 h.

The treated samples were centrifuged for 7 min at 10 000 rpm. The supernatant was filtered through a Chromafil AO-45/25 polyamide filter (Macherey-Nagel, Düren, Germany) and transferred to a vial prior to injection into a HPLC system. Samples were analysed using a Thermo Finnigan Surveyor HPLC system (Thermo Scientific, San Jose, CA) with a diode array detector at 280 nm (hydroxycinnamic acids, dihydrochalcones and flavan-3-ols), 350 nm (flavonols) and 530 nm (anthocyanins). 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 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 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; and finally washing and reconditioning of the column. Identification of compounds was achieved by comparing retention times and their UV–vis spectra from 200 to 600 nm, as well as by the addition of an external standard. Compounds were identified using a mass spectrometer (Thermo Scientific, LCQ Deca XP MAX) with an electrospray ionization (ESI) operating in negative ion mode (positive ion mode for anthocyanins). The analyses were carried out using full-scan data dependent MS^n scanning from *m/z* 115 to 2000. Quantification was achieved according to the concentrations of a corresponding external standard.

Concentrations of phenolic compounds were calculated from the peak areas of the sample and the corresponding standards. The concentrations were expressed as mg g^{-1} dry weight (DW) for leaves and as mg 100 g^{-1} fresh weight (FW) for fruit peel or pulp. For the compounds lacking standards, similar compounds as standards for the quantification were used. Thus, 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.

2.6. Determination of total phenolic content

The extraction of fruit and leaf samples for the determination of total phenolic content (TPC) was made according to the same protocol as for individual phenolics, with the difference that no BHT was added. TPC of the extracts was assessed using the Folin–Ciocalteu phenol reagent method (Singleton and Rossi, 1965). Six millilitre of twice-distilled water and 500 μL of Folin–Ciocalteau reagent were added to 100 μL of the sample extracts and left to stand between 8 s and 8 min at room temperature; 1.5 mL sodium carbonate (20%, w/v) and 1.9 mL twice-distilled water was than added. The extracts were mixed and allowed to stand for 30 min at 40 °C before measuring absorbance at 765 nm on a Lambda Bio 20 UV/vis spectrophotometer (Perkin Elmer, Waltham, MA). A mixture of water and reagents was used as a blank. The total phenolic content was expressed as gallic acid equivalents (GAE) in mg 100 g^{-1} fresh weight (FW) of fruit peel or pulp and as mg GAE g^{-1} dry weight (DW) in leaves. Absorptions were measured in three replications.

2.7. Statistical analysis

The data was analysed using the Statgraphics Plus 4.0 program (Manugistics. Inc.; Rockville, MD, USA). Data from all the analyses were tested for any differences among treatments using one-way analysis of variance (ANOVA). Significant differences among means were determined by Duncan's test with a significance level of 0.05.

3. Results and discussion

3.1. Primary metabolites, phenolic compounds and external fruit quality parameters

SBC and PBC treatments reduced the disease severity on fruit in comparison to the control fruit. However, no differences were observed between both bicarbonate salts (Table 1). Similarly, a reduced severity on fruit as an effect of the use of 1% SBC has been reported by Ilhan et al. (2006). Although sprayings with SBC and PBC were partly performed in a period, when russetting of the apples can increase, no russetting caused by the use of bicarbonate salts has been measured in our experiment (data not shown). Fruit mass was statistically lower in control plants (165.5 g), followed by fruits from SBC (174.4 g), PBC (183.1 g) and fungicide treatments (190.2 g) (Table 1). Statistically highest fruit mass was recorded in fruits from fungicide treatments (Table 1). These results are according to our expectations, because SBC or PBC reduce the infection and consequently the negative impact on fruit weight is lower compared to control plants. No statistical differences in fruit firmness were measured among treatments (Table 1). Hunsche et al. (2003) reported that potassium fertilization can reduce flesh firmness and this might be related to a simultaneous increase in fruit size. However, in our experiment fruit width and height were statistically higher in PBC treatment and fungicide treatment (Table 1). Higher colour parameter a^* indicates a more intense red fruit from these treatments (data not shown), and these results were confirmed with the analysis of anthocyanins in fruit peel (Table 2). Many studies have namely confirmed the correlation between anthocyanins content and red colour of fruit and flowers (Schmitzer et al., 2010; Usenik et al., 2009). Statistically higher content of individual

Table 1
The effect of different treatments on fruit quality parameters (mean ± SE, $n = 25$).

Treatment	Mass (g)	Firmness (kg cm^{-2})	Width (mm)	Height (mm)	Disease severity
Control	165.53 ± 3.00 a	8.54 ± 0.16 NS	70.22 ± 0.86 a	63.05 ± 0.93 a	3.50 ± 0.22 c
Fungicide	190.19 ± 2.40 d	8.38 ± 0.08 NS	73.35 ± 0.56 b	67.49 ± 0.71 b	0.17 ± 0.17 a
SBC	174.36 ± 3.23 b	8.64 ± 0.12 NS	71.18 ± 0.69 a	63.66 ± 0.91 a	1.00 ± 0.26 b
PBC	183.10 ± 3.12 c	8.29 ± 0.13 NS	73.55 ± 0.79 b	66.20 ± 0.68 b	1.00 ± 0.26 b

Different letters (a–d; NS: not significant) in rows denote statistically significant differences among treatments by Duncan's test at $P < 0.05$.

and total analysed anthocyanins was measured in the PBC treatment compared to the fungicide treatment (Table 2). Hunsche et al. (2003) observed that fruit colour was positively correlated with the amount of potassium applied to the soil and also with the fruit anthocyanin content, inferring that potassium might be important in the anthocyanin synthesis pathway and could be a cofactor in the activation of some specific enzymes, similar to the case of UDP-Galactose and flavanoide-3-o-glycosyltransferase (Ju et al., 1999). However, higher anthocyanin content levels in the SBC treatment could be explained with the potential potassium replacement by sodium at the cellular level, consequently affecting the activity of enzymes, which particularly respond to potassium. We can conclude that the PBC treatment has a positive effect on reducing the disease severity in fruit, on fruit mass, fruit width and height, and also on fruit colour.

Glucose, fructose, sucrose and total sugar content levels of different treatments are presented in Table 3. Generally, fructose was measured in highest concentrations (from 55.64 (fungicide) to 65.77 g kg^{-1} FW (control)), followed by sucrose (from 35.12 (fungicide) to 43.56 g kg^{-1} FW (control)) and glucose (from 7.52 (fungicide) to 10.24 g kg^{-1} FW (control)). In apple fruit two organic acids were determined, the predominant malic acid (from 6.20 (fungicide) to 8.33 g kg^{-1} FW (control)), followed by citric acid (from 1.12 (fungicide) to 1.61 g kg^{-1} FW (control)) (Table 3). Mikulic-Petkovsek et al. (2007) obtained similar results for this cultivar. Fruit of control and PBS treatments contained significantly higher levels of fructose and total sugars compared to the fungicide fruit. On the other hand, fruit of SBC and fungicide treatments had significantly lowest content of all analysed sugars (Table 3). No significant differences were detected in the content of citric acid among treatments. Statistically higher contents of total organic acids and malic acid were measured in control fruit, and PBS and SBC treatments compared to fruit from fungicide treatments. In general, higher content of total sugars and organic acids in PBC treated fruit could potentially be explained with better leaf status, which can lead to higher photosynthetic activity. Beside the condition and capacity of chloroplasts photosynthesis could additionally be affected by potassium level which influences enzyme activity and stomata function. Casero et al. (2004) also observed that fruit acidity of 'Goolden Smoothee' apples was positively correlated with both leaf and fruit potassium concentrations, which in our case was higher in the leaves. Hence, the application of PBC in our experiment does not just function as protection against apple scab, but at the same time presents additional source of potassium for the plants. In general, potassium fertilization is important in order to maintain the acidity balance of apple fruits and to allow a greater storage potential of fruit.

Phenolic compounds are a class of secondary metabolites; compounds that are not essential for the survival of plants but are produced to enhance the plant's ability to withstand stress factors

Table 2
The content of individual phenolic compounds (mean ± SE in mg 100 g^{-1} FW) and total phenolic content (mean ± SE in mg GAE 100 g^{-1} FW) in apple peel affected by different treatments.

	Control	Fungicide	SBC	PBC
4-*O*-*p*-Comaroylquinic acid	4.31 ± 0.23 b	3.40 ± 0.17 a	3.82 ± 0.31 ab	3.06 ± 0.34 a
Caffeic acid	1.65 ± 0.08 c	1.29 ± 0.06 a	1.57 ± 0.09 bc	1.31 ± 0.12 ab
Chlorogenic acid	5.06 ± 0.23 b	2.87 ± 0.19 a	3.54 ± 0.19 a	3.54 ± 0.27 a
Hydroxycinnamic acids	11.03 ± 0.33 b	7.56 ± 0.35 a	8.93 ± 0.47 a	8.44 ± 0.89 a
phloretin-2-*O*-xyloside	29.08 ± 1.53 b	22.58 ± 1.28 a	29.23 ± 0.47 b	26.92 ± 1.49 b
phloridzin	30.08 ± 1.35 bc	24.56 ± 1.30 a	33.31 ± 1.69 c	27.29 ± 1.52 ab
Dihydrochalcones	59.15 ± 2.33 bc	47.14 ± 2.51 a	62.54 ± 2.14 c	54.12 ± 2.63 ab
Catechin	16.52 ± 0.91 b	13.11 ± 0.59 a	15.78 ± 0.66 b	13.18 ± 0.84 a
Epicatechin	39.84 ± 2.68 NS	40.26 ± 2.57 NS	45.34 ± 3.02 NS	40.93 ± 3.81 NS
Procyanidin B2	25.48 ± 1.62 NS	25.89 ± 2.00 NS	26.56 ± 0.94 NS	26.76 ± 1.46 NS
Procyanidin B1	5.38 ± 0.22 b	3.04 ± 0.09 a	4.74 ± 0.27 b	5.43 ± 0.48 b
Flavan-3-ols	87.36 ± 5.18 NS	82.63 ± 5.09 NS	92.42 ± 4.61 NS	87.14 ± 6.35 NS
Q-3-*O*-arabinopyranoside	24.56 ± 1.73 b	15.28 ± 0.79 a	18.04 ± 1.76 a	19.27 ± 1.94 a
Q-3-*O*-galactoside	25.40 ± 1.06 b	16.63 ± 1.22 a	17.35 ± 2.25 a	19.93 ± 2.62 ab
Q-3-*O*-glucoside	3.57 ± 0.24 b	2.03 ± 0.09 a	2.28 ± 0.35 a	2.71 ± 0.37 a
Q-3-*O*-rhamnoside	9.93 ± 0.93 b	5.38 ± 0.29 a	6.09 ± 0.59 a	6.28 ± 0.75 a
Q-3-*O*-rutinoside	2.89 ± 0.37 b	1.13 ± 0.08 a	1.54 ± 0.39 a	1.98 ± 0.44 ab
Q-3-*O*-xyloside	7.94 ± 0.70 b	5.16 ± 0.23 a	5.51 ± 0.63a	6.12 ± 0.68 ab
Flavonols	74.28 ± 4.17 b	45.42 ± 2.67 a	50.80 ± 5.88 a	56.29 ± 6.66 a
Cy.-galactoside	13.34 ± 0.54 b	9.57 ± 1.16 a	13.53 ± 0.81 b	14.57 ± 1.85 b
Cy.-3-arabinoside	0.19 ± 0.00 b	0.14 ± 0.01 a	0.20 ± 0.01 b	0.21 ± 0.02 b
Cy.- xyloside	0.47 ± 0.03 b	0.34 ± 0.05 a	0.45 ± 0.01 ab	0.54 ± 0.04 b
Cy.-7-arabinoside	1.24 ± 0.09 a	1.08 ± 0.15 a	1.43 ± 0.09 ab	1.76 ± 0.17 b
Peonidin galactoside	1.32 ± 0.07 a	1.02 ± 0.11 a	1.38 ± 0.09 ab	1.69 ± 0.16 b
Anthocyanins	16.65 ± 0.70 ab	12.16 ± 1.47 a	16.99 ± 0.97 b	18.56 ± 2.30 b
TPC[a]	1467.36 ± 184.10 c	997.16 ± 25.47 a	1128.75 ± 53.93 ab	1346.13 ± 41.88 bc

Different letters (a–c; NS: not significant) among different treatments denote significant differences (Duncan's test, $P < 0.05$).
[a] TPC: total phenolic content determined by Folin–Ciocalteu method in mg gallic acid 100 g^{-1} fresh weight.

Table 3
The effect of different tretments on the content of sugars and organic acids in mature fruit (mean ± SE in g kg^{-1} FW, $n = 5$).

Treatments	Fructose	Sucrose	Glucose	Total sugars	Malic acid	Citric acid	Total organic acids
Control	65.77 ± 1.59 b	43.56 ± 2.73 c	10.24 ± 1.32 NS	119.56 ± 3.32 b	8.33 ± 0.41 b	1.61 ± 0.17 NS	10.15 ± 0.63 b
Fungicide	55.64 ± 1.70 a	35.12 ± 1.37 ab	7.52 ± 0.36 NS	98.28 ± 2.98 a	6.20 ± 0.26 a	1.12 ± 0.07 NS	7.32 ± 0.28 a
SBC	56.07 ± 1.23 a	34.96 ± 1.26 a	9.59 ± 1.03 NS	98.61 ± 2.03 a	7.52 ± 0.26 b	1.32 ± 0.11 NS	8.84 ± 0.36 b
PBC	63.99 ± 3.43 b	40.83 ± 2.07 bc	8.86 ± 0.77 NS	109.68 ± 5.11 b	7.81 ± 0.56 b	1.33 ± 0.12 NS	9.14 ± 0.63 b

Different letters (a–c) in rows denote statistically significant differences by Duncan's test at $P < 0.05$.

such as pathogens (Robards et al., 1999). The oxidation of phenols is a common event in host-pathogen interactions as shown by the browning of cells and tissue. The oxidation of phenols leads to formation of quinones and free radicals that can inactivate enzymes which may be a part of the arsenal of weapons used by the pathogen. In addition, these oxidized phenolic compounds have enhanced antimicrobial activity and may thus be directly involved in suspending pathogen development (Urs and Dunleavy, 1975). Principally, due to their antioxidant properties, there is much interest in the potential health benefits of phenolic compounds in our diets (Heim et al., 2002). These two reasons must be considered when new technological measures with possible effects on the fruit and leaf phenolic content are introduced.

