UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA

Nika WEBER

# SPREMEMBE IZBRANIH METABOLITOV V RAZLIČNIH ORGANIH ŽLAHTNEGA JAGODNJAKA (*Fragaria* × *ananassa* Duch.) OB OKUŽBI Z GLIVAMI IZ RODU *Colletotrichum* IN OB RAZLIČNIH REŽIMIH NAMAKANJA

DOKTORSKA DISERTACIJA

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DOKTORSKA DISERTACIJA

# CHANGES OF SELECTED METABOLITES IN DIFFERENT ORGANS OF STRAWBERRY (*Fragaria* × *ananassa* Duch.) INFECTED WITH *Colletotrichum* sp. AND SUBJECTED TO DIFFERENT IRRIGATION REGIMES

DOCTORAL DISSERTATION

Ljubljana, 2016

Na podlagi Statuta Univerze v Ljubljani ter po sklepu Senata Biotehniške fakultete in sklepa Komisije za doktorski študij Univerze v Ljubljani z dne 28. 10. 2014 je bilo potrjeno, da kandidatka izpolnjuje pogoje za opravljanje doktorata znanosti na Interdisciplinarnem doktorskem študijskem programu Bioznanosti, znanstveno področje hortikulture. Za mentorja je bil imenovan prof. dr. Franci ŠTAMPAR.

Praktičen del poskusa je bil opravljen na Poskusnem polju Kmetijskega inštituta Slovenije na Brdu, Kmetiji Osredkar v Polhovem Gradcu ter Kmetiji Weber v Loki pri Zidanem Mostu. Laboratorijske analize in obdelava podatkov je potekala na Katedri za sadjarstvo, vinogradništvo in vrtnarstvo Oddelka za agronomijo Biotehniške fakultete.

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Datum zagovora:

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# KLJUČNA DOKUMENTACIJSKA INFORMACIJA

ŠD Dd
DK UDK 634.75:632.4:631.67:581.19(043.3)
KG žlahtni jagodnjak/*Fragaria* × *ananassa* Duch./glive *Colletotrichum*/primarni metaboliti/fenoli
AV WEBER, Nika
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ZA Univerza v Ljubljani, Biotehniška fakulteta, Podiplomski študij bioloških in biotehnoloških znanosti, področje agronomije
LI 2016
IN SPREMEMBE IZBRANIH METABOLITOV V RAZLIČNIH ORGANIH ŽLAHTNEGA JAGODNJAKA (*Fragaria* × *ananassa* Duch.) OB OKUŽBI Z GLIVAMI IZ RODU *Colletotrichum* IN OB RAZLIČNIH REŽIMIH NAMAKANJA
TD Doktorska disertacija
OP VIII, 73, [6] str., 1 sl., 3 pregl., 3 pril., 99 vir.
IJ sl
JI sl/en
AL V štirih ločenih poskusih, zastavljenih na žlahtnem jagodnjaku (*Fragaria* × *ananassa* Duch.), smo proučevali biokemijske obrambne procese žlahtnega jagodnjaka ob okužbi s črno pegavostjo (*Colletotrichum nymphaeae* Pass.). Poleg odziva na okužbo smo pri zadnjem poskusu dve večkrat rodni sorti izpostavili različnim režimom namakanja. Izbrane primarne (sladkorji in organske kisline) in sekundarne metabolite (fenoli) v plodovih in pritlikah žlahtnega jagodnjaka smo analizirali s pomočjo visokoločljivostne tekočinske kromatografije v kombinaciji z masnim spektrometrom (HPLC-MS). Glede na odziv metabolitov smo ugotovili, da so že prvi znaki okužbe povzročili povečanje vsebnosti fruktoze in glukoze (za 1,5-krat) ter kar za 4-krat zmanjšanje vsebnosti saharoze, vsebnosti citronske in jabolčne kisline, ki sta v plodovih najbolj zastopani, pa sta se po okužbi zmanjšali. Vsebnost derivatov elagne kisline (1,9-krat), flavanolov (1,5-krat) in flavonolov (5,1-krat) se je po okužbi z glivo povečala, ne glede na stopnjo zrelosti plodov. Tolerantnejša sorta 'Honeoye' je že v zdravih plodovih vsebovala večje vsebnosti flavonolov (1,5-krat), flavanolov (1,3-krat) in antocianov (1,7-krat), po okužbi pa je bila sinteza le-teh večja kot v občutljivejši sorti 'Elsanta'. Ker tehnologija pridelave značilno vpliva na vsebnost primarnih in sekundarnih metabolitov, smo preverili, kako na njihovo vsebnost v plodovih vpliva tretiranje s kalcijem in fungicidom, ki se najpogosteje uporablja za zatiranje črne pegavosti. Za oba pripravka se je izkazalo, da nimata značilnega vpliva na vsebnost fenolov, je pa kalcij značilno povečal razmerje med sladkorji in organskimi kislinami. Namakanje rastlin z najmanjšo količino vode ni vplivalo na pridelek sorte 'Flamenco' je pa zmanjšalo pridelek pri sorti 'Eva's Delight'. Manjše količine vode so povzročile povečanje vsebnosti vseh identificiranih primarnih in sekundarnih metabolitov.

KEY WORDS DOCUMENTATION

ND Dd
DC UDK 634.75:632.4:631.67:581.19(043.3)
CX strawberry/*Fragaria* × *ananassa* Duch./fungus/*Colletotrichum*/secoundary metabolites/phenolics
AU WEBER Nika
AA ŠTAMPAR, Franci (supervisor)
PP SI-1000 Ljubljana, Jamnikarjev 101
PB University of Ljubljana, Biotehnical Faculty, Postgraduate study of Biological and Biotechnical Science, Scientific field: Agronomy
PY 2016
TI CHANGES OF SELECTED METABOLITES IN DIFFERENT ORGANS OF STRAWBERRY (*Fragaria* × *ananassa* Duch.) INFECTED WITH *Colletotrichum* sp. AND SUBJECTED TO DIFFERENT IRRIGATION REGIMES
DT Doctoral disertation
NO VIII, 73, [6] p., 1 fig., 3 tab., 3 ann., 99 ref.
LA sl
AL sl/en
AB Four separate experiments were set on strawberry (*Fragaria* × *ananassa* Duch.) in order to evaluate the plant's biochemical defense process induced by the anthracnose infection (*Colletotrichum nymphaeae* Pass.) In addition to the effect of fungal infection, plant's response to different irrigation regimes was monitored. Selected primary (sugars and organic acids) and secondary (phenolics) metabolites were analyzed in strawberry fruits and runners with the use of high performance liquid chromatography coupled with mass spectrometer (HPLC-MS). Significantly higher content of fructose and glucose (1.5-fold) and a 4-fold lower content of sucrose were detected at the beginning if infection. On the contrary, citric and malic acid mostly decreased in infected fruits. The majority of identified phenolics increased after the infection regardless of the ripeness stage. Ellagic acid derivatives increased 1.9-fold, flavanols 1.5-fold and flavonols 5.1-fold. The tolerant strawberry cultivar was characterized by higher levels of flavonols (1.5-fold), flavanols (1.3-fold) and anthocyanins (1.3-fold) even in non-infected fruits and their synthesis was increased more intensively after the infection in comparison to the susceptible cultivar. The impact of production systems on the content of primary and secondary metabolites has been frequently reported. These findings lead us to further investigate the application of calcium and most commonly used fungicide and their influence on the content of sugars, organic acids and phenolic. The experimental results showed that treatments with calcium and fungicide had no significant influence on total phenolic content, but calcium increased sugar/organic acid ratio. Irrigation (as one of the most important technological measures s) additionally influenced primary and secondary metabolism of strawberry. Deficit irrigation, where water was barely available to plants, had a negative impact on 'Eva's Delight' strawberry yield and also increased the content of primary and most secondary metabolites in its fruit.

KAZALO VSEBINE

# KAZALO ZNANSTVENIH DEL

# KAZALO SLIK

str.



# KAZALO PREGLEDNIC

str.



# KAZALO PRILOG

# OKRAJŠAVE IN SIMBOLI

| **Okrajšava/simbol** | **Pomen** |
|---|---|
| ANS | antocianidin sintaza |
| CHI | halkon izomeraza |
| CHS | halkon sintaza |
| DFR | dihidroflavonol 4-reduktaza |
| DSDG | dehidrošikimat dehidrogenaza |
| F3'H | flavonoid 3-hidroksilaza |
| FLS | flavonol sintaza |
| HHDP | heksa hidroksi difenil |
| PAL | fenilalanin amonijak liaza |
| UFGT | flavonoid 3-*O*-glukoziltransferaza |

# 1 UVOD

Plodovi žlahtnega jagodnjaka (*Fragaria* × *ananassa* Duch.) so pri nas prvo pomladansko sveže sadje in zaradi svojega okusa ter arome zelo priljubljeni. Spadajo med birne plodove, za katere je značilno, da nastanejo iz večpestičnega cveta, v katerem pestiči niso zrasli, pri čemer je vsak pestič iz enega plodnega lista. Posamezni pestiči se razvijejo v monokarpne oreške, ki jih povezuje omesenelo in sočno cvetišče. Izhajajo iz družine rožnic (Rosaceae) in so nastale z medvrstnim križanjem vrste *Fragaria chiloensis* (L.) Mill. in *Fragaria virginiana* Duch. Tehnologija pridelave je precej zapletena in zahteva od pridelovalca za uspešno gojenje veliko mero znanja. Ob pojavu vedno novih bolezni in krčenju uporabe fitofarmacevtskih sredstev je zelo pomembno poznavanje prvih znakov okužbe, napade škodljivcev in drugih fizioloških motenj. Črna pegavost ali antraknoza je glivična bolezen, ki jo povzročajo glive iz rodu *Colletotrichum*, je takoj za glivo *Botrytis cinerea* Fr. druga gospodarsko najpomembnejša bolezen jagod. Gliva okužuje vse dele žlahtnega jagodnjaka: plodove, pritlike, liste, steblo in korenine (Freeman in sod., 2008). Če so med zorenjem plodov pogostejše padavine, imajo lahko predvsem pridelovalci, ki jih gojijo na prostem, velike težave zaradi okužbe s prej omenjeno glivo. Ker se trosi glive z vodo širijo zelo hitro, je lahko nasad v parih dneh popolnoma uničen, okuženi plodovi pa tržno neprimerni. Največkrat se pri varstvu proti črni pegavosti uporabljata preventivni sistemični fungicid na bazi piroklostrobina in boskalida (Signum, BASF) in azoksistrobina (Ortiva, SYNGENTA). Ker so poglavitni vir okužbe okužene sadike in tla oziroma folija, ki se stika z rastlinami, sta dobra higiena in primeren kolobar poglavitna preventivna ukrepa. Razvoj bolezni je izredno hiter, saj se po pojavu prvih vdrtih črnih peg že v dveh dneh pojavijo rožnate gmote trosov. V latentnem stanju lahko spore gliv ob ugodnih razmerah prezimijo v tleh (Eastburn in Gubler, 1992) ali v odmrlih okuženih rastlinskih delih (nadzemnih in podzemnih), zato je ključno dobro kolobarjenje in uničevanje okuženih rastlin. Optimalna temperatura za okužbo z glivo je 25 °C (Wilson in sod., 1990), v kombinaciji z visoko zračno vlago in okuženimi rastlinami pa je okužba skoraj neobvladljiva za kurativno varstvo. Tudi tehnologija pridelave ima velik vpliv na pojav ter razvoj bolezni. Zmerno gnojenje z dušičnimi gnojili, uporaba zastirk, gojenje v zavarovanih prostorih, kapljično namakanje, izogibanje poplavnih območij in izbor tolerantnejših sort so bistveni preventivni ukrepi za zdrav nasad. Izbor sorte je izredno pomemben preventivni ukrep varstva rastlin, saj se tolerantnejše sorte na okužbo odzovejo drugače kot občutljivejše. Na primer tolerantna sorta žlahtnega jagodnjaka 'Apollo' se je prilagodila tako, da je po okužbi s črno pegavostjo v celične stene zdravih celic okoli okuženega mesta nalagala pektin in tanine. Na ta način je odebelila celične stene in omejila širjenje glive v zdrave celice (Milholland, 1982).