The analysed phenolics were classified into four groups: hydroxycinnamic acids, dihydrochalcones, flavan-3-ols and flavonols. Control apples contained significantly higher levels of all analysed hydroxycinnamic acids in apple peel compared to apples from other treatments (Table 2). However, no statistical differences in the content levels of almost all analysed hydroxycinnamic acids have been determined among treatments in apple pulp (Table 4). The increased content of hydroxycinnamic acids in control compared to the other treatments is probably caused by stronger infection with fungus *V. inaequalis*, which induces stress conditions in the plant. Similarly, increased amounts of hydroxycinnamic acids were reported in tissue infected with fungus *V. inaequalis* by Mikulic-Petkovsek et al. (2009) as a result of stress conditions.

Apples of the fungicide treatments contained significantly lower levels of phloretin-2-*O*-xyloside in the peel compared to apples from the other treatments, respectively (Table 2). No statistically significant differences in the content of dihydrochalcones in apple pulp were determined among treatments (Table 4). Chinnici et al. (2004) reported that phloridzin content in the apple peel was higher in the organic fruit production than in the integrated one. However, our results indicate that the content of individual phenols in the peel as well as their sum were different in the fungicide treated apples when compared to other treatments, which are similar to integrated and organic production.

No statistically significant differences in the content of flavan-3-ols in apple peel have been determined among treatments (Table 2). However, higher amounts of flavan-3-ols were measured in pulp of PBC treated apples and statistically lowest amounts were determined in control apples (Table 4). No significant differences in the content of flavan-3-ols between organic and integrated apple production have been reported by Mikulic-Petkovsek et al. (2010). Thus it can be concluded that the group of flavan-3-ols is generally less affected by stress conditions, or their synthesis is restricted directly to the infection point and its proximity.

The highest content levels of phenolic compounds were quantified in the group of flavonols where the majority of analysed phenolics were determined. In comparison to the control apple skin, all other treatments contained statistically lower amounts of flavonols (Table 2). Control peel contained more than 1.5 fold content of total flavonols compared to SBC treated peel. In pulp, flavonol content was lower compared to peel, but no uniform trend was observed (Table 4). Also, differences in flavonol content between treatments were only slight. Pathogenic pressures may explain higher quercetin levels in organic samples, since it is known that flavonols such as quercetins are antimicrobial compounds synthesized by plants in response to pathogen attack (Dixon and Paiva, 1995).

The increased levels of total phenolic content measured in the peel was noted with an amplified infection severity (control > PBC > SBC > fungicide treatments) (Tables 2 and 4). This additionally confirms that phenolics are affected by the pathogen infection and that the severity of the attack is directly connected to their higher content levels. Probably the infection with apple scab has a greater influence on TPC that the effect of different treatments.

Table 4
The content of individual phenolic compounds (mean ± SE in mg 100 g^{-1} FW), total phenolic content (mean ± SE in mg GAE 100 g^{-1} FW) in pulp of different type of treatment.

	Control	Fungicide	SBC	PBC
4-*O*-*p*-Comaroylquinic acid	2.16 ± 0.12 b	1.58 ± 0.05 a	2.17 ± 0.13 b	1.74 ± 0.08 a
Caffeic acid	0.15 ± 0.01 NS	0.13 ± 0.01 NS	0.12 ± 0.00 NS	0.15 ± 0.01 NS
Chlorogenic acid	6.63 ± 0.66 NS	6.12 ± 0.15 NS	6.02 ± 0.41 NS	5.24 ± 0.28 NS
Hydroxycinnamic acids	8.76 ± 0.86 NS	7.83 ± 0.17 NS	8.31 ± 0.51 NS	7.13 ± 0.31 NS
Phloretin-2-*O*-xyloside	1.96 ± 0.41 NS	1.77 ± 0.05 NS	2.33 ± 0.26 NS	1.82 ± 0.12 NS
Phloridzin	2.22 ± 0.11 b	1.55 ± 0.08 a	1.70 ± 0.16 a	1.63 ± 0.14 a
Dihydrochalcones	4.50 ± 0.38 b	3.32 ± 0.12 a	4.02 ± 0.37 ab	3.45 ± 0.26 a
Catechin	0.27 ± 0.05 ab	0.16 ± 0.01 a	0.38 ± 0.07 b	0.28 ± 0.03 ab
Epicatechin	2.66 ± 0.40 a	3.62 ± 0.23 b	3.96 ± 0.28 b	4.14 ± 0.28 b
Procyanidin B2	0.79 ± 0.10 a	1.10 ± 0.10 b	1.38 ± 0.13 b	1.35 ± 0.05 b
Procyanidin B1	3.82 ± 0.20 ab	3.44 ± 0.07 a	4.20 ± 0.26 bc	4.60 ± 0.24 c
Flavan-3-ols	8.02 ± 0.16 a	8.54 ± 0.23 b	9.91 ± 0.03 b	10.67 ± 0.51 c
Q-3-*O*-arabinofuranoside	0.02 ± 0.00 b	0.02 ± 0.00 b	0.01 ± 0.00 a	0.02 ± 0.00 b
Q-3-*O*-galactoside	0.02 ± 0.00 b	0.02 ± 0.00 ab	0.01 ± 0.00 a	0.01 ± 0.00 ab
Q-3-*O*-glucoside	0.02 ± 0.00 NS	0.03 ± 0.00 NS	0.02 ± 0.00 NS	0.02 ± 0.00 NS
Q-3-*O*-rhamnoside	0.04 ± 0.00 b	0.02 ± 0.00 a	0.02 ± 0.00 a	0.03 ± 0.00 a
Q-3-*O*-xyloside	0.01 ± 0.00 NS	0.01 ± 0.00 NS	0.01 ± 0.00 NS	0.01 ± 0.00 NS
Flavonols	0.12 ± 0.01 b	0.11 ± 0.02 b	0.07 ± 0.00 a	0.10 ± 0.01 ab
TPC[a]	142.29 ± 6.49 c	112.25 ± 5.28 a	123.92 ± 4.23 ab	135.14 ± 3.85 bc

Different letters (a, b; NS: not significant) among different treatments denote significant differences (Duncan's test, $P < 0.05$).
[a] TPC: total phenolic content determined by Folin–Ciocalteu method in mg gallic acid 100 g^{-1} fresh weight.

Table 5
Potassium content level (g kg^{-1} DW) in apple leaves during the growing period (mean ± SE, n = 5).

Treatments	Sampling date		
	20 May	20 July	20 September
Control	12.2 ± 0.6 a[a]	12.8 ± 0.8 a	9.7 ± 0.3 b
Fungicide	11.9 ± 0.3 a	12.4 ± 0.2 a	8.1 ± 0.4 a
PBC	14.0 ± 0.4 b	16.0 ± 0.9 c	12.0 ± 0.2 c
SBC	12.9 ± 0.4 ab	14.9 ± 0.2 b	10.4 ± 0.3 b

[a] Different letters (a–c) in rows denote statistically significant differences by Duncan's test at P < 0.05.

Also, Mikulic-Petkovsek et al. (2010) reported an increase of total phenolic content from integrated to organic apple production.

3.2. Phenolic compounds and the accumulation of potassium in apple leaves

Both 1% SBC and PBC treatments were effective in reducing the disease severity on leaves in all growing seasons in comparison to the control (data not shown). 1% concentration of SBC or PBC also did not appear to be phytotoxic and did not cause necrosis on the leaves. Prior to PBC application the potassium status of the apple leaves was determined to accurately monitor the potassium accumulation in the leaves afterwards. The results indicate that the level of potassium in the apple leaves was significantly affected by different treatments (Table 5). Potassium content of apple leaves increased from the first to the second sampling date in all treatments, irrespective of PBC application, but the increase was much higher in the PBC treatment. The high potassium level in the cytosol and chloroplasts neutralizes the soluble and insoluble macromolecular anions and stabilizes the pH between 7 and 8 in these compartments, which is the optimum for most enzyme reaction, such as the accumulation of soluble carbohydrates and a decrease in starch content.

The total content of hydroxycinnamic acids in apple leaves is presented in Table 6. Control leaves and leaves treated with 1% SBC or PBC contained statistically higher levels of hydroxycinnamic acids at all sampling dates compared to the fungicide treatment. It is known that the level of hydroxycinnamic acids increases under stress conditions (Mikulic-Petkovsek et al., 2009) such as pathogenic invasion. This has also been confirmed in our study as the infection with fungus *V. inaequalis* is stronger in SBC and PBC treatments compared to fungicide application and therefore the phenolic profile is altered. Lattanzio et al. (2001) reported that chlorogenic acid inhibited spore germination or the mycelia growth. The increase in hydroxycinnamic acids after the infection may also be a consequence of the stimulated activity of phenylalanine ammonia lyase.

Among dihydrochalcones phloridzin was analysed in apple leaves. According to Gosch et al. (2009) phloridzin (phloretin 2′-*O*-*β*-D-glucoside) is the main polyphenol in the leaves, representing more than 90% of the extractable phenolics. The content of dihydrohalcones was statistically higher in PBC treatments compared to other treatments, respectively. On average, the leaves from PBC treatment contained 112.3 mg g^{-1} DW dihydrochalcones, while the leaves from SBC treatments contained 103.5 mg g^{-1} DW, control leaves 104.0 mg g^{-1} DW dihydrochalcones and the leaves treated with fungicide 89.9 mg g^{-1} DW dihydrochalcones. Higher content levels of dihydrohalcones in the leaves are often connected with scab infection. It was reported that infection with *V. inaequalis* fungus promoted higher phloridzin accumulation (Lattanzio et al., 2001; Leser and Treutter, 2005; Mikulic-Petkovsek et al., 2009), and our results confirm this statement. This can be explained by the defence reaction triggered by the attack of the pathogen. It is known that phloretin is degraded into phloroglucinol, phloretic acid and p-hydroxybenzoic acid, which successfully inhibit the fungus development (Lattanzio et al., 2001). It can therefore be concluded that phloretin is an active compound against the fungi attack in the infected tissue.

The monomeric flavanols, catechin and epicatechin, and the polymeric flavanol procyanidin B2 were determined in apple leaves. The average flavanol content during the year was almost the same in SBC and PBC treatments; while their content was 0.5 times lower in fungicide treatments compared to the control. Statistically lower contents of flavanols were generally measured in the leaves treated with fungicides in comparison to SBC or PBC treatment or the control (Table 6). The higher contents of flavanols in SBC or PBS treatments could be explained by a triggered answer to the attack of the pathogen previously described by other authors (Mikulic-Petkovsek et al., 2009; Picinelli et al., 1995; Treutter and

Table 6
The content of individual phenolic groups (mean ± SE in mg g^{-1} DW) and total phenolic content (mean ± SE in mg GAE g^{-1} DW) in apple leaves affected by different treatments.

Compound	Treatment	Sampling date				
		20 May	20 June	20 July	20 August	20 September
Hydroxycinnamic acids	Control	1.68 ± 0.16 b	0.99 ± 0.03 d	2.32 ± 0.07 d	1.13 ± 0.05 b	1.99 ± 0.06 c
	Fungicide	1.06 ± 0.07 a	0.17 ± 0.01 a	0.26 ± 0.01 a	0.16 ± 0.01 a	0.26 ± 0.01 a
	PBC	1.50 ± 0.06 b	0.91 ± 0.03 c	1.21 ± 0.03 b	1.05 ± 0.04 b	1.45 ± 0.02 b
	SBC	1.39 ± 0.10 b	0.83 ± 0.02 b	1.42 ± 0.03 c	1.14 ± 0.03 b	1.47 ± 0.07 b
Dihydrochalcones	Control	123.02 ± 3.59 NS	129.88 ± 3.13 c	102.63 ± 3.32 b	78.05 ± 2.26 a	86.41 ± 1.49 b
	Fungicide	111.74 ± 7.63 NS	90.70 ± 1.75 a	89.95 ± 1.19 a	77.82 ± 2.29 a	79.39 ± 1.50 a
	PBC	122.95 ± 4.75 NS	138.91 ± 2.58 d	112.40 ± 2.68 c	96.70 ± 2.79 c	96.73 ± 1.45 d
	SBC	118.30 ± 7.06 NS	113.46 ± 2.77 b	107.56 ± 0.63 b	86.79 ± 1.31 b	91.26 ± 1.39 c
Flavan-3-ols	Control	0.66 ± 0.03 NS	0.96 ± 0.04 c	1.11 ± 0.07 c	1.31 ± 0.04 b	1.95 ± 0.07 c
	Fungicide	0.65 ± 0.05 NS	0.49 ± 0.04 a	0.73 ± 0.02 a	0.48 ± 0.05 a	0.55 ± 0.02 a
	PBC	0.64 ± 0.01 NS	0.91 ± 0.04 c	1.54 ± 0.04 d	1.34 ± 0.03 b	1.71 ± 0.03 b
	SBC	0.73 ± 0.05 NS	0.66 ± 0.04 b	0.96 ± 0.03 b	1.28 ± 0.03 b	1.91 ± 0.09c
Flavonols	Control	9.35 ± 0.58 ab	8.98 ± 0.24 b	8.54 ± 0.26 a	7.77 ± 0.13 NS	10.84 ± 0.22 b
	Fungicide	10.68 ± 0.10 b	8.99 ± 0.14 b	10.17 ± 0.26 b	7.51 ± 0.25 NS	9.44 ± 0.20 a
	PBC	9.27 ± 0.56 a	10.18 ± 0.15 c	13.14 ± 0.33 c	7.28 ± 0.19 NS	10.87 ± 0.22 b
	SBC	8.64 ± 0.29 a	8.31 ± 0.16 a	8.61 ± 0.23 a	7.12 ± 0.10 NS	10.54 ± 0.15 b
TPC[a]	Control	54.09 ± 1.58 NS	51.63 ± 1.07 b	48.25 ± 1.70 NS	42.61 ± 1.21 a	42.72 ± 0.13 a
	Fungicide	50.73 ± 0.84 NS	47.14 ± 0.99 a	45.63 ± 1.97 NS	41.49 ± 0.85 a	40.63 ± 0.86 a
	PBC	53.69 ± 1.37 NS	58.86 ± 1.57 c	52.18 ± 2.53 NS	49.06 ± 1.76 b	46.07 ± 0.23 b
	SBC	54.15 ± 1.97 NS	53.92 ± 0.66 b	51.99 ± 1.42 NS	47.79 ± 0.51 b	45.20 ± 1.75 b

Different letters (a–d; NS: not significant) among different treatments denote significant differences (Duncan's test, P < 0.05. n = 5).
[a] TPC: total phenolic content determined by Folin–Ciocalteu method in mg gallic acid g^{-1} dry weight (mg GAE g^{-1} DW).