Vrsta *Colletotrichum nymphaeae* spada v rod *Colletotrichum*, deblo Ascomycota, red Glomerellales in družino Glomerellaceae. Spolni stadij je *Glomerella cingulata* (Stoneman) Spauld. Et. Schtens, od drugih vrst pa se razlikuje predvsem po obliki trosov. Identifikacija glive *C. nymphaeae* je težavna zaradi pogoste zamenjave z ostalimi vrstami

istega rodu, zato so jih v zgodovini vedno znova opisovali kot novo vrsto. Rod *Colletotrichum* je bil zato v zadnjem času izpostavljen mnogim taksonomskim preureditvam in s tem povezanim preimenovanjem. V začetku našega raziskovanja smo črno pegavost jagod identificirali kot *Colletotrichum simmondsii*, po zadnjih podatkih pa žlahtni jagodnjak okužuje vrsta *Colletotrichum nymphaeae* (prej znana kot *Colletotrichum acutatum* molekulska skupina A2) (Damm in sod., 2012). Strategija okužbe omenjene bolezni je hemibiotropična, kar pomeni, da okužuje tako biotropično kot nekrotropično (Peres in sod., 2005). Za razliko od okužbe na plodovih, vegetativne dele jagodnjaka gliva okužuje izključno kot nekrotrop. Potek okužbe z glivo *C. nymphaeae* pa je odvisen tudi od stopnje zrelosti plodov. V zrelih plodovih po okužbi sledi značilna subkutikularna nekrotropična faza, kjer se patogen razvije znotraj celičnih sten kutikule ter se hitro razširi tako znotraj kot zunaj celic. V nezrelih plodovih razmere za razvoj glive zaradi majhnega pH niso primerne, zato gliva preide v fazo mirovanja (biotropična faza) in tvori le nabreklo konico hife (apresorij). Ardi in sod. (1998) so ugotovili, da tudi fenoli v nedozorelih plodovih, kot na primer katehin, ustvarijo neugodne razmere za razvoj glive. Ko je gliva v fazi mirovanja, pridobi od gostitelja minimalno količino hrane za svoj obstoj, rastlina okužbe ne zazna in na zunaj nima vidnih znakov okužbe. Z dozorevanjem se razmere za razvoj glive izboljšajo in okužba preide v subkutikularno, uničevalno nekrotropično fazo (Guidarelli in sod., 2011). Konidij pod ugodnimi pogoji (primerna vlaga, pH in temperatura) vzkali, požene apresorij (del, s katerim se gliva oprime površine rastline), prodorni klin, ki ob ploski površini apresorija prebode kutikulo ter celično steno gostitelja. Prvi znaki okužbe z glivo iz rodu *Colletotrichum* so vdrte črne pege in se po navadi najprej pojavijo na listnih pecljih, pritlikah in šele nato na plodovih, kamor se iz prej okuženih delov prenesejo s trosi. Konidiji so brezbarvni, v večjih skupinah, rožnati, enocelični, ravni, gladkih robov, večinoma vretenasti do koničasti. Glive iz rodu *Colletotrichum* spadajo med karantenske bolezni, zato je treba z vsakim uvozom sadilni material pregledati (Wharton in Dieguez-Uribeondo, 2004).

Glive za svoj obstoj potrebujejo energijo, ki jo dobijo od gostiteljskih rastlin. Interakcija med glivo in rastlino je zelo različna in odvisna od posamezne kombinacije rastlinske vrste/sorte in glive. Obrambne strategije rastline vključujejo sintezo različnih obrambnih snovi in patogeni se na njih ves čas prilagajajo ter tako izogibajo negativnemu vplivu. Za uspešno stimuliranje naravnih mehanizmov obrambe rastline je treba dobro razumeti interakcijo med rastlino in glivo. Poznamo tako imenovano pasivno in aktivno obrambo (Taiz in Zeiger, 2010). Pasivna obramba je v rastlini stalno prisotna. Rastlina na primer s strukturno spremembo ustvari fizične prepreke, ki onemogočijo vstop oziroma potovanje patogena po rastlini (npr. kristali kalcijevega oksalata, sklerenhim, periderm) oziroma sintetizira kemične snovi (npr. fitoanticipine), ki so lahko v rastlini prisotne kot založne ali konjugirane snovi ali pa so v obliki prekurzorjev za aktivne snovi, ki se aktivirajo ob napadu. Površina rastline je pokrita s kutikulo, voski in trihomi. Kutikula preprečuje okužbo z direktno penetracijo patogenov in predstavlja vodoodporno plast, ki preprečuje zastajanje vode na površini rastline. Odebeljene stene epidermalnih celic prav tako

zmanjšajo možnost penetracije spore. Ko gliva poškoduje tkivo, pride v rastlini do tako imenovane aktivne obrambe, ki poveča splošno zaščito rastline. Aktivna obramba zajema kombinacije različnih tako kemijskih kot fizikalnih mehanizmov. Rastlina skuša čim bolj omejiti obseg poškodbe s tem, da zapira ranjena mesta in v okolici poškodbe poveča sintezo fenolov. Prav tako lahko rastline po napadu sintetizirajo prej v rastlini ne obstoječe snovi, ki so toksične za patogen (npr. fitoaleksine). Ker pa fenoli niso nujno potrebni za razvoj celice, ampak omogočajo obstoj v danem okolju, rastline za njihovo sintezo ne porabljajo odvečne energije in jih tako sintetizirajo le toliko, da omogočijo preživetje rastline v danem okolju (Strack, 1997). Fenoli lahko povzročijo obarjanje beljakovin in tako onemogočijo delovanje določenih encimov, s tem prekinejo sprejem energije in onemogočijo preživetje patogena (Schwab in Feucht, 1999). Rastline lahko fenolne snovi nalagajo v celične stene in v tem primeru predstavljajo fizično prepreko pred vdorom patogena (Schwab in Feucht, 1999). Terry in sod. (2005) poročajo, da plodovi (oreški) žlahtnega jagodnjaka vsebujejo največ fenolnih snovi, ki imajo dokazano proti-glivično delovanje, poleg tega pa so zaščitena s trdim perikarpom, ki ga gliva ne more predreti. Rastlina za uspešno obrambo pred okužbami in napadi potrebuje veliko dodatne energije. Zato so pri obrambi rastline izredno pomembni tudi primarni metaboliti, saj predstavljajo vir energije, ki jo rastlina potrebuje za tvorjenje sekundarnih metabolitov (Broeckling in sod., 2005).

Sladkorji in organske kisline so primarni metaboliti, ki so nujno potrebni za delovanje celic in glavni rastlinski vir energije (Broeckling in sod., 2005). Plodovi žlahtnega jagodnjaka vsebujejo disaharid saharoza, ki se hidrolizira v monosaharida glukozo in fruktozo (Schweiterman in sod., 2014). Omenjeni trije sladkorji so tisti, ki so v plodovih žlahtnega jagodnjaka najbolj zastopani (Crespo in sod., 2010). Od organskih kislin sta najbolj zastopani citronska in jabolčna kislina, v manjši meri pa tudi šikimska in fumarna kislina (Sturm in sod., 2003). Razmerje med sladkorji in organskimi kislinami določa sladkost jagod, zato je to razmerje pomemben pokazatelj notranje kakovosti (Terry in sod., 2005) in posledično indeks potrošnikove sprejemljivosti pridelka (Keutgen in Pawelzik, 2007). Vsebnost sladkorjev in organskih kislin ter njunega razmerja se med sortami bistveno razlikuje (Sturm in sod., 2003), poleg sorte pa ima velik vpliv tudi okolje in tehnologija pridelave (Opstad in sod., 2011). Vsebnost sladkorjev in organskih kislin se med zorenjem plodov spreminja, znanstveniki pa so s svojimi študijami dokazali, da okužba z glivo *C. nymphaeae* prav tako vpliva na povečano sintezo ogljikovih hidratov (Crespo in sod., 2010). Pri proučevanju tolerantnih in občutljivih sort so ugotovili, da sorte, ki so občutljivejše na okužbo z glivo iz rodu *Colletotrichum*, sintetizirajo več sladkorjev kot tolerantnejše (Lobato in sod., 2009). Dokazali so, da je povečana vsebnost sladkorjev v rastlini v povezavi s sintezo snovi, ki lahko patogena uničijo ali pa zavirajo njegovo rast (Naqvi in sod., 2011).

V preteklih raziskavah so žlahtni jagodnjak škropili z različnimi pripravki, ki stimulirajo mehanizme tolerantnosti na patogene (Lara in sod., 2004; Wojcik in Lewandowski, 2003). Nanos kalcija je značilno povečal vsebnost sladkorjev v plodovih žlahtnega

jagodnjaka (Lara in sod., 2004; Wojcik in Lewandowski, 2003) in povečal trdoto mesa. Kalcij se je nalagal v celične stene in povezoval polisaharidne molekule pektina ter s tem povečal trdnost celičnih sten. Prav tako so ugotovili, da s tem, ko zmanjšamo vrednost pH celičnega soka, ustvarimo neugodne razmere za razvoj glive in le ta porabi več energije, da znotraj celice vzdržuje ugodno homeostazo za svoj razvoj (Bracey in sod., 1998; Salmond in sod., 1984). Glive namreč izločajo amonijak, ki ima zaradi prostega elektronskega para na dušiku bazičen značaj. S tem pride do povečanja vrednosti pH celičnega soka in ustvarijo se ugodne razmere za delovanje encima pektin liaza, ki razgrajuje pektin in posledično pride do zmanjšanja trdnosti celične stene. Aplicirane snovi, ki dokazano zavirajo razvoj mnogih mikrobov, gliv in bakterij, temeljijo na osnovi šibkih organskih kislin, kot so ocetna, mlečna, benzojska in sorbinska kislina (Lopez in sod., 2006; Valencia-Chamorro in sod., 2009).

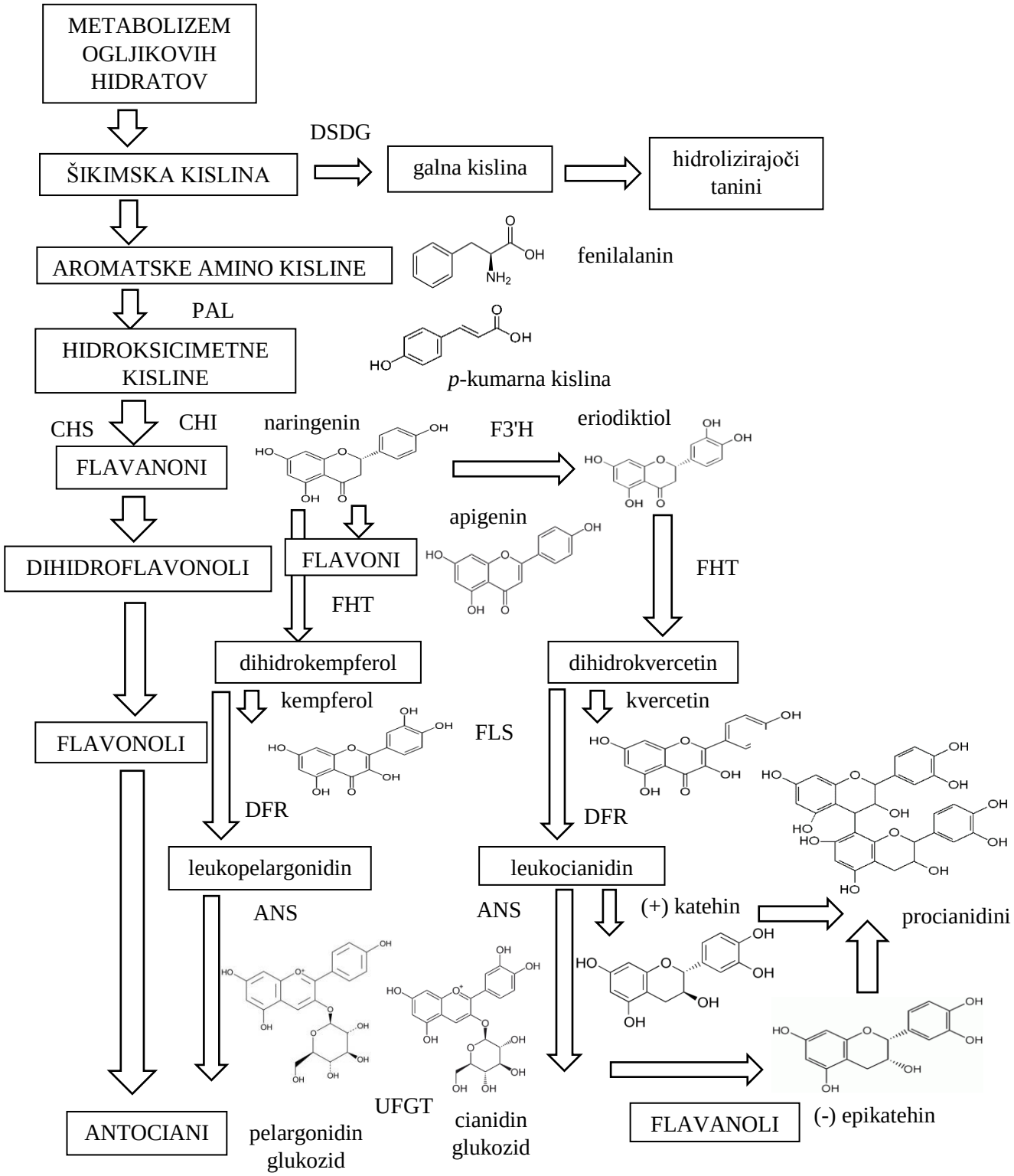
Fenoli so produkti fenilpropanoidne poti in imajo vsaj en aromatski obroč z vsaj eno ali več –OH skupin. Biosintezna pot se začne z encimom fenilalanin amonijak-liaza (PAL), ki katalizira pretvorbo fenilalanin v cimetno kislino (slika 1). Ta točka velja za povezavo med primarnim in sekundarnim metabolizmom rastlin (Lister in sod., 1996), hidroksicimetne kisline pa so prekurzorji za mnoge sekundarne metabolite. Encim halkon sintaza (CHS) je prvi encim, ki omogoča nastanek fenolnih spojin s petnajstimi ogljikovimi atomi in halkon izomeraza (CHI) katalizirata nastanek naringenina in eriodiktiola. Flavanon 3-hidroksilaza (FHT) katalizira pretvorbo flavanonov v dihidroflavonole in flavonol sintaza (FLS) naprej do kvercetina in kempferola. Pretvorbo dihidrokvercetina in dihidrokempferola katalizira dihidroflavonol reduktaza (DFR) v levkocinidin, ki je prekurzor za flavanole in antociane (cianidin), in levkopelargonidin, ki je prekurzor pelargonidina. Znanje o fenilpropanoidni poti se nenehno nadgrajuje, določajo se novi encimi in njihova vloga, vedno bolj se povezujejo z ekspresijami genov. Najpogosteje zastopani fenoli v plodovih žlahtnega jagodnjaka spadajo v skupino hidroksicimetnih kislin, flavonolov, flavanolov, elagitaninov in antocianov (Aaby in sod., 2012).

V plodovih in pritlikah žlahtnega jagodnjaka so dokazali vsebnost hidrolizirajočih taninov oziroma elagitaninov, ki so prisotni le v manjšem številu sadnih vrst (Aaby in sod., 2012). V žlahtnem jagodnjaku so prisotni v obliki polimerov – elagitaninov ali njihovih derivatov (Häkkinen in sod., 2000), najdemo jih v celični citoplazmi in vakuolah (Khadem in Marles, 2010). Elagitanini so polifenoli, ki spadajo med hidrolizirajoče tanine in imajo v sredini molekulo sladkorja (najpogosteje D-glukozo). Hidroksilne skupine teh sladkornih molekul tvorijo diestre z elagno kislino. Hidrolizirajoči tanini, v našem primeru elagitanini, predstavljajo polimere (sestavljeni iz heksahidroksidifenolnih kislin (HHDP)), ki hidrolizirajo ob prisotnosti šibkih kislin ali baz. so hidrolizirani s šibkimi kislinami ali bazami. Zaradi nestabilnosti HHDP je potrebna še laktonizacija, ki poveča količino elagne kisline (Veberic, 2010). Znano je, da rastline po napadu patogena sintetizirajo večje količine elagitaninov, ki imajo dokazano protimikrobno (Zhou in sod., 2007; Quave in sod., 2012) in proti-glivično delovanje (Seigler, 1998). Imajo namreč

sposobnost, da tvorijo močne komplekse z beljakovinami in polisaharidi (Haslam, 1996). Nedozoreli plodovi imajo velike vsebnosti elagitaninov , saj jih je večina prisotna v semenih, kjer z zaščito semenu omogočijo uspešen razvoj (Fait in sod., 2008). Derivati elagne kisline so prisotni v vseh rastlinskih delih, največ jih najdemo v vegetativnih delih rastlin – listih in pritlikah (Maas in sod., 1991). Med fenolne kisline pa spadajo tudi hidroksicimetne kisline, za katere so prav tako dokazali, da so izredno pomembne pri obrambi pred patogeni, saj naj bi uspešno zavirale razvoj in razmnoževanje glive (Sammi in sod., 2009).

Dokazano je bilo, da so flavonoli snovi, ki uspešno lovijo reaktivne oblike kisika ter tako zmanjšajo oksidativne poškodbe, do katerih pride, kadar je rastlina izpostavljena okoljskemu stresu (Baratto in sod., 2003). Prav tako so potrdili, da flavonoli in flavanoli zavirajo razvoj glive v listih in pritlikah žlahtnega jagodnjaka, Vincent in sod., (1999) so dokazali povezavo med omenjenimi snovmi in tolerantnostjo jagode na glivo iz rodu *Colletotrichum*. Potrdili so, da se količina flavanolov in flavonolov razlikuje med sortami, ki so na glivo občutljive, in sortami, ki so tolerantnejše. Hébert in sod. (2002) so ugotovili, da so sorte, ki sintetizirajo več katehina in epikatehina, tolerantnejše na glivične okužbe. Listi občutljivejše sorte so vsebovali manj flavonolov kot listi tolerantnejše, kar kaže na pomembno vlogo flavonolov v obrambnem mehanizemu rastline (Hanhineva in sod., 2009). Terry in sod. (2005) predvidevajo, da so snovi, ki so prisotne v zelenih delih rastline, podobne snovem, ki jih najdemo v nezrelih, zelenih plodovih. V listih žlahtnega jagodnjaka je skupina znanstvenikov odkrila snov fragarin, ki deluje proti bakterijam in glivam (Filippone in sod., 1999). Fragarin je snov, ki naj bi imela destruktivno delovanje proti širokemu spektru bakterij in gliv, tudi proti glivam iz rodu *Colletotrichum*. Dokazano je bilo, da rastlina predvsem okoli mesta okužbe kopiči več katehina in s tem prepreči razvoj patogena (Feucht in sod., 1992). Prav tako sta v zelenih plodovih sorte 'Elsanta' Puhl in Treutter (2008) dokazala povečano vsebnost katehina, zaradi katerega se je zaustavil razvoj patogena. Hitra sinteza flavanolov je tako odločilna v boju proti patogenom in povečani tolerantnosti na njih (Michalek in sod., 1999).

Rdeča obarvanost plodov žlahtnega jagodnjaka nastane zaradi vsebnosti antocianov. Dozoreli plodovi žlahtnega jagodnjaka vsebujejo največ pelargonidin in cianidin glukozidov (Wang in sod., 1996). Dokazali so, da so glikozidi cianidina odgovorni za rdečo obarvanost zrelih plodov jagod, glikozidi pelargonidina pa dajejo oranžen odtenek (Pineli in sod., 2011). Od nezrelih do zrelih plodov se vsebnost antocianov poveča kar za 9 do 13-krat, vendar pa je količina določenega antociana zopet odvisna od sorte (Aaby in sod., 2012) in drugih dejavnikov.




Slika 1: Sintezna pot pri žlahtnem jagodnjaku. PAL-fenilalanin amonijak liaza; CHI-halkon izomeraza; FLS-flavonol sintaza; DFR-dihidroflavonol 4-reduktaza; ANS-antocianidin sintaza; DSDG-dehidrošikimat dehidrogenaza; F3'H-flavonoid 3-hidroksilaza; CHS-halkon sintaza; UFGT-flavonoid 3-*O*-glukoziltransferaza

Figure 1: Biosynthesis pathway in strawberry. PAL-phenylalanine ammonia lyase; CHI-chalcone isomerase; FLS-flavonol synthase; DFR-dihidroflavonol 4-reductase; ANS-anthocyanidin synthase; DSDG-dehidroshikimate dehydrogenase; F3'H-flavonoid-3'-hydroxylase; CHS-chalcone synthase; UFGT-flavonoid 3-*O*-glucosyltransferase

Povečane potrebe po vodnih virih in širjenju kmetijskih pridelovalnih zemljišč sta dva izmed razlogov, zakaj so pridelovalci vedno bolj dolžni utemeljevati količino porabljene vode za namakanje. V okolju, kjer vodnih virov ne primanjkuje, so količine porabljene vode velikokrat neupravičene in okoljsko nesprejemljive. Pretekle raziskave so pokazale, da zmanjšano namakanje negativno vpliva na velikost ploda in posledično zmanjša pridelek jagod (Castellarin in sod. 2007). Vodni režim v rastlini v veliki meri določa kemijsko sestavo plodov žlahtnega jagodnjaka. Glede na to, da je žlahtni jagodnjak rastlina s plitvim koreninskim sistemom in veliko listno površino, je izredno občutljiva na pomanjkanje vode (Krüger in sod., 1999). Na območjih, kjer je voda relativno poceni, jo pridelovalci pogosto uporabljajo za namakanje v nerazumnih količinah (El-Farhan in Pritts, 1997) z namenom povečanja pridelka.

Pomanjkanja vode zaustavi rast rastlin (Klamkovski in Treder, 2008) in posledično zmanjša pridelek (Rahmati in sod., 2015). Ko se rastlina sooči s pomanjkanjem vode začne sintetizirati signalne snovi, ki sprožijo zapiranje listnih rež ali celo upočasnijo rast listov (Chaves in sod., 2010). Različni režimi namakanja vplivajo na maso ploda (Gibert in sod., 2005), delež suhe snovi (Fishman in sod., 1998), metabolizem sladkorjev in transport ogljikovih hidratov po rastlini (Lechaudel in sod., 2005). Preveč namakane rastline so podvržene koreninskim boleznim, prav tako pa se iz tal izpirajo hranila. Povzamemo lahko, da sta tako preveč kot premalo vode neprimerna za uspešno pridelavo, veliko pa je odvisno tudi od sorte. Gine-Bordonaba in Terry (2010) sta dokazala, da so se sorte različno odzvale na pomanjkanje vode in da točka, ki pomeni za rastlino pomanjkanje, ni enaka pri različnih sortah.

Pomanjkanje vode vpliva na sintezo sladkorjev, organskih kislin in fenolov ter kakovost plodov (Stefanelli in sod., 2010). Pomanjkanje vode in stres, ki ga povzroči, so mnogi povezali s povečano vsebnostjo fenolov (Hummel in sod., 2010), zato so ga nekateri znanstveniki že navedli kot možen ukrep k povečanju notranje kakovosti plodov (Kumar in Dev, 2010). Poročali so, da je odvisno tudi od tega, kdaj rastlino izpostavimo pomanjkanju vode, tako so Castellarin in sod. (2007) ugotovili, da se poveča vsebnost sladkorjev v plodovih žlahtnega jagodnjaka, če jih izpostavimo pomanjkanju vode v fazi nezrelih plodov. Spet drugi raziskovalci so povečane vsebnosti sladkorjev v plodovih žlahtnega jagodnjaka, ki so bili izpostavljeni pomanjkanju vode, pripisali vplivu razredčitve. Predpostavili so tudi, da je razlog spremenjene količine sladkorjev tudi v tem, da se vegetativna rast rastlin upočasni ali celo ustavi in rastlina zato povečano nalaga sladkorje in organske kisline v plodove (Gine-Bordonaba in Terry, 2010).

Poročali so, da namakanje žlahtnega jagodnjaka z različno količino vode bistveno spremeni fenolno sestavo plodov. Tovar in sodelavci (2002) so ugotovili, da pomanjkanje vode povzroči povečano delovanje encima fenilalanin amonijak liaza (PAL). Dokazali so tudi povečano ekspresijo določenih genov, ki so odgovorni za sintezo antocianov, kadar rastline nimajo na voljo dovolj dostopne vode (Deluc in sod., 2009). Glede na dejstvo, da

so plodovi rastlin, ki so izpostavljeni pomanjkanju vode, po navadi manjši, in da je večina antocianov prisotna v zunanjem delu ploda, je lahko razlog za povečanje količine antocianov morfološki dejavnik (Rahmati in sod., 2015). Povečane vsebnosti fenolnih kislin v rastlinah, izpostavljenih pomanjkanju vode, bi lahko pripeljale do povečane sinteze lignina v celičnih stenah (Ayaz in sod., 2000).

Kako se pomanjkanje vode odraža na podrobno fenolno sestavo plodov žlahtnega jagodnjaka, do sedaj še ni bilo natančno raziskano. Ali lahko z zmernim namakanjem celo izboljšamo notranjo kakovost plodov in še jih vedno pridelamo tržno zanimive? Prav tako ostajajo vprašanja o poteku okužbe z glivo *Colletotrichum* in povezavo s sintezo fenolnih snovi v različnih organih žlahtnega jagodnjaka. Zato smo se odločili, da zastavimo tri različne poskuse in poskusimo odgovoriti na čim več odprtih vprašanj. Z njimi bi radi bolje razložili odnos med žlahtnim jagodnjakom in glivo iz rodu *Colletotrichum* ter kako se odzove metabolizem rastline na različne režime namakanja.