Feucht, 1990). Catechin and epicatechin are probably involved in the defence reaction, and their content increases in the infected tissue, which consequently causes lower content of procyanidin B2 in the leaves (data not shown). This may be explained in terms of the biosynthetic pathway of flavonoids, in which catechin and epicatechin represent a precursor for procyanidins (Roemmelt et al., 2003).

Five different quercetin-glycosides were identified from the group of flavonols (quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-rhamnoside). Quercetins represented the second largest phenolic group in addition to phloridzin in apple leaves. The highest average content of flavonols was measured in the leaves, treated with PBC treatment (10.15 mg g^{-1} DW), while the leaves from the control contained 9.09 mg g^{-1} DW flavonols, leaves from fungicidal treatments 9.35 mg g^{-1} DW flavonols and leaves from SBC treatment 8.64 mg 100 g^{-1} DW flavanols. Our results are contrary to the results of some other authors who report higher contents of flavonols in the tissues infected with *V. inaequalis* (Dixon and Paiva, 1995). Nevertheless, Picinelli et al. (1995) did not find a clear connection between the flavonol content and apple scab resistance.

Total phenolic content was also significantly affected by different treatments. Statistically highest content of total phenolics were measured in leaves treated with SBC or PBC on the second, fourth and fifth sampling date (Table 6). Our results are similar to those reported by Mikulic-Petkovsek et al. (2008), who measured higher contents of TPC in the infected leaves compared to non-infected leaves.

4. Conclusion

Apple scab control requires a large number of treatments during the growing season and potentially toxic substances present significant chemical residues on apple fruit. Therefore, it is necessary to use new non-toxic substances in apple phytoprotection, such as PBC and SBC. The study points out the potential relevance of bicarbonate salts for controlling apple scab and includes the following points: (a) SBC or PBC used at 1% concentration were effective in controlling apple scab in our experiment; (b) the use of SBC and PBC did not show any phytotoxicity and influences on lower fruit quality; (c) the use of PBC probably led to higher sugar and organic acids accumulation in the mature fruit; (d) secondary metabolites in the analysed fruit were similar than in the fungicide treatment; (e) PBC can potentially also be used as fertilizers.

PBC and SBC application performed good protection against apple scab, and moreover these products gave comparable results to classical fungicidal treatments. Additionally, the advantage of PBC and SBC application is that these substances do not accumulate in the soil, are non toxic to human health and provide an additional foliar potassium source for the plant.

The results of this pilot test indicate the possible use of PBC and SBC in protection against apple scab. As an increased environmental awareness has been recorded by apple producers and consumers these alternative protective substances should be investigated in detail and tested in practical use in organic apple production.

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2.6 SPREMEMBA VSEBNOSTI FENOLNIH SNOVI V JABLANOVIH LISTIH PO NJIHOVI MEHANSKI POŠKODBI

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Content of phenolic compounds changes after mechanical injury of apple leaves
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Nabrani so bili polno razviti, zdravi listi, na jablanov škrlup občutljive sorte 'Zlati delišes' in odporne sorte 'Florina'. Listi za analizo so bili nabrani petkrat v razmaku od 1 do 15 dni po mehanski poškodbi lista. V listih smo s pomočjo masnega spektra (HPLC-MS) in tekočinske kromatografije visoke ločljivosti določili naslednje fenolne snovi: klorogensko kislino, floridzin, epikatehin, procianidin B2, kvercetin 3-*O*-galaktozid, kvercetin 3-*O*-glukozid in kvercetin 3-*O*-ramnozid. Količina skupnih fenolov je bila določena spektrometrično. Skozi opazovano obdobje se je vsebnost fenolnih snovi razlikovala med odporno in občutljivo sorto, kot tudi med opazovano periodo pri posamezni sorti. Vsaka izmed fenolnih snovi se je na mehansko poškodbo odzvala različno. Po mehanski poškodbi smo zaznali povečano vsebnost klorogenske kisline, procianidina B2 in skupnih fenolov. Po mehanski poškodbi listov je bilo naraščanje fenolnih snovi bolj izrazito pri listih občutljive sorte kot pri listih odporne sorte. Rezultati raziskave kažejo, da se rastlina na mehansko poškodbo odzove s spremenjeno vsebnostjo fenolnih snovi.

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Content of Phenolic Compounds Changes after Mechanical Injury of Apple Leaves

By

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With 1 Figure

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Summary

SLATNAR A., STAMPAR F. & VEBERIC R. 2013. Content of phenolic compounds changes after mechanical injury of apple leaves. – Phyton (Horn, Austria) 53 (1): 113–124, with 1 figure.

Fully developed healthy leaves were collected from two apple cultivars, apple scab susceptible 'Golden Delicious' and resistant 'Florina'. Leaves were analyzed 5 times in a period of 1–15 days after mechanical injury and phenolic compounds, using high-performance liquid chromatography with mass spectrometer (HPLC-MS), were determined. In apple leaves, the following phenolic compounds were detected: chlorogenic acid, phloridzin, epicatechin, procyanidin B2, quercetin-3-O-galactoside, quercetin-3-O-glucoside and quercetin-3-O-rhamnoside. Total phenolic compounds in apple leaves were determined spectrophotometrically. During the observed period, the content of phenolic compounds differed between resistant and susceptible cultivar and also changed in time. Each of the phenolic compounds responded differently to mechanical injury. An increase in chlorogenic acid, procyanidin B2 and total phenolic content was observed after mechanical injury. The increase of phenolic content was much more prominent in leaves of the susceptible cultivar compared to the resistant one after mechanical injury. The results of study show that plants respond similarly to mechanical injury with modified phenolic content and that the response is not the same compared to the pathogenic infection.

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Zusammenfassung

SLATNAR A., STAMPAR F. & VEBERIC R. 2013. Content of phenolic compounds changes after mechanical injury of apple leaves [Der Gehalt an phenolischen Verbindungen ändert sich nach mechanischer Verletzung in Blättern von Apfelbäumen]. – Phyton (Horn, Austria) 53 (1): 113–124, mit 1 Abbildung.

Von zwei Apfelsorten, einmal von der schorfempfindlichen Sorte "Golden Delicious" und einmal von der schorfresistenten Sorte "Florina", wurden voll entwickelte gesunde Blätter gesammelt. 5-mal wurden die Blätter auf phenolische Verbindungen während eines Zeitraums von 1 bis 15 Tage nach einer mechanischen Verletzung mittels high-performance Flüssigkeitschromatographie mit Massenspektrometrie-Koppelung (HPLC-MS) untersucht. Folgende phenolische Komponenten konnten in Blättern von Apfelbäumen gefunden werden: Chlorogensäure, Phloretin, Epicatechin, Procyanidin B2, Quercetin-3-O-Galaktose, Quercetin-3-O-Glucosid und Quercetin-3-O-Rhamnosid. Der Gesamtgehalt der phenolischen Komponenten in den Apfelblättern wurde spektralphotometrisch bestimmt. Während des Beobachtungszeitraums wurden Unterschiede im Phenolgehalt zwischen resistenten und empfindlichen Sorten festgestellt und auch der Zeitpunkt des Auftretens der einzelnen Phenole war nicht gleich. Jede der phenolischen Komponenten reagierte auf mechanische Verletzung anders. Ein Anstieg von Chlorogensäure, Procyanidin B2 und des Gesamt-Phenol-Gehaltes war nach mechanischer Verletzung zu beobachten. Im Vergleich zur resistenten Sorte war bei der empfindlichen Sorte der Anstieg des Phenolgehaltes in den Blättern nach mechanischer Verletzung wesentlich ausgeprägter. Die Ergebnisse zeigen, dass die Pflanzen ähnlich auf mechanische Verletzungen mit ihren Veränderungen im Phenolgehalt antworten, aber dass diese Reaktion nicht mit der einer Pathogeninfektion vergleichbar ist.

Introduction

Phenolic compounds in plant leaves are involved in several physiological mechanisms. They are well known UV-protecting agents in plant tissues (TAKEDA & al. 1994) and are often involved in plant-pathogen interactions, both constitutively and as newly induced compounds (CLÉRIVET & al. 1996). The composition of these phenols in plant tissues is markedly influenced by such environmental conditions as UV-light (MARKHAM & al. 1998), temperature (RIVERO & al. 2001) and nutrition (RUEHMANN & al. 2002).

Among phenolic compounds, flavonoids are known to play a key role in plant defence against both biotic and abiotic stresses (DIXION & al. 2002), and are one of several factors contributing to plant resistance (TREUTTER 2005). Many flavonoid compounds can function as passive or inducible barriers against herbivores or microbial pathogens, and the flavonoid content can increase or the flavonoid composition can change in response to pathogen attack (DIXION & PAIVA 1995, MIRANDA & al. 2007, CARLSEN & al. 2008). The involvement of flavonoids in plant defence greatly depends on the species. Annual grasses have low flavonoid content, and thus flavonoids are less significant in their defence (LOGEMANN & HAHLBROCK 2002).

Modified flavonoid profile can be detected due to plant wounding by herbivore, parasitism or by mechanical injury. It can result in retardation of plant growth and offers the easy penetration for pathogens into the plant tissue. Plants respond to wounding by different strategies. The biochemical changes and the activation of genes to produce defence related proteins have been detected in many studies (Bowles 1990, Liu & al. 2003).

However, after mechanical wounding of bilberry leaves flavonoid biosynthesis was not increased and therefore flavonoid compounds may represent only passive defences against herbivores (Jaakola & al. 2008). On the other hand, high irradiance, temperature extremes, UV radiation, mineral imbalances, water stress, mechanical injury and pathogen attack, have all been shown to enhance the production of anthocyanins in leaves (McClure 1975, Chalker-Scott 1999).

Plant defence against the pathogen occurs with a combination of different mechanisms: (1) structural characteristics, representing physical obstacles that prevent the pathogen to enter and spread throughout the plant, (2) biochemical reactions in the plant cells and tissues, resulting in formation of compounds toxic to the pathogen or that create conditions, which are unfavourable for the growth of the pathogen (suck as phenolic compounds). Many reports state that a higher amount of phenolic compounds can be detected after the pathogen attack. But reports on the modifications of the phenolic content due to mechanical injury, which it known to cause a higher content of phenolic compounds, are very scarce. In our study, we aim to investigate how the mechanical injury induces a higher amount of individual phenolic and total phenolic compounds in apple leaves with the intent to study biochemical responses of the plant to such forms of stress. Moreover, the goal of this study is to clarify, which types of stress (mechanical injury/pathogen attack) causes an increase of some crucial phenolic compounds in plants.

Abbreviations: BHT = 2.6-di-tert-butyl-4-methylphenol, DW = dry weight; GAE = gallic acid equivalents; HPLC-MS = high-performance liquid chromatography with mass spectrometer; TPC = total phenolic content

Materials and Methods

Plant Material and Experimental Design

Experiment was carried out in 2010 sampling leaves from ten-year-old apple trees (*Malus domestica* Borkh., *Rosaceae*) grafted on M9 rootstocks, located at the University experimental orchard in Ljubljana (latitude: 46° 2', longitude: 14° 28'). Samples were collected from apple scab susceptible cv. 'Golden Delicious' and resistant cv. 'Florina'. 250 fully developed healthy leaves of each cultivar were selected and one half of the leaves were mechanically injured with a scalpel. Leafs were sampled on the 1st, 3rd, 5th, 10th and 15th day after the injury. The collected leaves were immediately frozen in liquid nitrogen and stored at –20°C until analysis.