Poskus 1: Vpliv antraknoze (*Colletotrichum simmondsii*) na izbrane sekundarne in primarne metabolite v plodovih in pritlikah žlahtnega jagodnjaka (*Fragaria* × *ananassa* Duch.)

V poskus bo vključen vrtni jagodnjak sorte `Clery`. V analizo vsebnosti nekaterih izbranih fenolov, sladkorjev in organskih kislin bomo vključili zdrave plodove, plodove s prvimi znaki okužbe in okužene plodove žlahtnega jagodnjaka ter zdrave in okužene dele pritlik z istih rastlin. Okužbo z glivo in definicijo vrste glive bo laboratorijsko določil Kmetijski inštitut Slovenije. Cilj raziskave je ugotoviti, kaj se dogaja s primarnimi in sekundarnimi metaboliti v različnih stopnjah okužbe z glivo *C. simmondsii* na plodovih in pritlikah žlahtnega jagodnjaka. Rezultati nam bodo v veliko pomoč pri razumevanju procesov, ki se dogajajo v rastlini med glivično okužbo.

Poskus 2: Vpliv stopnje zrelosti plodov na pojav črne pegavosti in okužbe v različnih organih žlahtnega jagodnjaka

Na poskusnem polju pri pridelovalcu, ki ima vsakoletne težave zaradi okužbe vrtnega jagodnjaka z antraknozo, bomo pobrali plodove in pritlike sorte `Asia` v različnih stopnjah zrelosti. Vzorčili bomo (1) bele, (2) delno dozorele in (3) dozorele plodove ter okužene in zdrave pritlike. Do okužbe z glivo iz rodu *Colletotrichum* bo prišlo spontano, zato bomo lahko ugotovili, kaj se dejansko dogaja s snovmi v okuženem delu in v zdravem delu na isti rastlini. Prisotnost glive bodo potrdili v laboratoriju na Kmetijskem inštitutu Slovenije v Ljubljani. Vzorce pritlik in plodov žlahtnega jagodnjaka bomo pobirali na zunanjih pridelovalnih površinah. S tem bomo ugotovili, do kakšnih razlik pride v vsebnosti izbranih fenolov, sladkorjev in organskih kislin ob pojavu okužbe.

Poskus 3: Spremenjena vsebnost primarnih in sekundarnih metabolitov v plodovih žlahtnega jagodnjaka zaradi okužbe s *Colletotrichum nymphaeae*

Na dveh različnih sortah žlahtnega jagodnjaka bomo v poljskem poskusu proučevali vpliv okužbe z glivo *Colletotrichum nymphaeae*, ki povzroča črnolistno pegavost. Izbrali bomo sorto 'Elsanta', ki je občutljiva na okužbo s prej omenjeno glivo, in sorto 'Honeoye', ki je tolerantna. V poskus bomo vključili 4 obravnavanja: umetna okužba z glivo *C. nymphaeae*, škropljenje s fungicidom na osnovi piroklostrobina in boskalida (Signum), škropljenje s kalcijevim pripravkom (Stopit) in kontrolo, kjer bomo rastline poškropili samo z vodo. Ugotoviti želimo, ali izbrana pripravka in okužba vplivajo na vsebnost primarnih in sekundarih metabolitov v plodovih žlahntega jagodnjaka.

Poskus 4: Vpliv zmanjšane količine dodane vode za namakanje na pridelek in izbrane primarne ter sekundarne metabolite v plodovih žlahtnega jagodnjaka

Poskus bo potekal na novejših, večkrat rodnih sortah žlahtnega jagodnjaka `Flamenko` in 'Eva's Delight'. Omenjeni sorti sta novost na našem območju in se ju še ni pridelovalo na večjih površinah. V zavarovanem prostoru bomo rastlinam omejili namakanje (za 30 in

50 % vodne kapacitete tal) in ugotavljali, kaj se dogaja z izbranimi primarnimi in sekundarnimi metaboliti v različnih organih rastline. Ugotovili bi radi, kje je mejna potreba po namakanju za optimalen pridelek in kakovost plodov žlahtnega jagodnjaka. Z raziskavo bomo ugotovili, kakšne so možnosti za rentabilno pridelavo ob morebitnem zmanjšanju količine razpoložljive vode za namakanje.

## 1.1 RAZISKOVALNE HIPOTEZE

Postavili smo naslednje raziskovalne hipoteze:

1. Rastlina se na stres odzove s spremembo vsebnosti določenih metabolitov. Pri okužbi z glivo se vsebnost fenolnih snovi v plodovih in pritlikah žlahtnega jagodnjaka poveča.
2. Vsebnost fenolnih snovi v plodovih žlahtnega jagodnjaka se spreminja s stopnjo zrelosti plodu. Ob pojavu okužbe pa se vsebnost fenolnih spojin v plodovih poveča, neodvisno od stopnje zrelosti plodov.
3. Sladkorji in organske kisline so eden izmed parametrov, ki dajejo okus plodu žlahtnega jagodnjaka, okus pa je bistvenega pomena za kupca. Ob pojavu bolezni *Colletotrichum* se vsebnost sladkorjev poveča, vsebnost organskih kislin pa se z okužbo zmanjša.
4. Gliva *Colletotrichum* okužuje vse dele rastline žlahtnega jagodnjaka, tako plodove, pritlike in liste. Pritlike, okužene z antraknozo, bodo tvorile več fenolnih snovi kot zdrave.
5. Tretiranje s kalcijem in fungicidom spremeni fenolno sestavo plodov žlahtnega jagodnjaka.
6. Izpostavljanje rastlin omejenemu namakanju vpliva na izbrane primarne in sekundarne metabolite ter pridelek žlahtnega jagodnjaka. Rastline, ki imajo zagotovljen optimalen vodni potencial, imajo večji pridelek. Rastline, ki bodo namakane z manjšo količino vode, bodo tvorile več primarnih in sekundarnih metabolitov.

# 2 ZNANSTVENI ČLANKI

## 2.1 OBJAVLJENA ZNANSTVENA DELA

### 2.1.1 Vpliv okužbe *Colletotrichum simmondsii* R. G. Shives & Y. Y. Tan na izbrane primarne in sekundarne metabolite plodov in pritlik žlahtnega jagodnjaka (*Fragaria* × *ananassa* Duch.)

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INFLUENCE OF *Colletotrichum simmondsii* R. G. SHIVES & Y. P. TAN INFECTION ON SELECTED PRIMARY AND SECONDARY METABOLITES IN STRAWBERRY (*Fragaria* × *ananassa* DUCH.) FRUIT AND RUNNERS

European Journal of Plant Pathology, 2013, 136: 281–290

Na sorti 'Clery' smo ugotavljali, kako stopnja okužbe z glivo *Colletotrichum simmondsii* vpliva na vsebnost sladkorjev, organskih kislin in posameznih fenolov. Primarne metabolite smo identificirali s sistemom visokoločljivostne tekočinske kromatografije (HPLC), sekundarne metabolite pa smo dodatno potrdili z masnim spektrofotometrom. V okuženih plodovih se je zmanjšala vsebnost saharoze, jabolčne in citronske kisline ter povečala vsebnost fruktoze in glukoze. Uspešno smo identificirali 9 različnih derivatov elagne kisline, 6 flavanolov, 7 flavonolov in 4 antociane v plodovih žlahtnega jagodnjaka. V pritlikah smo identificirali 12 različnih derivatov elagne kisline, 9 flavanolov in 8 flavonolov. Vsebnost elagnih kislin se je povečala šele v popolnoma okuženih plodovih, vsebnost flavanolov in antocianov pa se je značilno povečala že v prvih stopnjah okužbe in se z nadaljnjim razvojem začela zmanjševati. Po analizi flavonolov smo ugotovili, da se je vsebnost izoramnetina in glukozid kvarcetina po okužbi značilno zmanjšala. Okužba pritlik z glivo *C. simmondsii* je imela zelo različen vpliv na vsebnost elagnih kislin in flavanolov, le ti so se nekateri značilno povečali, drugi zmanjšali. Vsebnost flavonolov pa se je v pritlikah za razliko od plodov 2,1-krat povečala po okužbi.

Eur J Plant Pathol (2013) 136:281–290
DOI 10.1007/s10658-012-0162-7

# Influence of *Colletotrichum simmondsii* R. G. Shives & Y. P. Tan infection on selected primary and secondary metabolites in strawberry (*Fragaria* x *ananassa* Duch.) fruit and runners

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Accepted: 13 December 2012 / Published online: 8 January 2013


**Abstract** The effect of *Colletotrichum simmondsii* infection on the contents of sugars, organic acids, and individual phenolic compounds was investigated in strawberry cultivar 'Clery'. Primary metabolites were determined with the use of HPLC and secondary metabolites further confirmed with HPLC-MS. *Colletotrichum simmondsii* caused a decrease in sucrose and an increase in fructose and glucose in strawberry fruit. A significant decrease in the content of malic and citric acids was recorded in infected fruit. 12 forms of ellagic acid, nine flavanols and eight flavonols were identified in strawberry runners and nine forms of ellagic acid, six flavanols, seven flavonols and four anthocyanins in strawberry fruit. Significant differences in individual phenolic compounds in strawberry fruit were detected at the beginning of the infection compared to non-infected fruit. Specifically, ellagic acids significantly increased, flavonols generally decreased, and flavanols and anthocyanins increased with the progression of infection. Similarly, some forms of ellagic acid increased and others decreased in infected runners, procyanidins generally decreased and flavonols, increased but the differences were much less prominent.

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**Keywords** *Fragaria* x *ananassa* Duch. · Anthracnose · Infection · Sugars · Organic acids · Phenolics

## Introduction

Strawberries are characterised by a unique, highly desirable taste and flavour and are one of the most popular summer fruits. Strawberry fruit biochemical composition has been extensively studied and due to their health promoting properties strawberries are often recommended in a balanced diet.

Until recent, *Colletotrichum acutatum* has been identified as the main agent causing anthracnose on strawberries. Some authors have proposed that the high genetic divergence of *C. acutatum* population indicated that it should be further divided into several distinct species (Vinnere et al. 2002; Guerber et al. 2003; Sreenivasaprasad and Talhinhas 2005). One of them is represented by *C. simmondsii* (Shivas and Tan 2009) causing anthracnose on many different plant groups such as ornamental plants and several fruit plants. However, the fungus is economically most significant on strawberries (*Fragaria* x *ananassa* Duch.). In strawberry worldwide production, *C. simmondsii* namely represents the second most important pathogen in addition to *Botrytis cinerea* Fr.

*C. simmondsii* behaves as a generalist invader (Curry et al. 2002) and causes damage both on vegetative and generative parts of strawberry plants. Therefore, runners and ripe fruits can significantly be affected by the fungus

and the response of different plant parts to the infection must be elucidated. The heaviest economic losses caused by *C. simmondsii* are the result of fruit infection appearing on immature fruits pre-harvest, on mature fruits at harvest or in the postharvest storage stage. In the past years different fruit has been treated with preservative agents, mostly weak organic acids such as acetic, lactic, benzoic and sorbic acid, which inhibit the microbial growth, various fungi and bacteria (Lopez et al. 2006; Valencia-Chamorro et al. 2009). In regard to the inhibitory mode of these organic acids, some mechanisms of protection against fungal growth have been suggested. Namely, higher content levels of organic acids detected in fungal cells inhibited their growth by lowering the pH level of cell sap causing high energy consumption in order to maintain the intracellular pH homeostasis (Bracey et al. 1998; Salmond et al. 1984). Other methods against fungal infection include the application of various fungicides but strawberry producers still struggle against *C. simmondsii* as most of the products are not demonstrating a good efficacy. Therefore new possibilities of protection against this fungus must be considered and plant cell defence mechanisms (such as production of various secondary metabolites induced by the infection) further investigated. However, up to now few studies closely examining the effect of *C. simmondsii* on the content of individual and total phenolics, sugars and organic acids in different strawberry tissues have been published.

The objective of this study was thus to establish sugar and organic acid content as well as identify the individual phenolic compounds at different stages of *C. simmondsii* infection of strawberry fruit and runners. The results will present information on the chemical compounds affected by the fungus, which can potentially be associated with induced strawberry tolerance against *C. simmondsii*. This knowledge could in future be used as a tool for developing natural plant protection strategies against *C. simmondsii* and help reduce negative effects of pesticides on the environment and human health.

## Material and methods

### Plant material

Strawberry (*Fragaria* x *ananassa* L. Duch) cultivar 'Clery' was included in the study. Samples were collected at Zdole production field located in central Slovenia (45°58′N, 15°32′E) on the 15th of September 2011. The experiment included samples from different plants cultivated according to the commercial guidelines for integrated production. Fruits were collected at three different stages of infection: (1) non-infected fruits, (2) fruit at the beginning of the infection, and (3) infected fruits and runners at two different stages of infection: (1) non-infected runners and (2) infected runners. All fruit were sampled in their technological maturity stage to ensure comparable measuring conditions for the contents of primary and secondary metabolites among different treatments. Fruit and runners were transferred to laboratory facility and stored at −20 °C until further analyses.

### Sugars and organic acids analysis

Ten grams of strawberry fruit were brought to 50 ml of total volume with twice-distilled water and homogenised with T-25 Ultra-Turrax (IKA®-labortechnik, Staufen, Germany) for one min at room temperature. Samples were left to extract for 30 min at 24 °C and centrifuged at 10,000 rpm for 7 min at 5 °C (Eppenceort 5810 R Centrifuge, Hamburg, Germany). The supernatant was filtered through a 0.45 μm cellulose filter (Machery-Nagel, Düren, Germany) into a vial and transferred to HPLC system, where it was analysed according to the method described by Mikulic-Petkovsek et al. (2007).

Three individual sugars were analysed (sucrose, glucose and fructose) using a Thermo Separation Products HPLC (high-performance liquid chromatography) with a refractive index (RI) detector (Thermo Scientific, Waltham, MA). The mobile phase was twice-distilled water. For the separation of the sugars, a Rezex RCM-monosaccharide column (300× 7.8 mm; Phenomenex) maintained at 65 °C with sample flow rate of 0.6 $ml min^{-1}$ was used. All individual sugars were identified by the addition of corresponding external standards and expressed in g $kg^{-1}$ fresh weight (FW). Analysis of individual organic acids was performed by the same equipment differing in the column (Rezex ROA—organic acid H+, 300×7.8 mm; Phenomenex, Torrance, CA) and mobile phase (4 mM $H_2SO_4$ aqueous solution). The flow rate of the mobile phase was 0.6 $ml min^{-1}$ and the detector was set at 210 nm. Organic acids content levels were expressed in g or mg $kg^{-1}$FW.

Sugar and organic acid ratio

*Extraction and analysis of individual phenolic compounds*

Samples of strawberry fruit and runners were cut in small pieces with a ceramic knife and 2 g of mezocarp or 1 g of runner tissue were extracted with 10 ml of methanol containing 3 % (v/v) formic acid and 1 % (w/v) 2,6-di-tert-butyl-4-methylphenol (BHT) for 60 min. After extraction, the treated samples were centrifuged for 7 min at 10,000 rpm at 5 °C. The supernatant was filtered through a 0.45 μm Chromafil A0-45/25 polyamide filter produced by Macherey-Nagel (Düren, Germany) and transferred into a vial prior to injection to the HPLC system.

Samples were analysed using a Thermo Finningan Surveyor HPLC system (Thermo Scientific, San Jose, USA) with a diode array detector at 280 nm (ellagic acid pentoside, bis HHDP (6,6′-dicarbonyl-2,2′,3,3′,4,4′- hexahydroxybiphenyl moiety) glucose isomer 1, bis HHDP glucose isomers, procyanidin dimers, procyanidin trimers, ellagic acid hexoside, galloyl bis HHDP glucoses, ellagic acid deoxyhexoside, catechin, procyanidin tetramer); 350 nm (quercetin hexoside, ellagic acid deoxyhexoside, ellagic acid, quercetin 3-glucoside, quercetin 3-glucuronide, kaempferol 3-glucoside, kaempferol 3-glucuronide, isorhamnetin 3-glucuronide, kaempferol 3-malonylglucoside, kaempferol 3-coumaroylglucoside, ellagic acid pentoside, isorhamnetin 3-galactoside); and 530 nm (cyanidin 3-glucoside, pelargonidin 3-glucoside, cyanidin 3-malonylglucoside, pelargonidin 3-malonylglucoside).

The elution solvents used were 1 % formic acid in twice-distilled water (A) and 100 % acetonitrile (B) maintained at 1 $ml\,min^{-1}$ using the linear gradient method 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 then washing and reconditioning of the column. Identification of compounds was achieved by comparing retention times and their UV–VIS spectra from 220 to 550 nm, as well as by the addition of external standards and comparison with literature reports. Unknown compounds were further identified using a mass spectrometer (Thermo Scientific LCQ Deca XP MAX) with electrospray ionization (ESI) operating in positive mode (for anthocyanins) and negative mode (for other phenols). The analysis was carried out using full-scan data dependent $MS^n$ scanning *m/z* from 115 to 2000. Capillary temperature was 250 °C, the sheath gas and auxiliary gas were 20 and 7 units, respectively, and the source voltage was 0.1 kV. Injection volume was 10 μl and the flow rate maintained at 1 $ml\,min^{-1}$. Individual phenolic compounds were expressed in mg $kg^{-1}$ FW.

*Chemicals*

Chemicals for the identification and quantification of individual sugars (fructose, glucose and sucrose) were obtained from Fluka (Buchs, Switzerland). For the quantification of organic acids, malic acid was purchased from Merck (Darmstandt, Germany), and citric, shikimic and fumaric acids were purchased from Fluka.

Standards for the quantification of individual phenolic compounds were ellagic acid, procyanidin B2, quercetin 3-galactoside, quercetin 3-glucuronide and pelargonidin from Fluka, and kaempferol, isorhamnetin and cyanidin from Sigma (Steinheim, Germany). Methanol for the 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 twice-distilled and purified with the Milli-Q system (Millipore, Bedford, MA, USA).

*Statistical analysis*

Statistical analysis was conducted using the Statgraphics Plus 5.1 program (Statgraphics, Herdon, VA). The data were subjected to one-way analysis of variance and differences among means were estimated at $p<0.05$. Five replications were performed for each individual primary and secondary metabolite analysed in strawberry fruit and runners.

## Results

Sugars and organic acids in strawberry fruit

Content levels of individual sugars and organic acids in strawberry fruit were assessed in three different stages of infection with *C. simmondsii* (Table 1). The most abundant sugars in non-infected strawberry fruit were fructose and glucose, whereas sucrose was

**Table 1** The content of sugars (g $kg^{-1}$FW) in strawberry fruit at three stages of *C. simmondsii* infection

| *Fragaria x ananassa* L. 'Clery' | Non-infected | The beginning of infection | Infected |
|---|---|---|---|
| Sucrose | 20.3±1.3 b[a] | 5.9±1.0 a | 3.8±0.5 a |
| Fructose | 47.6±3.9 a | 73.9±2.9 b | 67.4±2.7 b |
| Glucose | 45.6±3.8 a | 69.6±3.0 b | 59.6±3.9 b |
| Total sugars | 113.6±9.1a | 149.5±7 b | 131.0±7.2 b |

[a] Different letters denote statistically significant differences among treatments at significance level ($p<0.05$)

detected in somewhat lower amounts. Quite oppositely, the highest content of sucrose was measured in non-infected strawberries and a significant decrease was detected in both stages of the infection. Similar to individual sugar turnover, total sugar content was lowest in non-infected fruit (113.6 g$kg^{-1}$FW), significantly increased at the beginning of infection (149.5 g $kg^{-1}$FW) and dropped slightly in infected strawberry fruit (131.0 g$kg^{-1}$FW). Generally, total sugars level was significantly higher in both stages of infection for over 15 % compared to non-infected fruits.

The highest proportion of total analysed organic acids in strawberries was represented by citric acid (Table 2) and its content was highest in non-infected fruit. The second prevailing organic acid was malic acid followed by two minor organic acids; shikimic and fumaric acid. Citric and malic acid combined represented 99.8 % total organic acids in non-infected fruit. The content of malic acid was highest in non-infected fruit and was significantly lower in infected fruit. The sugars/organic acids ratio of non-infected fruit was 5.0. With progressed infection the sugars/organic acids ratio was increased from 6.8 at the beginning of infection to 7.2 in fully infected fruit (data not shown). With the use of this index the potential influence of infection with *C. simmondsii* on fruit taste can be detected prior to visual indication of the infection.

## The influence of *C. simmondsii* infection on strawberry fruit phenolic content

For a more systematic presentation of the results and discussion, the phenolic compounds in strawberries were grouped according to their structure into derivates of ellagic acid, flavanols, flavonols and anthocyanins. Pelargonidin 3-glucoside and pelargonidin 3-malonylglucoside were the most abundant anthocyanins in the analysed fruit. With the beginning of the infection the area adjacent to the infection site became darker than the rest of the fruit. Therefore, as expected, significant differences in the content of almost all anthocyanins have been measured among different treatments (Table 3). Anthocyanins increased with the progression of the infection and the content levels of pelargonidin 3-glucoside were significantly lower in non-infected fruit compared to fruit sampled at the beginning of infection. Similarly, cyanidin glycosides significantly increased after *C. simmondsii* infection. Quercetin 3-glucuronide and kaempferol 3-glucuronide were the prevailing flavonols in the analysed strawberry fruit. The level of flavonols was not significantly different among treatments. Six flavanols were identified at all three stages of fruit infection, three procyanidin dimers and three procyanidin trimers, The highest content levels were determined at the beginning of infection and a minor

**Table 2** The content of organic acids in strawberry fruit (g $kg^{-1}$FW or mg $kg^{-1}$FW for shikimic and fumaric acid) at three stages of *C. simmondsii* infection

| *Fragaria x ananassa* L. 'Clery' | | Non-infected | The beginning of infection | Infected |
|---|---|---|---|---|
| g $kg^{-1}$FW | Citric acid | 12.1±0.6 ns[a] | 11.4±0.3 ns | 10.0±0.7 ns |
| | Malic acid | 10.6±0.4 b | 10.3±0.4 b | 8.1±0.2 a |
| mg $kg^{-1}$FW | Shikimic acid | 33±2 ns | 39±3 ns | 42±4 ns |
| | Fumaric acid | 16±0.1a | 24±1b | 28±2 b |

[a] Different letters denote statistically significant differences among treatments at significance level ($p<0.05$)

**Table 3** The content of individual anthocyanins in strawberry fruit (mg $kg^{-1}$FW) at two stages of *C. simmondsii* infection

| *Fragaria x ananassa* L. 'Clery' | Non-infected fruits | Begining of infection | Infected fruit |
|---|---|---|---|
| Cyanidin 3-glucoside | 10.9±0.7 a[a] | 13.7±0.5 b | 13.0±0.9 ab |
| Pelargonidin 3- glucoside | 742.1±40.5 a | 903.9±32.5 b | 856.3±40.6 ab |
| Cyanidin 3-malonylglucoside | 7.3±0.6 a | 10.2±0.4b | 10.0±0.3 b |
| Pelargonidin 3-malonylglucoside | 238.3±25.9 a | 311.1±26.9 ab | 335.5±17.3 b |
| Total anthocyanins | 998.6 a | 1238.9 b | 1214.8 ab |

[a] Different letters denote statistically significant differences among treatments at significance level ($p<0.05$)

decrease was measured in infected fruits, but it was not significant. Twelve different forms of ellagic acid were detected in strawberry fruit; all forms of ellagic acid increased (Fig. 1a, d) significantly with infection.