Extraction and Determination of Individual Phenolic Compounds

Frozen leaves were lyophilized and ground in a mortar. For each sampling date, 5 repetitions of 5 leaves per group were extracted as described by MIKULIC-PETKOVSEK & al. 2008 with some modification. The fine powder (70 mg) was extracted with methanol (5 ml) containing 1% (w/v) 2.6-di-*tert*-butyl-4-methylphenol (BHT) in a cooled ultrasonic bath for 1 hour. The treated samples were centrifuged for 7 min at 10000 *rpm*. The supernatant was filtered through a Chromafil AO-45/25 polyamide filter (Macherey-Nagel, Düren, Germany) and transferred to a vial prior to injection into a HPLC system. Samples were analyzed using a Thermo Finnigan Surveyor HPLC system (Thermo Scientific, San Jose, CA) with a diode array detector at 280 nm (hydroxycinnamic acids, dihydrochalcones and flavan-3-ols), 350 nm (flavonols). A Phenomenex (Torrance, CA) HPLC column C18 (150 x 4.6 mm, Gemini 3 μ) protected with a Phenomenex security guard column operated at 25°C was used. The injection volume was 20 μl and the flow rate maintained at 1 ml/min. The elution solvents were aqueous 1% formic acid (A) and 100% acetonitrile (B). Samples were eluted according to the gradient described by MARKS & 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; and finally washing and reconditioning of the column. Identification of compounds was achieved by comparing retention times and their UV-VIS spectra from 200 to 600 nm, as well as by the addition of an external standard. Compounds were further identified using a mass spectrometer (Thermo Scientific, LCQ Deca XP MAX) with an electrospray interface (ESI) operating in negative ion mode. The analyses were carried out using full-scan data dependent MSn scanning from m/z 115 to 2000. Quantification was achieved according to the concentrations of a corresponding external standard.

Concentrations of phenolic compounds were calculated from the peak areas of the sample and the corresponding standards and expressed in mg/g dry weight (DW).

Determination of Total Phenolic Content

The extraction of leaf samples for the determination of total phenolic content (TPC) was made according to the same protocol as for individual phenolics, with the difference that no BHT was added. TPC of the extracts was assessed using the Folin-Ciocalteu phenol reagent method (SINGLETON & ROSSI 1965). Six ml of twice-distilled water and 500 μl of Folin-Ciocalteu reagent were added to 100 μl of the sample extracts and after waiting for between 8 s and 8 min at room temperature, 1.5 ml of sodium carbonate (20% w/v) and 1.9 ml twice-distilled water was added. The extracts were mixed and allowed to stand for 30 min at 40°C before measuring absorbance at 765 nm on a Lambda Bio 20 UV/VIS spectrophotometer (Perkin Elmer, Waltham, MA). A mixture of water and reagents was used as a blank. The total phenolic content was expressed as gallic acid equivalents (GAE) in g/kg DW. Absorptions were measured in three replicates.

Statistical Analysis

The data was analyzed using the Statgraphics Plus 4.0 program (Manugistics. Inc.; Rockville, Maryland, USA). Data from the analyses were tested for differences among injured and non-injured leafs using one-way analysis of variance (ANOVA).

Data from the analyses were tested for differences among sampling dates using one-way analysis of variance (ANOVA). Significant differences among means were determined by the least significant difference (LSD) with a significance level of $p \leqslant 0.05$ for differences between the injured and non-injured leaves; and by Duncan's multiple range test for differences among sampling dates with a significance level of $p \leqslant 0.05$.

Results and Discussion

Among the hydroxycinnamic acids, chlorogenic acid was the most abundant in apple leaves. No significant differences in the content of chlorogenic acid were detected between the cultivars. The injured leaves of cv.´Golden Delicious' contained statistically higher amounts (1.03–1.88 mg/g DW) of this phenolic compound compared to non-injured (1.83–3.62 mg/g DW) on the 1st, 3rd and 5th day after the injury (Table 1). The injured leaves of cv. 'Florina' contained statistically higher amounts (0.74–2.38 mg/g DW) of chlorogenic acid on all analyzed days (Table 2). Generally, similar changes were detected in both cultivars, but the leaves from cv. 'Florina' contained higher amounts in chlorogenic acid through the sampled period. A higher amount of chlorogenic acid in resistant cultivar through the season was also reported by Mikulic-Petkovsek & al. 2009. Hahlbrock & Scheel 1989 and Bernards & Lewis 1992 reported that wounding can induce an increase of chlorogenic acid, and that cell wall-bound phenolic esters may act directly as defence compounds or may serve as precursors for the synthesis of lignin, suberin, and other wound-induced polyphenolic barriers.

In apple trees, the major sub-family of flavonoids is represented by dihydrochalcones, found in high amounts (up to 5% DW) in the leaves (Hunter & Hull 1993, Treutter 2001). Among known dihydrochalcones, phloridzin and its aglycone, phloretin, are simple forms and their biosynthesis in *Malus* species has recently been deciphered (Judgé & al. 2008, Gosch & al. 2009). However, their function in plants remains unresolved. They have been hypothesized to act as UV filters in leaves (Treutter 2006) and their role in resistance to pathogens has also been suggested (Picinelli & al. 1995, Pontais & al. 2008). A statistically higher amount (139.67 mg/g DW) of phloridzin on the 1st day after the injury was observed in cv. 'Golden Delicious'. In injured leaves of cv. 'Golden Delicious', on the 3rd and 5th day a higher amount was noted, but it was not statistically significant and it disappeared in the last two sampling days (Table 1). In resistant cultivar no differences between injured and non-injured leaves were detected (Table 2). Higher amounts of phloridzin in apple peel infected with *Venturia inaequalis* was reported by Slatnar & al. 2010. Our experiment shows that mechanical injury has no influence on the content of phloridzin, so it can be hypothesized that the increase of this compound is a response to other stress factors.

Table 1. The content of individual phenolic compounds (mean ± SE in mg/g DW) after mechanical injury in apple leafs of cv. 'Golden Delicious'.

		Days after injury				
		1	3	5	10	15
Chlorogenic acid	no-injury[a]	1.42 ± 0.09**a**	1.88 ± 0.14**a**	1.03 ± 0.11**a**	0.84 ± 0.03 **NS**	0.73 ± 0.04 **NS**
	injury	2.64 ± 0.14**b**	3.62 ± 0.44**b**	1.83 ± 0.16**b**	0.84 ± 0.02 **NS**	0.72 ± 0.02 **NS**
	no-injury[b]	c	d	b	ab	a
	injury	c	d	b	a	a
Phloridzin	no-injury	101.00 ± 3.37**a**	135.40 ± 7.06 **NS**	118.08 ± 7.58 **NS**	74.98 ± 8.94 **NS**	85.52 ± 7.97 **NS**
	injury	139.67 ± 4.42**b**	143.21 ± 14.76 **NS**	125.20 ± 4.65 **NS**	65.41 ± 7.13 **NS**	85.07 ± 6.98 **NS**
	no-injury	bc	d	cd	a	ab
	injury	b	b	b	a	a
Epicatechin	no-injury	0.37 ± 0.04**a**	0.58 ± 0.11 **NS**	0.55 ± 0.04 **NS**	1.60 ± 0.19 **NS**	2.07 ± 0.09**a**
	injury	0.68 ± 0.03**b**	0.81 ± 0.13 **NS**	0.71 ± 0.07 **NS**	1.19 ± 0.20 **NS**	1.40 ± 0.13**b**
	no-injury	a	a	a	b	c
	injury	a	a	a	b	b
Procyanidin B2	no-injury	0.03 ± 0.00**a**	0.05 ± 0.00**a**	0.05 ± 0.00 **NS**	0.02 ± 0.00 **NS**	0.03 ± 0.00 **NS**
	injury	0.04 ± 0.00**b**	0.05 ± 0.00**b**	0.04 ± 0.00 **NS**	0.03 ± 0.00 **NS**	0.03 ± 0.00 **NS**
	no-injury	bc	c	c	a	b
	injury	b	c	b	a	a
Q-galactoside	no-injury	2.24 ± 0.04 **NS**	2.24 ± 0.04**a**	2.59 ± 0.12**a**	1.97 ± 0.15 **NS**	1.96 ± 0.10 **NS**
	injury	2.21 ± 0.08 **NS**	2.21 ± 0.04**b**	1.83 ± 0.12**b**	1.65 ± 0.26 **NS**	2.08 ± 0.26 **NS**
	no-injury	ab	ab	b	a	a
	injury	a	b	a	a	a
Q-glucoside	no-injury	1.97 ± 0.02**b**	1.94 ± 0.09**a**	1.93 ± 0.09**b**	1.59 ± 0.15 **NS**	1.87 ± 0.19 **NS**
	injury	1.87 ± 0.03**a**	2.58 ± 0.25**b**	1.53 ± 0.13**a**	1.31 ± 0.22 **NS**	1.80 ± 0.17 **NS**
	no-injury	NS	NS	NS	NS	NS
	injury	a	b	a	a	a
Q-rhamnoside	no-injury	7.21 ± 0.24 **NS**	7.45 ± 0.12 **NS**	7.38 ± 0.16**b**	7.12 ± 0.57 **NS**	7.15 ± 0.51 **NS**
	injury	7.47 ± 0.34 **NS**	8.19 ± 0.52 **NS**	5.91 ± 0.25**a**	5.21 ± 0.63 **NS**	7.23 ± 0.55 **NS**
	no-injury	NS	NS	NS	NS	NS
	injury	c	c	ab	a	bc

[a] Different letters in columns (a-b; NS-not significant) denote significant differences in phenolic compounds among treatments for each set of sampling dates separately (LSD test, $P \leqslant 0.05$).
[b] Different letters in lines (a–d; NS- not significant) denote significant differences in phenolic compounds among sampling dates for each treatment separately (Duncan's test, $P \leqslant 0.05$).

Table 2. The content of individual phenolic compounds (mean ± SE in mg/g DW) after mechanical injury in apple leafs of cv. 'Florina'.

		Days after injury				
		1	3	5	10	15
Chlorogenic acid	no-injury[a]	1.54 ± 0.15**a**	1.82 ± 0.18**a**	1.39 ± 0.07**a**	0.64 ± 0.03**a**	0.82 ± 0.06**a**
	injury	2.04 ± 0.13**b**	2.38 ± 0.10**b**	1.69 ± 0.04**b**	0.74 ± 0.02**b**	1.17 ± 0.01**b**
	no-injury[b]	bc	c	b	a	a
	injury	d	e	c	a	b
Phloridzin	no-injury	142.51 ± 5.78 **NS**	143.22 ± 5.64 **NS**	140.09 ± 8.14 **NS**	69.10 ± 3.92 **NS**	78.38 ± 4.29 **NS**
	injury	141.52 ± 2.23 **NS**	140.73 ± 7.63 **NS**	146.25 ± 6.18 **NS**	74.34 ± 6.91 **NS**	86.35 ± 8.27 **NS**
	no-injury	b	b	b	a	a
	injury	b	b	b	a	a
Epicatechin	no-injury	0.22 ± 0.02 **NS**	0.21 ± 0.01 **NS**	0.21 ± 0.01**a**	1.38 ± 0.06 **NS**	2.00 ± 0.06**a**
	injury	0.20 ± 0.02 **NS**	0.23 ± 0.01 **NS**	0.25 ± 0.00**b**	1.12 ± 0.15 **NS**	1.70 ± 0.10**b**
	no-injury	a	a	a	b	c
	injury	a	a	a	b	c
Procyanidin B2	no-injury	0.05 ± 0.01**a**	0.07 ± 0.00**a**	0.07 ± 0.00**a**	0.03 ± 0.00 **NS**	0.03 ± 0.00**a**
	injury	0.07 ± 0.01**b**	0.10 ± 0.00**b**	0.09 ± 0.00**b**	0.03 ± 0.00 **NS**	0.04 ± 0.00**b**
	no-injury	b	c	c	a	a
	injury	c	d	d	a	b
Q-galactoside	no-injury	1.79 ± 0.11 **NS**	2.18 ± 0.18 **NS**	2.41 ± 0.29 **NS**	1.58 ± 0.08 **NS**	2.17 ± 0.08 **NS**
	injury	1.76 ± 0.06 **NS**	2.33 ± 0.13 **NS**	2.49 ± 0.27 **NS**	1.59 ± 0.12 **NS**	1.93 ± 0.18 **NS**
	no-injury	ab	bc	c	a	Bc
	injury	a	bc	c	a	Ab
Q-glucoside	no-injury	1.63 ± 0.08 **NS**	2.01 ± 0.12 **NS**	2.11 ± 0.18 **NS**	1.58 ± 0.06 **NS**	2.02 ± 0.07 **NS**
	injury	2.01 ± 0.12 **NS**	2.18 ± 0.12 **NS**	2.32 ± 0.18 **NS**	1.55 ± 0.09 **NS**	2.11 ± 0.17 **NS**
	no-injury	a	b	b	a	B
	injury	ab	bc	c	a	Bc
Q-rhamnoside	no-injury	6.67 ± 0.45 **NS**	6.74 ± 0.39 **NS**	6.42 ± 0.33 **NS**	6.59 ± 0.11 **NS**	7.86 ± 0.24 **NS**
	injury	7.33 ± 0.48 **NS**	7.12 ± 0.41 **NS**	7.11 ± 0.60 **NS**	7.88 ± 0.23 **NS**	8.26 ± 0.53 **NS**
	no-injury	NS	NS	NS	NS	NS
	injury	NS	NS	NS	NS	NS

[a] Different letters in columns (a–b; NS-not significant) denote significant differences in phenolic compounds among treatments for each set of sampling dates separately (LSD test, $P \leqslant 0.05$).
[b] Different letters in lines (a–d; NS- not significant) denote significant differences in phenolic compounds among sampling dates for each treatment separately (Duncan's test, $P \leqslant 0.05$).