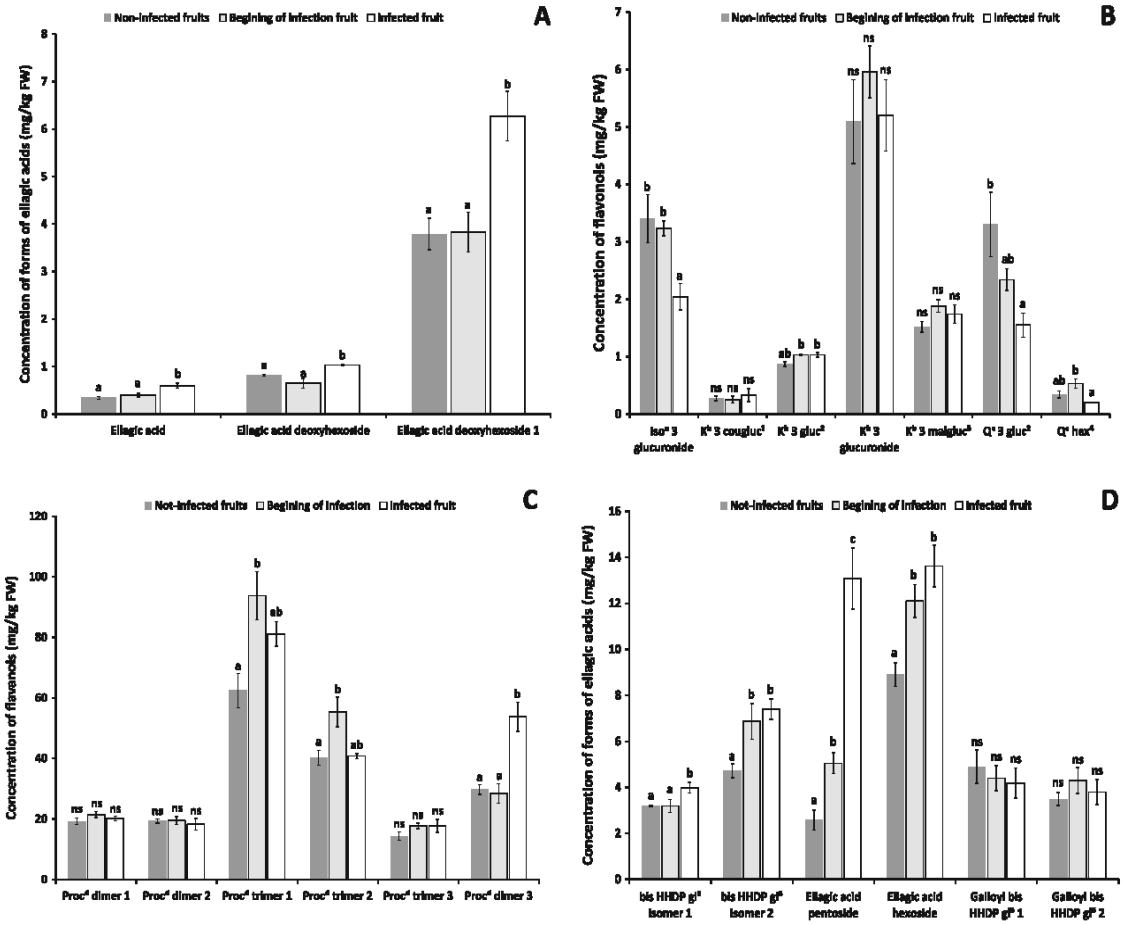


**Fig. 1** Content levels of forms of ellagic acids (**a**, **d**), flavonols (**b**) and flavanols (**c**) in non-infected fruit in the beginning of infection and in the infected strawberry fruit. Different letters denote statistically significant differences between the treatments at significance level ($p<0.05$); ns denotes statistically non-significant differences. Iso[a] - Isorhamnetin; K[b] - Kaempferol; Q[c] – Quercetin; Proc[d] – Procyanidin; cougluc[1] – coumaroylglucoside; gluc[2] – glucoside; malgluc[3] – malonylglucoside; hex[4] – hexoside; gl[5] – glucose

### The influence of *C. simmondsii* infection on strawberry runner phenolic content

Three different derivatives of quercetin, three kaempferol glycosides and two isorhamnetin derivatives were determined in non-infected and infected runners (Table 4). Generally, the content of flavonols was twofold lower in non-infected runners compared to the infected runners. Ten flavanols were confirmed in non-infected and infected strawberry runners, catehin being the prevailing one. Total flavanols content increased after the infection but the content of several individual flavanols decreased also. Ellagic acid dehexoside, ellagic acid pentoside, ellagic acid deoxyhexoside 1, ellagic acid deoxyhexoside and ellagic acid significantly increased after *C. simmondsii* infection. All other forms of ellagic acid decreased or only increased non-significantly (Fig. 2a, b).

## Discussion

### Sugars and organic acids in strawberry fruit

According to Crespo et al. (2010), Milivojevic et al. (2011) and Basson et al. (2010) glucose and fructose are the major sugars in strawberry fruit, which is in accordance with our results. Namely, sugar content and sugar ratio are modified during the ripening process and several studies report a decrease of sucrose content and an increase of fructose and glucose levels in strawberries (Sturm et al. 2003) and in pear (Lindon et al. 2012). The results of other authors also indicate that *C. simmondsii* infection alters carbohydrate content levels in different parts of the host strawberry plant (Crespo et al. 2010; Basson et al. 2010). Lobato et al. (2009), who studied the effect of *C. lindemuthianum* infection on different bean cultivars, observed a total sugar level increase for over 10 % in 'Mexico' cultivar and a 26 % increase in 'Widusa' cultivar compared to non-infected beans. These results indicate that the level of total sugars significantly increased in the susceptible bean cultivar compared to the more resistant 'Mexico' bean cultivar. These results are in accordance to our results and to the data reported by Sturm et al. (2003) and Basson et al. (2010) in different strawberry cultivars.

Because of the effect of sugars/organic acids ratio on perceived sweetness of strawberries and other berries this can act as an important indicator of fruit quality (Terry et al. 2005; Bordonaba and Terry 2008), ripeness (Perez et al. 1997) or even as an index for consumer acceptability (Keutgen and Pawelzik 2007). The sugars/organic acids ratio of non-infected fruit in our research was similar to those found in literature (Davik et al. 2006; Terry et al. 2007) but slightly lower than the one reported by Crespo et al. (2010). That was mainly due to higher organic acid content levels measured in our fruit, which could be related to different growing conditions or cultivars analysed. The increase of sugars/organic acids ratio in our research can be well correlated to a significant decrease of the major organic acids and an increase of the prevailing sugars caused by the fungus infection. With the use of this index the potential influence of infection with *C. simmondsii* on fruit taste can be detected prior to visual indication of the infection.

**Table 4** The content of individual flavonols in strawberry runners (mg $kg^{-1}$FW) at two stages of *C. simmondsii* infection

| *Fragaria x ananassa* L. 'Clery' | Non-infected runners | Infected runners |
|---|---|---|
| Quercetin 3-glucoside | 7.6±0.9 b[a] | 24.2±2.5 a |
| Quercetin 3-glucuronide | 157.2±18.6 ns | 145.2±19.8 ns |
| Quercetin hexoside | 1.7±0.2 b | 5.3±0.6 a |
| Kaempferol 3-glucoside | 0.6±0.1 ns | 0.7±0.2 ns |
| Isorhamnetin 3-galactoside | 2.7±0.1 b | 1.8±0.3 a |
| Kaempferol 3-glucuronide | 0.5±0.0 b | 20.5±2.3 a |
| Isorhamnetin 3-glucuronide | 0.1±0.0 ns | 1±0.4 ns |
| Kaempferol 3-coumaroylglucoside | 0.6±0.0 b | 0.9±0.1 a |
| Total flavonols | 118.6±40.4 a | 242.0±15.9 b |

[a]Different letters denote statistically significant differences between the treatments at significance level ($p<0.05$)

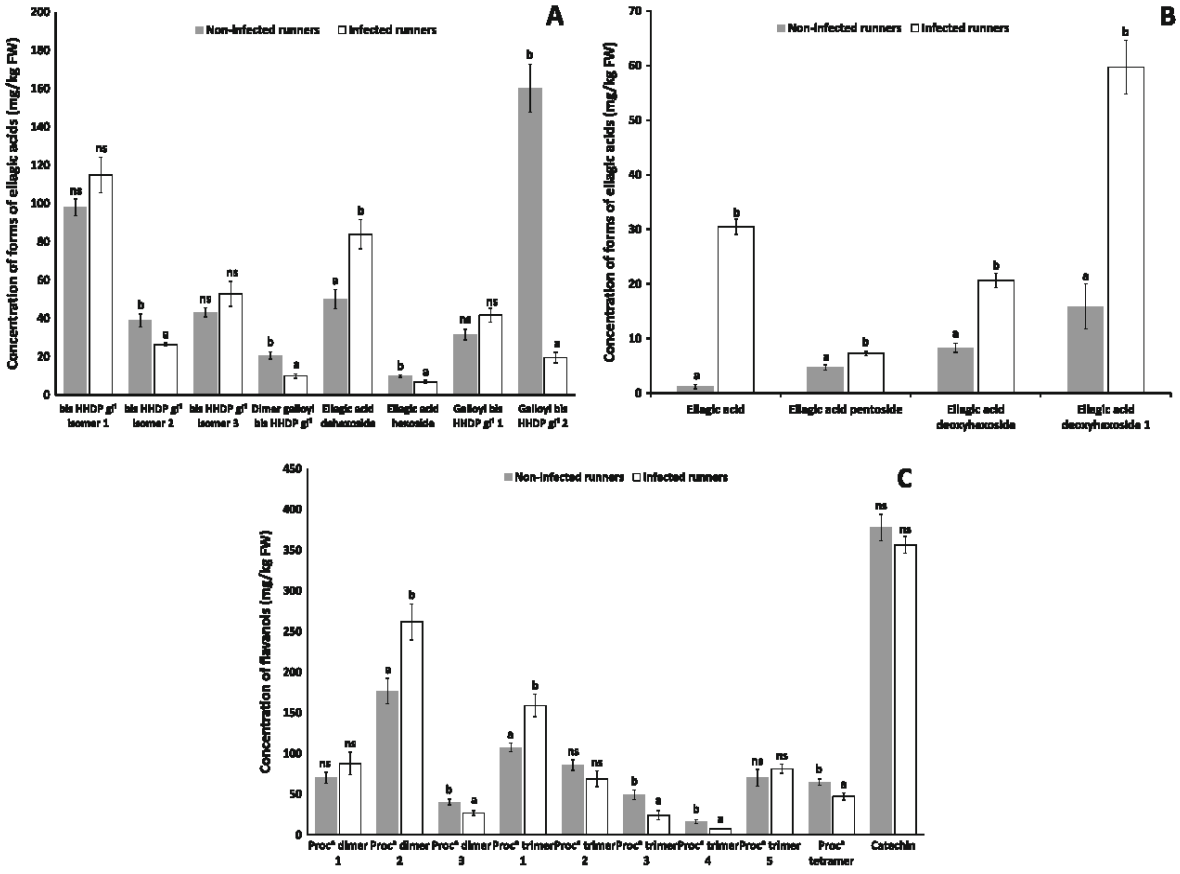


**Fig. 2** Content level of forms of ellagic acid (**a**, **b**) and flavanols (**c**) in infected and non-infected runners of strawberry. Different letters denote statistically significant differences between the treatments at significance level ($p<0.05$); ns denotes statistically non-significant differences. $gl^1$ – Glucose; $Proc^a$ – Procyanidin

## The influence of *C. simmondsii* infection on strawberry fruit phenolic content

The major class of phenolic compounds in strawberry fruit is represented by hydrolysable tannins, i.e. ellagitannins, which are only present in a few other berry fruit species (Aaby et al. 2012). Anthocyanins are the second prevailing class of phenolics in pigmented berries, and hydroxycinnamic acids, flavonols, flavanols, and proanthocyanidins are far less abundant (Määttä-Riihinen et al. 2004; Aaby et al. 2012). The characteristic red colour of strawberries can be linked to the content levels of anthocyanins; mostly glycosides of pelargonidin and cyanidin (Määttä-Riihinen et al. 2004; Aaby et al. 2007; Aaby et al. 2012). Pineli et al. (2011) analysed anthocyanins in 'Camino Real' strawberry and demonstrated that cyanidin glycosides give reddish hues to the mature fruit whereas pelargonidin glycosides a more distinct orange-red shades. Moreover, they reported an approximately 9–13 fold increase of total anthocyanins from green to pink stage of strawberries and similar results have been obtained in a study on 27 different strawberry cultivars (Aaby et al. 2012). Quercetin and kaempferol derivatives are the major flavonols present in strawberries (Vasco et al. 2009). As shown by Halbwirth et al. (2006) flavonols sharply increase at fourth developmental stage of strawberry fruit and then decrease in the fully ripe stage. This can be related to our results, as the content of some flavonols decreased in the full infection stage when the mechanisms of fruit maturation are reportedly stimulated.

Mikulic-Petkovsek et al. (2008; 2009) studied the response of apple fruit to the scab pathogen and measured a significant increase in flavanols as a response to infection, similar to the effect of *C. simmondsii* fungus on strawberries. Twelve different forms of ellagic acid

were detected in strawberry fruit previously reported by Määttä-Riihinen et al. (2004). Strawberries are one of the main sources of ellagic acid, which is normally present as a polymer (ellagitannin) or glycosylated derivatives (Häkkinen et al. 2000).

Solar et al. (2012) reported tight correlations between *Xanthomonas arboricola* pv. *juglandis* and phenolic acids in walnut and found significant differences in ellagic acid content at different stages of infection. It seems that different forms of ellagic acid are increased with infection in strawberry fruits similar to bacterial infection on walnut. As a result of microbial attack, phenolics such as aglicomes or in conjugated forms, may accumulate post-infection or do so constitutively. In the first case, they can rapidly accumulate upon attack, although they may already be present in the plant at low concentrations. In the second case, these compounds are already present in healthy tissues at concentrations high enough for defence, either as free elements or in conjugated forms, from which they are released after the pathogen attack (Strack 1997).

### The influence of *C. simmondsii* infection on strawberry runner phenolic content

The involvement of flavonol glycosides in fruit defence against different pathogens has frequently been reported for apple infected by *Venturia inaequalis* (Mayr et al. 1997; Usenik et al. 2004; Mikulic-Petkovsek et al. 2008, 2009). Mikulic-Petkovsek et al. (2009) studied the response of apple leaf cells induced by the infection with scab pathogen and reported a significant increase of flavonols as a response to the infection. A similar increase was observed in strawberry runners after *C. simmondsii* infection suggesting that flavonols play an important role in plant defence. Aaby et al. (2012) similarly reported a decrease in catehin content during ripening process of different strawberry cultivars which is again in concordance with the fungus accelerating the maturation of strawberry fruit. Flavanols may also interact with proteins and inhibit the enzymes secreted by diverse pathogenic fungi, which is probably the reason for the presence of catechins in defence mechanisms of plants (Vasco et al. 2009). Phenolic derivatives can react and oxidize proteins, thus causing the loss of enzyme function and restricting the viability of aggressors. They can be deposited inside cell walls as an important first line of defence against infection (Schwalb and Feucht 1999). Previous studies (Mayr et al. 1997; Michalek et al. 1999) demonstrated that rapid biosynthesis of flavanols is crucial for successful protection and enhanced resistance.

## Conclusion

The results indicate that the infection with *C. simmondsii* fungus significantly alters the synthesis of primary and secondary metabolites in strawberry runners and fruit. Sucrose decreased by 80.9 %, and fructose and glucose increased in strawberry fruit suggesting that the fungus infection accelerates the ripening process in the infected tissue. Moreover, this can be further confirmed by a significant decrease in the content of malic and citric acids. It is also known that the synthesis and accumulation of several phenols are increased under the influence of different stress conditions. Content levels of different forms of ellagic acids increased in infected strawberry runners (ellagic acid dehexoside, ellagic acid, ellagic acid pentoside and two ellagic acid deoxyhexosides) and a decrease of bis HHDP glucose isomer, dimer of galloyl bis HHDP glucose, ellagic acid hexoside and one of analysed galloyl bis HHDP glucoses was measured. However, in strawberry fruit, all identified forms of ellagic acid increased significantly after *C. simmondsii* infection. Among flavanols, two procyanidin trimers and one procyanidin dimer increased with infection and anthocyanins increased at the beginning of the infection and did not change significantly with the progression of fungal attack.

However, high accumulation of individual phenolics could be a part of mechanisms of general response to a stress situation caused by *C. simmondsii*. Therefore, further studies of the rate of the synthesis, enzymatic studies and the abundance of phenolic compounds as a response to antrachnose infection should be carried out. This could also yield clues to the difference between the responses of antrachnose-susceptible and resistant strawberry cultivars.

**Acknowledgements** The research is part of program Horticulture No. P4-0013-0481 and the project No J4-4187 funded by the Slovenian Research Agency (ARRS).

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### 2.1.2 Spremenjena vsebnost primarnih in sekundarnih metabolitov v plodovih žlahtnega jagodnjaka zaradi okužbe s *Colletotrichum nymphaeae*

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ALTERATION OF THE CONTENT OF PRIMARY AND SECONDARY METABOLITES IN STRAWBERRY FRUIT BY *Colletotrichum nymphaeae* INFECTION

Journal of Agricultural and Food Chemistry, 2013, 61: 5987–5995

Na dveh različnih sortah žlahtnega jagodnjaka smo v poljskem poskusu proučevali vpliv okužbe z glivo *Colletotrichum nymphaeae*, ki povzroča črnolistno pegavost. Izbrali smo sorto 'Elsanta', ki je občutljiva na okužbo s prej omenjeno glivo, in sorto 'Honeoye', ki je tolerantna. V poskus smo vključili 4 obravnavanja: umetna okužba z glivo *C. nymphaeae*, škropljenje s fungicidom na osnovi piroklostrobina in boskalida (Signum), škropljenje s kalcijevim pripravkom (Stopit) in kontrolo, kjer smo rastline poškropili samo z vodo. Z uporabo visokoločljivostne tekočinske kromatografije (HPLC) smo identificirali primarne metabolite v plodovih žlahtnega jagodnjaka (sladkorje in organske kisline), sekundarne metabolite pa smo dodatno potrdili z uporabo masnega spektrofotometra. Okuženi plodovi so vsebovali povečane vsebnosti skupnih sladkorjev in zmanjšane vsebnosti organskih kislin. Razmerje med sladkorji in organskimi kislinami je bilo bistveno večje v okuženih plodovih in tistih, ki smo jih tretirali s kalcijevim pripravkom. Med fenolnimi snovmi, ki smo jih določili, so se vsebnosti derivatov elagne kisline, flavonolov, procianidinov in flavanolov značilno povečale po napadu patogena. Prav tako se je povečala vsebnost skupnih fenolov v okuženih plodovih. V raziskavi nismo zaznali statističnih razlik v vsebnosti analiziranih fenolov med fungicidom in tretiranjem s kalcijem.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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# Alteration of the Content of Primary and Secondary Metabolites in Strawberry Fruit by *Colletotrichum nymphaeae* Infection

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**ABSTRACT:** The effects of infection with *Colletotrichum nymphaeae*, the causative agent of strawberry black spot, were studied on two strawberry cultivars: susceptible 'Elsanta' and tolerant 'Honeoye' cultivars. Four treatments were established: (1) artificial inoculation; (2) spray application of pyraclostrobin + boscalid (Signum); (3) foliar spraying with calcium (Stopit); and (4) control (spraying with water). Primary metabolites (sugars and organic acids) and secondary metabolites (phenolic compounds) were determined in strawberry fruit with the use of HPLC-MS$^n$. Infected fruit accumulated large amounts of total sugars and low levels of organic acids. The sugar/acid ratio was higher in the infected and in Ca-treated strawberries. The contents of ellagic acid derivatives, flavonols, oligomeric procyanidins, flavan-3-ols, and total phenolics were highest in inoculated strawberry fruit. Results indicated that fungicide and calcium sprayings did not alter polyphenolic levels in plant tissue.

**KEYWORDS:** *Fragaria × ananassa, strawberry anthracnose, fungus, inoculation, sugars, organic acids, phenolics, resistance*

## INTRODUCTION

Strawberry anthracnose, also known as strawberry black spot, is a severe disease in commercial strawberry production all over the world, second only to gray mold. The disease predominantly infects fruit, stolons, and crown but can also affect strawberry roots.[1] The heaviest economic losses result from fruit infection, which can occur on immature fruit preharvest, on mature fruit at harvest, or in the postharvest storage stage. In Europe, *Colletotrichum acutatum* has been recognized as predominant and most important causative agent of strawberry anthracnose.[2] The species has been subjected to thorough taxonomic reassessment and is now recognized as a species complex, comprising 31 species. Several species within the *C. acutatum* species complex cause strawberry anthracnose; however, *Collectotrichum nymphaeae* (formerly known as *C. acutatum* molecular group A2 or *C. simmondsii*) is the most important.[3] The best way to control strawberry anthracnose is to prevent the introduction of the pathogen into the field by using anthracnose-free transplants. Different key management strategies, including methods for reduction of pathogen occurrence and spread, cultural practices (cultivation in plastic tunnels), and chemical and biological control measures have been suggested against *C. nymphaeae* infection. However, no chemical methods are effectively controlling the disease. Among cultural techniques, drip irrigation and the use of plastic tunnels considerably limit inoculum dispersal and therefore greatly reduce fruit losses.[1]

Strawberry is an important fruit crop in temperate regions including Central Europe. Attractive fruits are favored for their excellent taste and health-promoting properties due to their richness in vitamins, minerals, and antioxidative compounds.[4] Sugars and organic acids are regarded as significant quality factors defining strawberry fruit taste. Ripeness stage, plant vitality, pedoclimatic conditions, and genotype are known to affect the quantitative variations in sugars and organic acids in strawberry fruit.[5] Some organic acids also have an inhibitory effect on pathogenic organisms. Namely, a low pH level resulting from high content of organic acids produced by plants may require high energy inputs of pathogenic organism to maintain a favorable intracellular pH.[6]

Resistance to diseases may be related to the content and diversity of phenolic compounds in plant cells, as has been reported for some economically important pests and diseases of plants in general and also for several fruit species.[7,8] Phenolic compounds are toxic to the pathogens, and many of them, such as flavanols and hydroxycinnamic acids, can act as passive or inducible barriers against herbivores or microbes. In response to the pathogen attack, the content and composition of polyphenols can change, playing an active role in induced resistance to the pathogens.[8,9]

The most frequently identified phenolic groups in strawberries are hydroxybenzoic and hydroxycinnamic acids, hydrolyzable tannins, flavonols, flavan-3-ols, and anthocyanins. In strawberry fruit high content levels of flavan-3-ols, epicatechin, and procyanidin derivatives have been determined.[10,11] These compounds are beneficial as they increase plant antioxidative capacity and presumably contribute to the restriction of infection caused by plant pathogens such as *Botrytis cinerea*.[12,13] However, the presence of procyanidins in fruits results in an astringent and unfavorable taste.

Only two fungicides can be used against strawberry anthracnose in Slovenian production orchards.[14] Preventive control measures (healthy plant material), site selection,

Received: February 22, 2013
Revised: May 29, 2013
Accepted: June 5, 2013
Published: June 5, 2013





dx.doi.org/10.1021/jf402105g | J. Agric. Food Chem. 2013, 61, 5987−5995

optimal nutrition, and air circulation are therefore very important. One possible way to effectively protect against strawberry anthracnose is the use of resistant or tolerant strawberry cultivars. Therefore, the aim of the study was to determine possible connections between the infection and content levels of primary and secondary metabolites in strawberry plants. The two studied cultivars ('Honeoye' and 'Elsanta') also differ in their susceptibility to *C. nymphaeae* infection.[15,16] Several papers indicate that fungicide sprayings alter polyphenolic levels in plant cells,[17] but other studies[18] suggest that no modification occurs. Therefore, the effects of Signum fungicide application on polyphenolic composition has been studied to determine the effect on strawberry phenolic profile. In the same way, calcium application has been evaluated. Finally, inoculation with *C. nymphaeae* can cause changes in the synthesis of primary and secondary metabolites as some studies have reported for different fungus−plant interactions.[8] The present study highlights individual phenolic compounds that could be involved in defense mechanisms against specific fungal pathogens in addition to other plant strategies, for example, morphological, biochemical, physical, and other mechanisms. With this knowledge breeding strategies can focus on providing plants with high levels of various compounds and, consequently, new anthracnose-resistant strawberry cultivars could be developed. Economic losses in strawberry production due to strawberry anthracnose can potentially be diminished and negative environmental impacts of fungicide treatments greatly reduced.

## MATERIALS AND METHODS

**Fungal Material.** Samples of diseased strawberries showing symptoms of anthracnose were collected in 2009 from strawberry plantations near Ljubljana, central Slovenia. Fungal isolates were obtained by culturing pieces of necrotic tissue from rotten strawberry fruit on potato dextrose agar (PDA). They were identified as *C. nymphaeae* (Pass.) Aa on the basis of cultural characteristics and sequence analysis of ITS and TUB2 according to the method of Damm et al.[3] Single-spore isolate was prepared by spreading spore suspension on PDA plates and isolating individual germinating conidia. Prior to plant inoculation, the single-spore isolate was subcultured on PDA plates and incubated at 24 °C in the dark for 10 days. Spores were then scraped from the plates and dispersed in sterile distilled water. Spore suspension was adjusted to $10^6$ spores/mL.

**Plant Material and Growing Conditions.** The open field trial was conducted in 2011 and 2012 at the experimental station of the Agricultural Institute of Slovenia, located at Brdo pri Lukovici (latitude, 46° 10′ N; longitude, 14° 41′ E). The soil texture is silty loam, rich in potassium and nitrogen and poor in phosphorus. The organic matter is high. Frigo plants of susceptible 'Elsanta' cultivar and tolerant 'Honeoye' cultivars[15,16] were planted on August 3, 2011, on slightly elevated beds covered with black polyethylene at a spacing of 0.25 m × 0.25 m in double rows. The experimental site was equipped with a drip irrigation system.