Epicatechin and procyanidin B2 were determined from the group of flavan-3-ols. MIKULIC-PETKOVSEK & al. 2009 reported a higher content of epicatechin in the resistant cultivar compared to the susceptible cultivar. However differences in the content of flavan 3-ols were not detected between the susceptible and resistant cultivar in our study. An increase in the content of epicatechin from 0.37 to 0.68 mg/g DW in cv. 'Golden Delicious' was detected on the 1st day after the injury but as soon as on the 10th day after the injury a turnabout occurred. After that, the non-injured leafs contained higher levels of epicatechin (0.71–1.19 mg/g DW), and a further increase was detected on 15th day after the injury (Table 1). A similar pattern was also recorded in the resistant cultivar (Table 2). The content of procyanidin B2 significantly increased in the first days after the injury (from 0.03 to 0.04 mg/g DW in cv. 'Golden Delicious' and from 0.05 to 0.07 mg/g DW in cv. 'Florina') in both cultivars analyzed. In the leaves of the susceptible cultivar this increase was not detected at later samplings, while in the resistant cultivar this difference was visible throughout the observed period (Tables 1 and 2). The same increase of dimeric procyanidins and epicatechin after the mechanical injury in the first leave of pear was reported by ANDREOTTI & al. 2006. Similarly FEUCHT 1994 showed that mechanical wounds induce the accumulation of oligomeric procyanidins, during defence barrier formation close to the injured tissue.

The following compounds from the group of flavonols were determined: quercetin-3-O-galactoside, quercetin-3-O-glucoside and quercetin-3-O-rhamnoside, all present in high content in apple leaves. On increase in the content of flavonols after the mechanical injury was detected in the cv. 'Florina' throughout the observed period (Table 2). On the 3rd and 5th day after the injury a statistically higher content (2.24–2.59 mg/g DW) of quercetin-3-O-galactoside was noticed in non-injured leaves. Quercetin-3-O-glucoside and quercetin-3-O-rhamnoside showed a different pattern. On the 5th day after the injury a statistically higher content of querercetin-3-O-rhamnozide was measured in healthy leaves. Likewise, statistically higher contents of quercetin-3-O-glucoside were determined on the 1st (1.97 mg/g DW) and 5th (1.93 mg/g DW) day after injury in healthy leaf tissue (Table 1). Many authors (TREUTTER & FEUCHT 1990, PICCINELI & al. 1995, MIKULIC-PETKOVSEK & al. 2009) have already shown that resistant cultivars contain greater amounts of flavonols in leaf tissue than susceptible ones. But in our case higher amount of flavonols were not observed in the leaves of the resistant cultivar. Our study shows that mechanical injury has no influence on the changes in quercetin content, and that higher amounts of quercetin can accumulate due to some other factors, such as pathogenic invasion. Pathogenic invasion may explain higher quercetin levels in infected tissues, because it is known that flavonols are synthesized by plants in response to the pathogen attack (DIXION & PAIVA 1995).

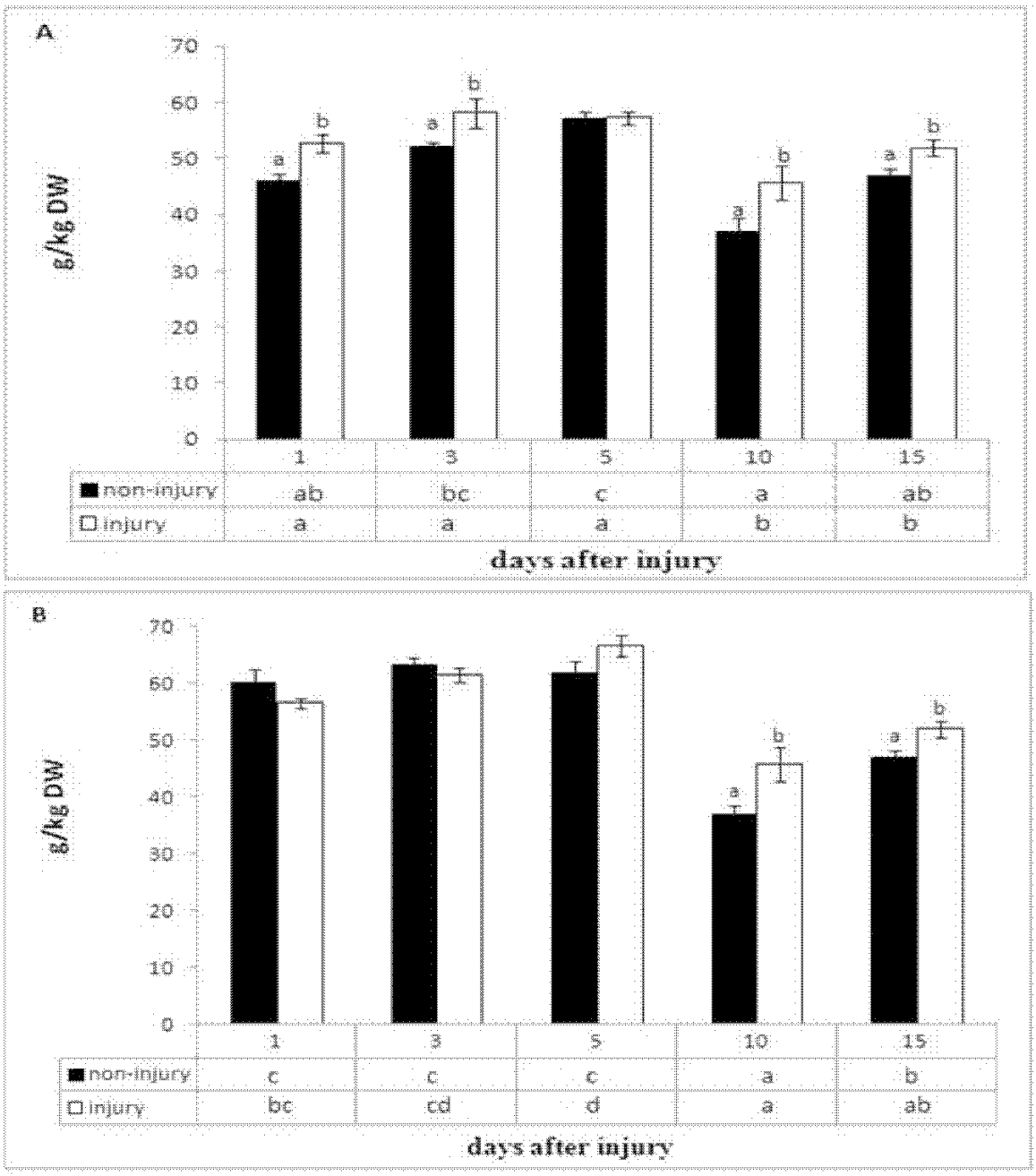

Fig. 1. Content of TPC (mean ± SE in g/kg DW) of cv. 'Golden Delicious' (A) and cv. 'Florina' (B) determined at different days after injury in injured and non-injured leaves (above each column) and among sampling dates (in the Table).

Statistically significant differences in the total phenolic content (TPC) between the injured and non-injured leaves of cv. 'Golden Delicious' occured on almost every sampling date (Fig. 1A). In the resistant cultivar 'Florina' a statistically significant increase occurred only on the last two samplings (Fig. 1B). Total phenolic content of injured leaves can thus be a

good tool for distinguishing resistant and susceptible apple cultivars, however it is probably not valid for other plant parts, such as fruit, as reported by LATA & al. 2005. Differences in TPC between the resistant and susceptible cultivar were minimal. A previous experiment with cotton leaves indicated that mechanically injured leaves contained less phenolics compared to the control leaves (PERVEEN & al. 2001). Our results contrast those made on cotton, which can be explained with a vast number of internal and external factors influencing TPC (stress conditions, species, and cultivar). A higher amount of total phenolic content in apple leaves infected with *Venturia inaequalis* was reported by MIKULIC-PETKOVSEK & al. 2008, similar to our results.

Conclusion

The content of leaf phenolic compounds was also greatly affected by wounding. Plant metabolic reaction to localized stress was detectable in specific compound classes: hydroxycinnamic acids, flavan-3-ols, flavonols and total phenolic content. The inhibitory effects of polyphenols as a response to wounding have been investigated in depth in the plant-herbivore relationship (FRITZ & al. 2001). This accumulation of flavanols is associated with new biosynthesis (FEUCHT & al. 1996, BECKAM 2000) and, specifically for procyanidins, may be related to the marked oxidation process triggered by the wounding that occurs soon after the stress event on the leaves. As far as we have researched the matter we can establish that the mechanical injury induces changes in the content of phenolic compounds, which differ in relation to the changes that are provoked by the infection with the pathogen. With this study we intended to elucidate the types of changes in the phenol content after the mechanical reaction and add to the knowledge we have attained during the research of the interaction plant-pathogen.

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3 RAZPRAVA IN SKLEPI

3.1 RAZPRAVA

Kombinacije strukturnih posebnosti in biokemičnih reakcij, vključenih v obrambo rastline, so v različnih sistemih gostitelj-patogen različne. Kombinacija se lahko celo pri istem gostitelju in patogenu spreminja s starostjo rastline, vrsto rastlinskega organa in tkiva, prehranskega statusa rastline in podnebjem. Čeprav strukturne značilnosti do neke mere varujejo rastlino pred patogeni, pa je danes jasno, da je odpornost poleg fizičnih preprek odvisna tudi od snovi, ki jih celice izdelajo pred oziroma po okužbi. Na to kaže dejstvo, da določen patogen nekaterih varietet ne okužuje, čeprav te nimajo nikakršnih strukturnih posebnosti. V takih primerih je obramba kemične narave in je pri zdravih rastlinah posledica številnih sekundarnih metabolitov s protimikrobnim delovanjem. Take snovi so v rastlini lahko prisotne v aktivni obliki ali pa so shranjene v obliki neaktivnih prekurzorjev. Prekurzorje nato v aktivno obliko pretvorijo encimi gostitelja kot odgovor na okužbo patogena ali kot odgovor na poškodbo tkiva (Agrios, 1997).

Da bi poskusili odgovoriti na številna odprta vprašanja v povezavi z odzivom rastline na okužbo s patogenom, smo v letu 2008 zastavili različne poskuse, s katerimi smo želeli ugotoviti kakšen je odziv kožice jabolka na okužbo z jablanovim škrlupom z vidika fenolnega profila kot tudi še popolnoma nepoznane aktivnosti encimov, ki so v nastanek fenolnih spojin vključeni.

Več avtorjev poroča, da okužba z jablanovim škrlupom poveča akumulacijo fenolnih snovi, ki pa je lahko glede na oddaljenost od mesta okužbe različna (Treutter in Feucht, 1990; Feucht in sod., 1998; Mayr in Treutter, 1998; Mikulič-Petkovšek in sod., 2008, 2009a). Zato smo v prvem poskusu v tehnološko zrelih plodovih sorte 'Zlati delišes' preučevali akumulacijo fenolnih snovi in aktivnost pripadajočih encimov fenilpropanoidne poti v povezavi z oddaljenostjo od mesta okužbe. Tkiva smo ločili na tri različna obravnavanja: pega, okolica pege, ki je zajemala tkivo 1-2 mm okoli škrlupaste pege in zdravo tkivo.

V skupini hidroksicimetnih kislin smo opazili, da je pega v primerjavi z zdravim tkivom vsebovala statistično značilno večjo vsebnost tako klorogenske kakor tudi ferulne kisline. Izraženo v vsebnosti je to pomenilo kar 5 do 6,7-krat večjo vsebnost ferulne kisline (0,2 mg/100 g sveže mase (SM)) in 8,5 do 13,6-krat večjo vsebnost klorogenske kisline (10,5 mg/100 g SM) v primerjavi z zdravim tkivom. Dihidrohalkoni so skupina fenolnih snovi, ki se jo največkrat povezuje z odpornostjo na različne bolezni. Študije na jablani kažejo, da prisotnost floridzina in njegovih aglikonov lahko povezujemo z odpornostjo na jablanov škrlup (Hamauzu, 2006). Rezultati dihidrohalkonov so pokazali podobno sliko kot hidroksicimetne kisline, saj smo najnižjo vsebnost floridzina določili v zdravem tkivu (168,4 mg/100g SM), ki sta mu sledila pega in okolica pege. Vsebnost floretina je bila v zelo nizki koncentraciji določena le v pegi. Treutter in Feucht (1990) poročata, da tkivo, okuženo z jablanovim škrlupom, kaže povečano akumulacijo nekaterih flavan 3-olov (epikatehina, katehina, procianidina B2, procianidina B5, procianidina E-B5). Njuna poročanja se skladajo z dobljenimi rezultati v naši študiji, saj smo tudi mi v okuženem tkivu zaznali večje vsebnosti epikatehina, katehina in procianidina B2, nismo pa med

okolico pege in zdravim tkivom zaznali razlik v vsebnosti vseh treh določenih flavan 3-olov. V skupini flavonolov smo določili kvercetin 3-*O*-ramnoglukozid, kvercetin 3-*O*-galaktozid, kvercetin 3-*O*-glukozid in kvercetin 3-*O*-ramnozid. V povprečju je okolica pege vsebovala največ flavonolov (238,3 mg/100 g SM), sledilo ji je zdravo tkivo in pega. O povečani akumulaciji flavonolov po okužbi z jablanovim škrlupom, kot tvorbi zaščitne bariere poročajo že Feucht in sod. (1998) in Mayr in Treutter (1998).

Ker glede na prisotno količino analiziranih fenolov težko govorimo kdaj je do njihove sinteze prišlo, smo študijo nadgradili z določanjem delovanja nekaterih ključnih encimov fenilpropanoidne poti. Aktivnost PAL-a smo uspeli določiti le v škrlupasti pegi. Rezultati so pokazali, da je najvišjo aktivnost delovanja encimov v analiziranih tkivih kazal encim CHS/CHI, katerega aktivnost je glede na oddaljenost od mesta okužbe padala. To nakazuje, da okužba z jablanovim škrlupom vodi do povečane sinteze različnih fenolov. Pri določanju encimske aktivnosti FHT-ja smo bili neuspešni, saj njegove aktivnosti nismo zaznali. FHT je odgovoren za pretvorbo flavanonov v dihidroflavonole, ki se nato reducirajo v potrebne leukocianidine. Redukcijo opravi encim DFR, čigar aktivnost je bila največja v pegi. Odsotnost delovanja FHT-ja bi lahko razložili z dejstvom, da v kožici analizirane sorte antociani niso prisotni in zato tudi encim ni aktiven. Vendar se moramo zavedati, da je za sintezo flavonolov delovanje encima FHT ključno, saj so dihidroflavonoli njegovi intermediati. To bi lahko pomenilo, da je encim aktiven v drugi razvojni stopnji ploda ali pa da je njegova aktivnost nižja od detekcije naše metode. Kot pri ostalih encimih je tudi encim FLS, ki je odgovoren za nastanek flavonolov (kvercetin glikozidov), je v pegi kazal večjo aktivnost kot v okolici pege oziroma zdravem tkivu. A vendar smo ravno v pegi zaznali nižje koncentracije flavonolov. To bi lahko pojasnili z navedbami nekaterih avtorjev (Feucht in sod., 1998; Mayr in Treutter, 1998), ki trdijo, da nalagaje flavonolov v okolici okužbe ustvari zaščitno bariero ali celo, da prihaja do sedaj še nepojasnjenih kasnejših procesov v tkivu. Razlago bi lahko iskali tudi v zmanjšani oskrbi z intermediati, saj nismo uspeli določiti aktivnosti FHT-ja. Prav nasprotno sliko v aktivnosti kot pri ostalih encimih smo dobili pri D2'GT-ju. Slednji encim je ključen za pretvorbo floretina v floridzin, njegova aktivnost pa je z oddaljevanjem od mesta okužbe naraščala. Padec v aktivnosti encima lahko pojasnimo z upoštevajočim dejstvom, da kot aktivna snov pri obrambi z glivo deluje aglikon in ne 2'-O-glukozid. To dejstvo je potrdila tudi sama koncentracija aglikona floretina določenega v pegi. Prav tako je bila v pegi visoka tudi količina floridzina, vendar se njegova vsebnost od ostalih tkiv ni statistično razlikovala.

Drugi encim fenolne oksidacije je POD, ki oksidira fenole do kinonov in katalizira nastanek peroksida. Peroksid ni le protimikrobna spojina, temveč tudi sprošča visokoreaktivne proste radikale in tako povečuje stopnjo polimerizacije fenolnih spojin v ligninu podobne snovi. Te spojine se nato nalagajo v celične stene, s čimer motijo nadaljnjo rast in razvoj patogena (Zhao in sod., 2008; Harrison in sod., 1995). To smo potrdili tudi v naši raziskavi, saj se je aktivnost encima POD z oddaljenostjo od mesta sinteze zmanjševala.

Rezultati poskusov so pokazali, da v fenilpropanoidni poti prihaja do sprememb po okužbi z jablanovim škrlupom, kar je bilo prvič potrjeno tudi s stopnjo aktivnosti encimov. Ker pa je bila uvodna raziskava opravljena le na tehnološko zrelih plodovih, smo že v istem letu

želeli preveriti, kaj se s samo količino fenolnih snovi in aktivnostjo pripadajočih encimov dogaja v času razvoja oziroma dozorevanja plodov. V naslednji študiji smo tako vzorčenje jabolk sorte 'Braeburn 'opravili v dveh terminih, medtem ko so obravnavanja za analizo ostala enaka kot v prvem poskusu.

S poskusom smo potrdili, da se kožica jabolk na okužbo z jablanovim škrlupom odzove s povečano akumulacijo fenolnih snovi, prav tako pa smo zaznali večje aktivnosti določenih pripadajočih encimov fenilpropanoidne poti v pegi in njeni okolici. Rezultati raziskave med posameznimi tkivi so bili primerljivi in v enakih trendih, kot jih Slatnar in sod. (2010a) poročajo v svoji predhodni raziskavi. Med dozorevanjem smo v zrelih plodovih zaznali nižjo aktivnost encimov kot v tehnološko zrelih plodovih. Izjema sta bila le encima DFR in D2'GT, katerih aktivnost se je z dozorevanjem povečevala. Tudi pri koncentraciji fenolnih snovi smo zaznali nespremenjene koncentracije vseh analiziranih fenolov z izjemo kavne kisline in epikatehina, kjer je prišlo do zmanjšanja vsebnosti.

Rezultati prvih raziskav so bili povod za nadaljnje raziskave v letu 2009, ki so poleg treh različnih tkiv kožice zajele še: (1) primerjavo odporne ('Florina') in na jablanov škrlup občutljive sorte ('Zlati delišes') in (2) odziv tkiva izraženega tako v akumulaciji fenolnih snovi kot aktivnosti encimov tekom celotnega razvoja ploda. Pogosto opazimo, da v odpornih sortah prihaja do hitrejše sinteze nekaterih fenolnih spojin toksičnih za patogene, kot se to dogaja v občutljivih varietetah. Nekatere od teh snovi lahko dosežejo koncentracijo, ki je toksična za patogena. Kljub temu se večina pojavlja v istem prizadetem tkivu, njihova kombinacija pa je verjetno povezana z inhibicijo okužbe v odpornih varietetah.

Več avtorjev (Mikulič-Petkovšek in sod., 2007; Picinelli in sod., 1995; Michalek in sod., 1999) v svojih študijah poročajo o višjih vsebnostih nekaterih posameznih fenolov kot tudi skupnih fenolov v odpornih sortah v primerjavi z občutljivimi sortami, vendar pa mehanizem njihovega delovanja pri odpornosti še vedno precej slabo razumemo. Rezultati raziskave niso pokazali statistično značilnih razlik v skupinah hidroksicimetnih kislin, dihidrohalkonov, flavan 3-olov in flavonolov med odporno in na jablanov škrlup občutljivo sorto z izjemo drugega termina vzorčenja, kjer smo v kožici na jablanov škrlup odporne sorte 'Florina' zaznali večji vsebnosti flavan 3-olov in flavonolov kot v kožici občutljive sorte. V fenolnem profilu so bile največje razlike med sortama pri skupini antocianov, kjer smo pri sorti 'Florina' skozi vso rastno sezono beležili nizke koncentracije te skupine fenolnih spojin. Kar se tiče aktivnosti encimov, pri encimih PAL, CHS/CHI in FLS med odporno in na jablanov škrlup občutljivo sorto tekom opazovane periode nismo zaznali razlik. Smo pa pri odporni sorti v petem terminu zabeležili statistično večjo aktivnost encimov FHT in DFR Večjo aktivnost teh dveh encimov lahko pojasnimo s povečano sintezo antocianov, ki so značilni za kožico izbranega odpornega kultivarja ali pa celo flavan 3-olov. Encim DFR je NADPH odvisna oksireduktaza, ki reducira kisikovo skupino na poziciji 4, s čimer omogoči nastanek flavan 3,4-diolov, le ti pa so prekurzorji za sintezo katehina, epikatehina in antocianidinov (Forkman in Heller, 1999).

Drugi del poskusa je zajemal spremljanje akumulacije fenolnih snovi in aktivnost delovanja pripadajočih encimov tekom rastne dobe v tkivih različno oddaljenih od mesta okužbe. Znano je, da se vsebnost fenolnih snovi tekom razvoja ploda spreminja. O večji

vsebnosti hidroksicimetnih kislin, dihidrohalkonov in flavan 3-olov v pegi v primerjavi z okoliškim tkivom in zdravim tkivom na zrelih plodovih že poročajo Slatnar in sod. (2010a, 2010b). Višjo koncentracijo hidroksicimetnih kislin lahko povežemo s povečanim stresom, ki mu je tkivo izpostavljeno po okužbi z jablanovim škrlupom (Mikulič-Petkovšek in sod., 2009a). Koncentracija dihidrohalkonov v pegi je bila od 2,3 (zrel plod) do 5-krat (nezrel plod) višja kot koncentracija v zdravem tkivu. Hipotezo, da je aglikon raje vključen v obrambo pred patogenom kot pa kot 2'-*O*-glukozid, podpirata tudi raziskavi Slatnar in sod. (2010a, 2010b), ki prav tako poročata o padcu UDP-glukoze in manjši aktivnosti encima G2'TP v pegi v primerjavi z zdravim tkivom. V skupini flavonolov smo statistično večje vsebnosti v pegi zaznali v prvem in drugem terminu vzorčenja, medtem ko v kasnejših terminih teh razlik nismo več zaznali.

Tkivo okuženo z glivo (pega) je tekom rastne sezone kazalo povečano aktivnost encimov PAL, DFR, FHT in POD, medtem ko se aktivnost encimov CHI/CHS in FLS tekom sezone med različnimi tkivi oziroma obravnavanji ni razlikovala. Največji vpliv na aktivnost delovanja se je pokazal pri PAL-u, kjer je tekom rastne sezone prišlo med 2,6 do 21,4-kratnega povečanja aktivnosti v pegi v primerjavi z zdravim tkivom. Opazili smo trend povečane aktivnosti encima PAL v okolici pege. To samo še dodatno potrjuje naše predhodne študije (Slatnar in sod., 2010a, 2010b) in študije nekaterih drugih avtorjev (Fuenfgelder in sod., 1994; Vidhyasekaran 1997; Anand in sod., 2009). Prav tako smo zaznali med 6,7 in 44,2-krat večjo aktivnost encima DFR v pegi v primerjavi z zdravim tkivom. Te ugotovitve lahko podpremo s predhodnimi študijami (Slatnar in sod., 2010a, 2010b; Mayr in sod., 1997), ki domnevajo, da hitra akumulacija katehina in procianidinov, na okuženih delih lahko zaustavi oz. upočasni nadaljnje širjenje glive. Veliko manjši odziv na okužbo pa je kazal encim FHT, pri katerem smo v prvem terminu vzorčenja zaznali 2,8-krat večjo aktivnost v pegi kot v zdravem tkivu.

Naraščajoča aktivnost encimov PAL in DFR v poznih stopnjah razvoja ploda je sovpadala s povečano akumulacijo hidroksicimetnih kislin in flavan 3-olov kot tudi padcem flavonolov. Kljub temu pa povečana koncentracija hidroksicimetnih kislin, dihidrohalkonov, flavan 3-olov in antocianov v zgodnjih fazah razvoja ploda ni sovpadala z aktivnostjo encimov v teh fazah. Razlago lahko iščemo v tem, da so mladi plodiči bolj občutljivi na jablanov škrlup. Do okužbe z jablanovim škrlupom pride že zelo zgodaj po cvetenju, simptomi pa se pojavijo mnogo kasneje. V naši študiji je bila ob visokih koncentracijah fenolov zabeležena aktivnost pripadajočih encimov precej nizka, kar bi lahko pomenilo, da je aktivnost pripadajočih encimov pred oziroma ob pojavu simptomov že upadla.

Spremljanje sezonskega odziva je tako pokazalo, da okužba z jablanovim škrlupom tekom celotnega razvoja ploda spremeni oz. vpliva na fenilpropanoidno pot tako preko aktivnosti encimov kot tudi vsebnosti fenolnih snovi. Čeprav več avtorjev poroča o različnosti sprememb med odporno in na jablanov škrlup občutljivo sorto, pa teh razlik mi v naši študiji nismo zaznali. V naši študiji je bila akumulacija vseh fenolnih snovi razen flavonolov bolj izrazita v zgodnji fazi razvoja ploda, medtem ko je bila aktivnost encimov bolj pestra in je kazala višjo oz. v nekaterih primerih nespremenjeno aktivnost v pozni fazi razvoja ploda.

Poleg raziskav odziva kožice na jablanov škrlup smo vzporedno izvajali poskuse, v katerih smo želeli preveriti, kako lahko različni načini pridelave oz. pripravki, ki so okolju in zdravju ljudi bolj prijazni od sintetičnih fitofarmacevtskih sredstev, vplivajo na sekundarni metabolizem, kakor tudi na nekatere druge zelo pomembne zunanje parametre kakovosti ploda. Zato smo v letih 2008 in 2009 spremljali odziv sekundarnega metabolizma v dveh načinih pridelave, ekološkem in integriranem.

Ugotovili smo, da je bila vsebnost skupnih analiziranih hidroksicimetnih kislin večja v listih dreves iz ekološke pridelave. Rezultati so kazali isti trend v obeh letih, s tem da so bile vrednosti nekoliko večje v letu 2009, kar smo pripisali predvsem povečanemu sončnemu obsevanju, ki lahko povzroči večjo sintezo določenih posameznih fenolov znotraj te skupine fenolnih spojin. Listi iz ekološke pridelave so septembra vsebovali kar 3-krat večje vsebnosti hidroksicimetnih kislin, kot listi iz IP. V letu 2008 je bila razlika v vsebnosti dihidrohalkonov med načinoma pridelave minimalna. Toda že v letu 2009 smo v začetku rastne sezone v listih iz ekološke pridelave zabeležili značilno večjo vsebnost dihidrohalkonov, in sicer je bila vsebnost v primerjavi z listi iz integrirane pridelave višja za 7-12 %. Kasneje v rastni sezoni 2009 pa razlika med preučevanima načinoma pridelave ni bila več statistično različna. Tudi pri skupini flavan 3-olov smo v obeh letih zaznali za 10 do 38 % večjo vsebnost znotraj ekološke pridelave v primerjavi z IP. Razlog za večjo vsebnost je najverjetneje večji pritisk patogenov iz okolja pri ekološki pridelavi kot pa pri IP. Znano je namreč, da večji pritisk patogenov poveča akumulacijo flavan 3-olov. Tudi skupina flavonolov ni odstopala od trenda, ki smo ga ugotovili pri ostalih skupinah. Tako so listi iz ekološke pridelave vsebovali za 10 do 30 % večje vsebnosti flavonolov. Kot pri ostalih skupinah tudi pri flavonolih povečanje vsebnosti lahko pripišemo pritisku patogenov, predvsem jablanovem škrlupu, saj o tem že poročajo tudi drugi avtorji (Feucht, 1994). Pri analizi skupnih fenolnih snovi smo pričakovali podobne vsebnosti pri obeh načinih pridelave. Tako smo v letu 2008 zabeležili enake koncentracije skupnih fenolnih snovi pri obeh načinih pridelave, medtem ko smo leta 2009 pri ekološki pridelavi zaznali za 10 do 20 % višjo vsebnost skupnih fenolnih snovi kot v IP jabolk.

Rezultati analize plodov so bili zelo različni in so pokazali predvsem to, da prihaja do razlik med načini pridelave, leti pridelave in sortami, ki smo jih v analizo zajeli. Tako so plodovi sort 'Florina' in 'Šampanska reneta' iz ekološke pridelave v letu 2008 vsebovali večje vsebnosti hidroksicimetnih kislin tako v kožici kot tudi mesu plodov. V mesu ekoloških jabolk je bila vsebnost za 1,1 do 2,6-krat večja, medtem ko je bila v kožici za 1,2 do 3,2-krat večja kot v plodovih iz IP. Sorta 'Carjevič' v nobenem letu ni kazala razlik v vsebnosti hidroksicimetnih kislin med načinoma pridelave.

V naši raziskavi smo v kožici pri sorti 'Topaz' in 'Šampanska reneta' v ekološki pridelavi leta 2008 ugotovili statistično značilno večjo vsebnost dihidrohalkonov v primerjavi z IP. Naši rezultati se tako ujemajo z ugotovitvami Chinnicija in sod. (2004), ki v kožici ekološko pridelanih jabolk poročajo o višjih vsebnostih floridzina, ki predstavlja enega od glavnih dihidrohalkonov prisotnih v jabolkih.

V kožici ekološko pridelanih jabolk pa smo pri sorti 'Šampanska reneta' v letu 2008 in sorti 'Carjevič' v letu 2009, zabeležili tudi višje vsebnosti flavan 3-olov kot v jabolkih IP. Podobne zaključke lahko podamo za kožico sorte 'Florina' v obeh letih in za meso sorte

'Topaz' v letu 2008 in kožico iste sorte v letu 2009. Hitra sinteza oz. akumulacija flavan 3-olov po okužbi s patogenom je že bila potrjena s strani več raziskav (Mayer in sod., 1997; Feucht in sod., 1998).

Kožica sorte 'Florina' in meso sort 'Topaz' in 'Šampanska reneta' so v ekološki pridelavi vsebovali statistično večji vsebnosti flavonolov v obeh opazovanih letih. Razlog je odziv rastline na patogena, ki je pri ekološki pridelavi večji kot pri IP. Pri tej skupini fenolov smo v letu 2009 zabeležili tudi nasproten pojav, ko je prišlo do večjih vsebnosti flavonolov v mesu IP sort 'Florina' in 'Carjevič'. O takšnem odzivu so pri različnih načinih pridelave jabolk sorte 'Zlati delišes' že poročali Chinnici in sod. (2004). Prav tako kot v skupinah posameznih fenolov smo tudi pri analizi skupnih fenolov potrdili hipotezo, da pridelava (ekološka) pri kateri je stres za rastlino večji, lahko vpliva na povečano vsebnost fenolnih snovi. Tako smo višjo vsebnost skupnih fenolnih snovi v ekološko pridelanih plodovih (kožica in meso ločeno) zabeležili pri sortah 'Florina' in 'Šampanska reneta' v letu 2008 in pri sortah 'Topaz' in 'Carjevič' v letu 2009.

Dva različna načina pridelave sta pokazala, da rastlina, ki jo izpostavimo stresu, tvori več fenolnih snovi. Ob tem pa ne smemo zanemariti drugih dejavnikov (leto, sorta). V letu 2010 smo zasnovali poskus z bikarbonati. Do sedaj znani viri že poročajo o njihovem ugodnem vplivu na zmanjšanje okužbe z jablanovim škrlupom v laboratorijskih poskusih, nismo pa zasledili nobenega podatka o tem, kako bikarbonati vplivajo tako na parametre kakovosti ploda kot tudi na vsebnost primarnih in sekundarnih metabolitov.

Na podlagi rezultatov poskusa z NaBK v letu 2009 (Slatnar in sod., 2011) smo se odločili, da v letu (2010) v poskus dodatno vključimo še kalijev bikarbonat (KBK), kateremu prav tako pripisujejo dobre učinke pri zatiranju jablanovega škrlupa. V poskusu smo preverjali vpliv teh dveh sredstev na zmanjšanje okužbe z jablanovim škrlupom, vpliv na parametre kakovosti plodov, ter vsebnost primarnih in sekundarnih metabolitov. Poleg tega smo želeli preveriti še možnost uporabe KBK kot foliarnega gnojila.

Rezultati so pokazali, da se je okužba z jablanovih škrlupom na plodovih tretiranih z bikarbonati zmanjšala v primerjavi s kontrolo, medtem ko med uporabljenima bikarbonatoma nismo zaznali razlik v zmanjšanju okužbe. Mase plodov so si sledile v sledečem vrstnem redu: IP > KBK > NaBK > kontrola, kar samo še dodatno potrjuje našo predhodno raziskavo (Slatnar in sod., 2011). Med obravnavanji nismo zaznali razlik v trdoti plodov. Čeprav Hunsche in sod. (2003) poročajo, da foliarna aplikacija kalija lahko povzroči padec trdote jabolk, pa je bil padec v njihovi študiji najverjetneje posledica povečanja velikosti ploda. V naši študiji razlik v velikosti ploda (višina, širina) med jabolki iz IP in jabolki tretirani s KBK nismo zaznali. Smo pa pri plodovih, ki so bili tretirani s KBK zabeležili večje vrednosti parametra *a**, kar nakazuje na bolj intenzivno rdeče obarvano barvo ploda kot pri ostalih obravnavanjih. Ugotovitev smo potrdili tudi z določitvijo večje vsebnosti antocianov v plodovih tretiranih s KBK. Rezultati naše študije se ujemajo z rezultati raziskave Hunscheja in sod. (2003), ki poročajo, da večje količine kalija v tleh lahko povežemo z večjo vsebnostjo antocianov v plodovih. Po ugotovitvah Ju-a in sod. (1999), naj bi bil kalij pomemben kofaktor nekaterih specifičnih encimov, ki so pomembni za sintezo antocianov.

Kar se tiče rezultatov analiz primarnih metabolitov, so plodovi tretirani s KBK vsebovali več skupnih sladkorjev kot tisti iz obravnavanja z NaBK oz. IP. Prav tako so imeli plodovi vseh obravnavanj statistično večjo vsebnost ekoloških kislin v primerjavi z IP. Med samimi obravnavanji razlik v vsebnosti ekoloških kislin nismo zabeležili.

Vsebnost hidroksicimetnih kislin je bila v kožici ploda pri kontroli višja kot pri ostalih obravnavanjih, medtem ko v mesu ploda razlik med obravnavanji pri tej skupini fenolnih spojin nismo ugotovili. Chinnici in sod. (2004) poročajo, da je vsebnost dihidrohalkonov večja v ekološko pridelanem sadju kot v sadju iz IP. Podobne zaključke lahko naredimo tudi iz naših rezultatov, ki pa se razlikujejo od tistih z poskusom z NaBK v letu 2009 (Slatnar in sod., 2011), kjer pri skupini flavan 3-olov v kožici ni prišlo do razlik med obravnavanji. V mesu jabolk pa je vsebnost flavan 3-olov naraščala v sledečem vrstnem redu kontrola < IP = NaBK < KBK. Rezultati se ujemajo z našimi predhodnimi raziskavami (Mikulič-Petkovšek in sod., 2010), kjer smo ugotovili, da je stopnja flavan 3-olov v kožici in mesu ploda v IP manjša kot v ekološki pridelavi. Znano je, da se po okužbi pojavi večja akumulacija posameznih fenolov iz te skupine, kar naj bi omejilo širjenje glive (Mayr in sod., 1997). Kakorkoli, naši rezultati tega ne potrjujejo. Plodovi iz IP so imeli v jablanovi kožici večjo vsebnost flavonolov v primerjavi z ostalimi obravnavanji, večja vsebnost fenolnih spojin v jabolkih iz IP pa je bila ugotovljena tudi v mesu plodov. Kot poročajo Mikulič-Petkovšek in sod. (2010) in Chinnici in sod. (2004), imajo plodovi iz ekološke pridelave večjo vsebnost skupnih fenolov kot tisti iz IP. To se je pokazalo tudi v našem poskusu, saj je z zmanjšanjem okužbe/stresa prišlo do manjše vsebnosti skupnih fenolov tako v kožici kot mesu ploda (kontrola > KBK > NaBK > IP).

Tako kot v plodovih smo tudi na listih ugotovili zmanjšano stopnjo okužbe ob uporabi 1 % raztopine NaKB oz. KBK. Uporaba tega sredstva ni povzročila nikakršnih vidnih znamenj fitotoksičnosti na listih. Med rastno sezono smo spremljali vsebnost kalija v listih. Rezultati so pokazali, da se je koncentracija kalija v listih med obravnavanji razlikovala in pri vseh obravnavanjih med prvim in drugim terminom vzorčenja narasla, kasneje pa padla. Uporaba KBK ni vplivala le na zmanjšano okužbo z jablanovim škrlupom, ampak tudi na višjo vsebnost kalija v listih. Večja vsebnost kalija v citosolu in kloroplastih celic nevtralizira topne in netopne anione makromolekul in stabilizira pH med 7 in 8, to pa je optimalni pH za delovanje večine encimskih reakcij, kot je na primer sinteza topnih ogljikovih hidratov.

Vsebnost hidroksicimetnih kislin v listih kaže na to, da tretiranje s KBK oz. NaBK pripomore k večji vsebnosti fenolov iz te skupine v primerjavi z listi iz IP in nižje kot pri kontrolnem obravnavanju. Skupina dihidrohalkonov je kazala statistično večje vsebnosti pri obravnavnaju s KBK. Povprečna vrednost flavanolov skozi leto je kazala podobne vrednosti pri tretiranju s KBK oz. NaBK, medtem ko smo pri IP opazili za 0,5 krat nižjo vsebnost kot pri kontroli. Največjo vsebnost flavonolov smo izmerili v listih tretiranih s KBK (10,15 mg/g suhe mase (SUM)), medtem ko je kontrola vsebovala 9,09 mg/g SUM, IP 9,35 mg/g SUM in tretiranje z NaBK 8,64 mg/g SUM flavonolov.

Sklop več raziskav je pokazal, da po interakciji jablanov škrlup-kožica jabolk prihaja do odziva v aktivnosti encimov fenilpropanoidne poti in posledično tudi do večje akumulacije nekaterih skupin fenolov. Znano je, da spremembo fenolnega profila ne povzroči le gliva,

ampak tudi drugi stresni dejavniki, med katere štejemo tudi mehansko poškodbo. Da bi ugotovili, kakšen odziv sproži mehanska poškodba tkiva pri jablani v primerjavi z odzivom ki ga povzroči patogen, ki ob prodoru v tkivo tudi povzroči poškodbo, smo v letu 2010 zastavili poskus, kjer smo preverjali odziv listov jablane na mehansko povzročeno poškodbo. V poskus smo vključili odporno sorto 'Florina' in na jablanov škrlup občutljivo sorto 'Zlati delišes', s čimer smo želeli preveriti ali je odziv pri različno odpornih/občutljivih sortah na jablanov škrlup enak. Odziv na mehansko poškodbo smo spremljali od 1. do 15. dneva po mehanski poškodbi.

Poškodovani listi sorte 'Zlati delišes' so kazali statistično večjo vsebnost klorogenske kisline kot pa nepoškodovani listi prvi, tretji in peti dan po poškodbi. Pri sorti 'Florina' smo v poškodovanih listih večjo vsebnost klorogenske kisline zaznali v vseh analiziranih terminih. Predstavnikom iz skupine dihidrohalkonov pripisujejo, da lahko delujejo kot UV filtri listov (Treutter, 2006) ali da so vključeni v mehanizem odpornosti na patogena (Picinelli in sod., 1995; Pontais in sod., 2008). Rezultati pri sorti 'Zlati delišes' so pokazali višjo vsebnost floridzina pri poškodovanem listu le v prvem terminu vzorčenja, medtem ko kasneje razlik med poškodovanim in nepoškodovanim listom nismo več zabeležili. Pri odporni sorti značilnih razlik med poškodovanim in nepoškodovanim listom ni bilo. Iz pridobljenih rezultatov lahko zaključimo, da na količino spojin iz skupine dihidrohalkonov ne vpliva le mehanska poškodba, ampak se skupina dihidrohalkonov najverjetneje odzove tudi na druge oblike stresa. Skupina flavan 3-olov je prva skupina, kjer smo zaznali razliko v koncentraciji fenolnih snovi med preučevanima sortama. Pri sorti 'Zlati delišes' smo večjo vsebnost epikatehina ugotovili 1. in 15. dan po mehanski poškodbi, podoben trend pa smo ugotovili tudi pri sorti 'Florina'. Odziv na mehansko poškodbo smo zaznali pri spremljanju procianida B2, kjer je prvi dan po mehanski poškodbi prišlo do povečane koncentracije pri obeh sortah. Povečanje je bilo pri sorti 'Florina' značilno skozi vso opazovano dobo, medtem ko pri sorti 'Zlati delišes' razlike v kasnejših terminih vzorčenja niso bile več značilne. O podobnem odzivu epikatehina in procianidin dimerov v svoji raziskavi poročajo tudi Andreotti in sod. (2006), ki so vpliv mehanske poškodbe na tvorbo fenolnih spojin preučevali v hruškovih listih. Prav tako je že Feucht (1994) pokazal, da mehanske poškodbe izzovejo akumulacijo oligomernih procianidinov, saj služijo kot bariera v okolici poškodovanega tkiva. Pri flavonolih, v kateri smo določili kvercetin-3-*O*-galaktozid, kvercetin-3-*O*-glukozid in kvercetin-3-*O*-ramnozid, je bila njihova vsebnost večja kot v nepoškodovanih listih, vendar pa razlika ni bila statistično značilna. Pri sorti 'Zlati delišes' pa je prihajalo do celo statistično značilno večjih vsebnosti v določenih terminih pri nepoškodovanih listih pri posameznih kvercetinih. Tako so flavonoli skupina, pri kateri na podlagi naših rezultatov zelo težko ocenimo, ali ima mehanska poškodba vpliv na povečanje njihove koncentracije. Kakorkoli, nekateri avtorji že poročajo, da okužba s patogenom izzove višjo vsebnost kvercetinov v poškodovanem tkivu, saj se flavonoli začno sintetizirati v rastlini kot odgovor na okužbo s patogenom (Dixion in Paiva, 1995).

Po poročanju Perveena in sod. (2001) naj bi mehanska poškodba listov bombaževca izzvala zmanjšanje vsebnosti skupnih fenolnih snovi, medtem ko Mikulič-Petkovšek in sod. (2008) poročajo o naraščanju skupnih fenolov pri jablani po okužbi z jablanovim škrlupom. V naši raziskavi je pri sorti 'Zlati delišes' v poškodovanih listih prišlo do povečanja skupnih fenolov v večini terminov vzorčenja, medtem ko smo pri sorti 'Florina' povečanje v poškodovanih listih zaznali le 10. in 15. dan po poškodbi.

Opravljene raziskave interakcije med jablano in jablanovim škrlupom bodo prispevale k poznavanju mehanizmov odpornosti in biokemijskih odzivov na okužbo. Vključevanje analize sekundarnih metabolitov-fenolov in natančno poznavanje sintezne poti bo koristilo nadaljnjim raziskavam s področja sekundarnega metabolizma sadnih rastlin. Dobljeni bazični rezultati pa bodo vplivali na razvoj stroke oziroma znanosti na tem področju.

3.2 SKLEPI

Na podlagi opravljenih poskusov lahko podamo sledeče zaključke:

- Okužba z glivo *V. inaequalis* povzroči spremembe v aktivnosti encimov fenilpropanoidne poti, kar se posledično kaže tudi v količini določenih fenolnih snovi.

- Encimska aktivnost se po okužbi poveča pri vseh analiziranih encimih, izjema je le encim D2'GT, ki ima večjo aktivnost v zdravem tkivu. To lahko nakazuje, da so fenolne snovi brez vezanega sladkorja del obrambnega mehanizma rastline, saj delujejo kot aktivne molekule.

- V raziskavi, kjer smo spremljali spremembe v aktivnosti encimov fenilpropanoidne poti in vsebnosti fenolnih snovi med rastno dobo, lahko zaključimo, da nismo zaznali razlik med na jablanov škrlup občutljivo in odporno sorto v preučevanih parametrih.

- Akumulacija fenolnih snovi je največja v zgodnjih fazah razvoja ploda, vendar ni zadostna, da bi zaustavila nadaljnje širjenje bolezni.

- Ekološko pridelani plodovi vsebujejo večjo vsebnost analiziranih sekundarnih metabolitov kot plodovi iz IP. Razlog za večjo vsebnost fenolov lahko razlagamo z večjim stresom (večji pritisk bolezni in škodljivcev, pomanjkanje rastlinskih hranil), ki so mu izpostavljene rastline iz ekološke pridelave. Odziv na vse te dejavnike pa se kaže v povečani sintezi fenolnih snovi, ki delujejo kot obrambne snovi v rastlini.

- NaBK in KBK uspešno zmanjšata okužbo listov in plodov z glivo *V. inaequalis*. Vitalnost listov je nujno potrebna za normalni razvoj plodov in cvetnega nastavka za prihodnjo rastno sezono. Merjeni parametri kakovosti ploda so pokazali primerljive rezultate v primerjavi s fungicidnim obravnavanjem. S tema dvema pripravkoma okolja ne obremenjujemo v tolikšni meri kot z nekaterimi drugimi sredstvi za varstvo pred jablanovim škrlupom.

- Vsebnost fenolnih snovi v plodovih tretiranih s NaBK oz. KBK je bila manjša kot pri kontroli, kar nakazuje da uporaba 1-% raztopine NaBK oz. KBK zmanjša stres, ki mu je rastlina izpostavljena na račun okuženosti z glivo *V. inaequalis.*

- Mehanska poškodba listov je povzročila večjo akumulacijo določenih fenolnih snovi. Če primerjamo ta odziv z odzivom na okužbo z glivo *V. inaequalis* lahko ugotovimo, da se odziva na ta dva stresna dejavnika (mehanska poškodba/gliva) razlikujeta. To lahko nakazuje na specifičnost odziva rastline na različne vrste stresa.

Z raziskavami smo osvetlili do sedaj še neraziskane odzive jablane prek aktivnosti encimov fenilpropanoidne poti na okužbo z glivo *V. inaequalis*. Pridobljeno znanje bo v prihodnje služilo kot osnova za nadaljnje študije. Pomembno bo preučiti posamezne snovi, ki so produkt fenilpropanoidne poti in najti možne vzorce odgovorov na posamezne vrste stresa. V prihodnje bo pomembno, da bomo znanje o sintezi fenolnih snovi kot obrambnih snoveh poskusili prenesti v prakso in rastline stimulirati, da se na spremembe v okolju odzovejo s povečano sintezo le teh.

Trenutni trend uporabe okolju prijaznih pripravkov narekuje vse večjo osveščenost kupcev o možnosti okolju prijaznejših načinov pridelave. Prav to nas je vodilo do temeljitih raziskav o vplivu uporabe NaBK in KBK kot dveh »okolju prijaznih« sredstev za zatiranje jablanovega škrlupa. S pomočjo poljskih poskusov smo pridobili rezultate, ki kažejo, da ti dve sredstvi zmanjšujeta okužbo z jablanovim škrlupom. Prav tako pa pozitivno vplivata tudi na nekatere ostale parametri kakovosti jabolk.

4 POVZETEK (SUMMARY)

4.1 POVZETEK

V štirih ločenih poskusih, zastavljenih v več rastnih sezonah, smo poskusili pojasniti del odprtih vprašanj o biokemijskih procesih obrambe rastline, ki nastane ob interakciji s patogenom. Poskusi so bili zasnovani tako, da smo z dobljenimi rezultati nadgradili nekatera že poznana dejstva predhodnih raziskav. Osredotočili smo se predvsem na aktivnost nekaterih encimov fenilpropanoidne poti, ki vplivajo na sintezo in vsebnost fenolnih snovi, ki do sedaj še niso bili podrobneje raziskani.

Do sedaj je veljalo, da odporne sorte vsebujejo večje količine fenolnih snovi, njihovo večjo vsebnost pa so povezovali z večjo odpornostjo proti boleznim. Naše študije značilnih razlik v aktivnosti encimov fenilpropanoidne poti med odpornimi in občutljivimi sortami sicer niso dokazale, so pa ovrednotile pomembne razlike tako v aktivnosti encimov kot tudi v vsebnosti fenolnih snovi med tkivi, ki so različno oddaljeni od mesta okužbe s patogenom. Najbolj aktivni so bili encimi v pegi, njihova aktivnost pa je upadala z oddaljevanjem od mesta okužbe. Edini encim, ki je odstopal, je bil encim D2'GT, katerega aktivnost je z oddaljevanjem od mesta okužbe naraščala. To lahko pojasnimo z dejstvom, da so produkti tega encima neposredno vključeni v obrambo proti glivi *V.* inaequalis, saj smo aktivnost tega encima povezali s povišano vsebnostjo floridzina v preučevanih tkivih.

Tekom rastne dobe je prihajalo do sprememb v aktivnosti encimov in posledično tudi vsebnosti fenolnih snovi. V začetni fazi okužbe z jablanovim škrlupom (na začetku sezone) je bila akumulacija fenolnih snovi povečana, medtem ko je bila naraščajoča encimska aktivnost zaznana tudi v poznih fazah razvoja ploda.

Začetne raziskave so potrdile dejstvo, da okužba s patogenom spremeni aktivnost encimov fenilpropanoidne poti. Ugotovili smo, da na vsebnost fenolnih snovi v tkivu ne vpliva le okužba s patogenom, ampak tudi številni drugi dejavniki, med katerimi smo natančneje preučili način pridelave. Določitev številnih fenolov je pokazala, da so imeli listi iz organske pridelave 10-20 % višjo vsebnost skupnih fenolov v primerjavi z listi iz IP. Prav tako so plodovi organske pridelave vsebovali večjo vsebnost skupnih fenolov kot plodovi, pridelani na integriran način.

Ker vemo, da način pridelave vpliva na vsebnost fenolnih snovi, smo se odločili, da preverimo, kako na vsebnost primarnih in sekundarnih metabolitov vplivata NaBK in KBK. Znano je namreč, da ti dve snovi zmanjšujeta okužbo z glivo *V. inaequalis,* ni pa še bil preučen njun morebiten vpliv na vsebnost sekundarnih metabolitov v rastlinskih tkivih. Oba pripravka sta se izkazala kot uspešni sredstvi za zmanjšanje okužbe z jablanovim škrlupom. Parametri kakovosti ploda so bili primerljivi s tistimi, kjer smo bolezen nadzirali po navodilih za IP. Prav tako je bila primerljiva tudi vsebnost primarnih in sekundarnih metabolitov. Poleg vpliva na višjo vsebnost antocianov v kožici ploda, je KBK tekom rastne sezone deloval tudi kot foliarno gnojilo.

Fenolne snovi se ne sintetizirajo samo po okužbi s patogenom, temveč njihovo sintezo izzovejo tudi drugi dejavniki v okolju. Zanimalo nas je, ali je odziv na patogena različen v

primerjavi z odzivom rastline na mehansko poškodbo. Ker pri okužbi patogen tkivo poškoduje, smo v enem izmed poskusov analizirali, kako se rastlina odzove na okužbo s patogenom in kako le na mehansko poškodbo. Rezultati so pokazali, da je odziv rastline na mehansko poškodbo drugačen, kot tisti ki ga dobimo pri odzivu na okužbo s patogenom, kar nakazuje na specifičnost posameznih skupin (v preučevanem primeru dihidrohalkonov) pri obrambi rastline ob okužbi z glivo *V. inaequalis.*

4.2 SUMMARY

In four separate experiments set in several growing seasons we strived to answer some questions regarding plant's biochemical defence process induced by the pathogen infection. The experimental results supplemented the known facts reported by previous research studies. We mainly investigated the activity of selected enzymes of the phenylpropanoid pathway, which affect the synthesis and content levels of specific phenolic compounds. These have not been investigated in this aspect to the present day.

Previous reports show that resistant cultivars contain higher levels of phenolic compounds compared to susceptible cultivars. The increased content of phenolics has frequently been linked with the resistance. However, the results of our study did not confirm significant differences in enzyme activities between resistant and susceptible cultivars. Tissue analysis revealed that enzyme activity and phenolic content depend on the location on the fruit. Enzyme activity was highest in the spot and decreased with the distance from the infection site. The only exception was the G2'GT enzyme. Its activity was lowest in the spot and increased with the distance from the infection site. This could be linked with the importance of the synthesized product in defence mechanism against *V. inaequalis* infection. Higher enzyme activity of D2'GT was namely correlated with higher content of phloridzin in investigated tissue.

Enzyme activity and consequently phenolic content also differs through the growing season. At the onset of the infection with apple scab (beginning of the growing season) high content levels of phenolic compounds have been observed. On the other hand, enzyme activity was also increased in the end of the growing season.

Initial studies have demonstrated that pathogen infection alters the activity of PAL enzymes. We determined that the content of phenolic compounds in plant tissue is influenced not only by the pathogen infection but many other factors, among which we specifically studied the production technology. Identification of individual and total phenolic compounds revealed that leaves of trees managed according to organic production contained 10-20% higher levels of phenolics compared with those of integrated production. Also, organically grown fruit contained higher levels of total phenolics in comparison to integrated fruit.

It is known that production system influences to content of phenolic compounds, which are strong antioxidants. Those facts lead us to investigate the influence of sodium and potassium bicarbonate application on the content of selected primary and secondary metabolites. These compounds namely successfully decrease the occurrence of apple scab infection, which was also confirmed in our study. The fruit quality parameters were

comparable with fruit grown according to integrated pest management guidelines. Also, the content of primary and secondary metabolites was similar in fruit treated with sodium and potassium bicarbonate and fruit from integrated production. Potassium bicarbonate also caused higher accumulation of anthocyanidins and acted as a foliar fertilizer.

Phenolic compounds are synthesized not only as a result of pathogen infection, but their synthesis can be induced by other environmental factors. The aim of our research was to investigate whether the phenolic response to the pathogen infection differs in comparison with the response to mechanical damage. Namely, the pathogen can only successfully enter the plant cells if it causes mechanical damage to surface tissue. The results indicate that the accumulation of phenolic compounds is indeed different between artificially induced mechanical injury and pathogen infection. This demonstrates that individual groups of phenolic compounds (in the investigated plant-pathogen reaction it was the group of dihydrochalcones) are specific for defence mechanisms against *V. inaequalis* infection.

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