Six blocks with four treatments were established; each treatment per block included 10 plants: (1) artificial inoculation with *C. nymphaeae*; (2) spray application of pyraclostrobin + boscalid (Signum, BASF); (3) foliar spraying with calcium (Stopit, Yara); and (4) control (spraying with water). The first artificial inoculation was performed on May 21 and the second one on May 28. Spore suspension was applied to the plants by spraying till runoff with a hand sprayer. Immediately after inoculation, the plants were covered with a transparent polyethylene cover to maintain 100% relative humidity. After the first inoculation, no infection occurred due to very dry weather, and therefore a second inoculation was performed. Plants were sprayed with Signum after the second harvest on May 17 and with Stopit (concentration = 14 mL/L) on May 15 and 21. The control treatment plants were sprayed with water.

All plants were treated against diseases with cuprum (Champion, Nufarm) on March 20, 2012. Plant vigor was assessed in spring, and no differences were recorded between cultivars or blocks. The first flowers opened early in April and froze due to low temperatures. On April 20, 2012, all plants were covered with polyethylene to prevent further damage. The weather in the period between full flowering (May 3) and the beginning of harvest (May 17) was very hot and dry. Fruits were harvested two to three times a week, and fruit yield (mass and quantity) and number of infected fruits and runners per plant were evaluated each time. Ninety fruits were sampled per treatment (15 in each block), except for the inoculation treatment for which all fruits with visible symptoms were used. Fruits were recorded as diseased when lesions were visible. Disease incidence was determined as the percentage of the total number of ripe fruits harvested throughout the fruiting season. These percentages were calculated for each section and genotype. The fruits were immediately frozen in liquid nitrogen and stored for up to 1 month at −20 °C until chemical analyses.

**Chemicals.** The following standards were used for the determination of sugars and organic acids: sucrose, fructose, and glucose and citric, malic, and fumaric acid from Fluka Chemie (Buchs, Switzerland); shikimic acid from Sigma-Aldrich (Steinheim, Germany). For the quantitation of phenolic compounds the following standards were used: ellagic acid, cyanidin-3-glucoside, and pelargonidin-3-glucoside from Sigma-Aldrich Chemicals (St. Louis, MO, USA); quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-glucuronide, kaempferol-3-glucoside, (−)-epicatechin, *p*-coumaric acid, and procyanidin B2 from Fluka Chemie. Methanol for the extraction of phenolics was acquired from Sigma-Aldrich Chemicals. The chemicals for the mobile phases were HPLC-MS grade acetonitrile and formic acid from Fluka Chemie GmBH. Water for the mobile phase was double-distilled and purified with a Milli-Q system (Millipore, Bedford, MA, USA). For total phenolic content, Folin−Ciocalteu phenol reagent (Fluka Chemie GmBH), sodium carbonate (Merck, Darmstadt, Germany), and gallic acid and ethanol (Sigma-Aldrich) were used.

**Extraction and Determination of Sugars and Organic Acids.** Primary metabolites (sugars and organic acids) were analyzed in whole strawberry fruit. For each treatment, six repetitions were carried out ($n$ = 6); each repetition included several fruits. For the extraction of primary metabolites, 5 g of fruit was homogenized in 25 mL of double-distilled water using an Ultra-Turrax T-25 (Ika-Labortechnik) and left for 30 min at room temperature as reported by Mikulic-Petkovsek et al.[19] After the extraction, the homogenate was centrifuged (Eppendorf Centrifuge 5810 R) at 12000 rpm for 7 min at 10 °C. The supernatant was filtered through a 0.20 μm cellulose ester filter (Macherey-Nagel) and transferred into a vial, and 20 μL of the sample was used for the analysis. The analysis of primary metabolites was carried out using a high-performance liquid chromatograph (HPLC) of Thermo Separation Products (San Jose, CA, USA). The separation of sugars was carried out using a 300 mm × 7.8 mm i.d. Rezex RCM-monosaccharide Ca+ 2% column from Phenomenex operated at 65 °C. The mobile phase was double-distilled water, and the flow rate was 0.6 mL/min; the total run time was 30 min, and a refractive index (RI) detector was used to monitor the eluted carbohydrates as described by Mikulic-Petkovsek et al.[19] Organic acids were analyzed with the same HPLC system, equipped with a UV detector set at 210 nm, using a 300 mm × 7.8 mm i.d. Rezex ROA-organic acid $H^+$ (8%) column from Phenomenex, as described by Mikulic-Petkovsek et al.[19] The column temperature was set at 65 °C. The elution solvent was 4 mM sulfuric acid in double-distilled water at a flow rate of 0.6 mL/min. The duration of the analysis was 30 min. The sugars and organic acids in strawberry extracts were identified by their retention time characteristics; the concentrations were calculated with the help of the corresponding external standard and expressed as grams per kilogram fresh weight (FW) for sugars and grams per kilogram or milligrams per kilogram FW for organic acids, respectively. The content of all analyzed sugars was summed and presented as total analyzed sugars. In a similar way total analyzed organic acids were calculated. Both values were used for the determination of the sugar/organic acid ratio.

**Table 1. Two-Way ANOVA for Yield per Plant, Number of Fruits per Plant, Number of Infected Fruits, and Number of Infected Runners per Plant of Two Cultivars, Four Treatments, and the Interaction Cultivar × Treatment[a]**

| | | yield | | | |
|---|---|---|---|---|---|
| cultivar | treatment | mass per plant (g) | no. of fruits per plant | no. of infected fruits per plant | no. of infected runners per plant |
| 'Elsanta' | control | 150.1 ± 14.4 abc | 12.6 ± 1.3 | 0 ± 0.00 d | 0.23 ± 0.06 |
| | Signum | 144.7 ± 16.1 abc | 12.7 ± 1.8 | 0.05 ± 0.02 d | 0.09 ± 0.02 |
| | Ca | 158.4 ± 21.9 ab | 13.2 ± 1.5 | 0 ± 0.00 d | 0.15 ± 0.03 |
| | infected | 86.7 ± 6.7 d | 8.1 ± 0.9 | 0.54 ± 0.11 b | 0.33 ± 0.01 |
| 'Honeoye' | control | 78.9 ± 9.9 d | 9.3 ± 1.3 | 0 ± 0.00 d | 0.05 ± 0.02 |
| | Signum | 176.9 ± 17.2 a | 13.7 ± 1.2 | 0.08 ± 0.01 cd | 0.04 ± 0.03 |
| | Ca | 105.1 ± 9.12 bc | 9.8 ± 1.1 | 0 ± 0.00 d | 0.03 ± 0.01 |
| | infected | 98.3 ± 12.3 cd | 8.9 ± 1.1 | 0.23 ± 0.03 c | 0.17 ± 0.01 |
| cultivar × treatment[b] | | * | NS | * | NS |
| cultivar[b] | | NS | NS | NS | * |
| treatment[b] | | ** | * | *** | * |

[a]Different letters in a column denote significant differences (Duncan's test, $p < 0.05$). [b]*, statistically significant differences at $P$ value <0.05; **, statistically significant differences at $P$ value <0.01; ***, statistically significant differences at $P$ value <0.001.

**Extraction of Phenolic Compounds.** The extraction of fruit samples was performed as described by Mikulic-Petkovsek et al.,[20] with some modification. Phenolic compounds (flavonoids and phenolic acids) were analyzed in whole strawberry fruit. For each treatment, six repetitions were carried out ($n = 6$); each repetition included several fruits. Frozen fruits were ground to a fine powder in a mortar chilled with liquid nitrogen, and 5 g was extracted with 10 mL of methanol containing 3% (v/v) formic acid and 1% (w/v) 2,6-di-*tert*-butyl-4-methylphenol (BHT) in a cooled ultrasonic bath for 1 h. BHT was added to the samples to prevent oxidation.

After extraction, the fruit extracts were centrifuged for 10 min at 10000 rpm. Each supernatant was filtered through a Chromafil AO-20/25 polyamide filter produced by Macherey-Nagel (Düren, Germany) and transferred to a vial prior to injection into the high-performance liquid chromatography (HPLC) system.

**Determination of Individual Phenolic Compounds Using HPLC-DAD-MS$^n$ Analysis.** Phenolic compounds were analyzed on a Thermo Finnigan Surveyor HPLC system (Thermo Scientific) with a diode array detector at 280 nm (flavan-3-ols, cinnamic acid derivatives), 350 nm (flavonols), and 530 nm (anthocyanins). Spectra of the compounds were recorded between 200 and 600 nm. The column was a 150 × 4.6 mm i.d., 3 μm, Gemini $C_{18}$ (Phenomenex, Torrance, CA, USA) operated at 25 °C. The elution solvents were aqueous 0.1% formic acid in double-distilled water (A) and 0.1% formic acid in acetonitrile (B). Samples were eluted according to the linear gradient from 5 to 20% B in the first 15 min, followed by a linear gradient from 20 to 30% B for 5 min, then an isocratic mixture for 5 min, followed by a linear gradient from 30 to 90% B for 5 min, and then an isocratic mixture for 15 min before returning to the initial conditions.[21] The injection amount was 20 μL and flow rate, 0.6 mL/min.

All phenolic compounds were identified using a mass spectrometer (Thermo Scientific, LCQ Deca XP MAX) with electrospray ionization (ESI) operating in negative ion mode (all phenolic groups except for anthocyanins) and positive ion mode (anthocyanins). The analyses were carried out using full scan data-dependent MS$^n$ scanning from $m/z$ 115 to 1500. The injection volume was 10 μL, and the flow rate was maintained at 0.6 mL/min. The capillary temperature was 250 °C, the sheath gas and auxiliary gas were 20 and 8 units, respectively, and the source voltage was 4 kV for negative ionization and 0.1 kV for positive ionization. Spectrometric data were elaborated using Excalibur software (Thermo Scientific). The identification of compounds was confirmed by comparing retention times and their spectra as well as by adding the standard solution to the sample and by fragmentation.

Concentrations of phenolic compounds were calculated from peak areas of the sample and the corresponding standards and expressed in milligrams per kilogram FW of strawberry fruit. For compounds lacking standards, quantitation was carried out using similar compounds as standards. Thus, glycosides of kaempferol were quantitated in equivalents of kaempferol-3-glucoside and all procyanidin dimers and trimers in equivalents of procyanidin B2; *p*-coumaroyl glucoside was quantitated in equivalents of *p*-coumaric acid and pelargonidin-3-malonylglucoside in equivalents of pelargonidin-3-glucoside; all ellagic acid derivatives were quantitated in equivalents of ellagic acid.

**Determination of Total Phenolic Content.** The extraction of 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. Total phenolic content (TPC) of extracts was assessed according to the Folin−Ciocalteu phenol reagent method.[22] To 100 μL of the sample extracts (diluted 1: 4 (v/v) with MeOH) were added 6 mL of double-distilled water and 500 μL of Folin−Ciocalteu reagent; after between 8 s and 8 min at room temperature, 1.5 mL of sodium carbonate (20% w/v) was added. The extracts were mixed and kept for 30 min at 40 °C before the absorbance was measured on a UV−vis Lambda Bio spectrophotometer (Perkin-Elmer, Waltham, MA, USA) at 765 nm. A mixture of water and reagents was used as a blank. TPC was expressed as gallic acid equivalents (GAE) in milligrams per kilogram FA of tissue. Absorption was measured in three replications.

**Statistical Analysis.** The data were analyzed using the Statgraphics Plus 4.0 program (Manugistics, Inc., Rockville, MD, USA). A two-way analysis of variance was carried out to determine the significance of cultivar and treatment (control, calcium, Signum, and inoculation treatment) on strawberry fruit phenolic profile. The significance of the treatment on the content of individual phenolic compounds, sugars, organic acids, and total phenolic content was tested using one-way analysis of variance (ANOVA). Differences among treatments were tested with Duncan's test at a 0.05 significance level. Multivariate statistical analysis (hierarchical cluster analysis, discriminate analysis, and classification) was conducted to interpret the differences in average values of all analyzed parameters (sugars, organic acids, and phenolics) for the two strawberry cultivars among different treatments. Ward's method based on squared Euclidean distance was used to interpret the difference or similarity in determined compounds among treatments analyzed.

## ■ RESULTS AND DISCUSSION

**Yield and Fruit Infection Assessment.** As strawberry yield is greatly influenced by fungal attack, the infection rate of *C. nymphaeae* was assessed during strawberry fruit maturation. The numbers of infected runners and fruits among different treatments are presented in Table 1. The highest numbers of

**Table 2. Content of Individual and Total Analyzed Sugars and Organic Acids, Sugars/Organic Acids Ratio of Strawberry Fruit, and Two-Way ANOVA of Cultivar (C), Treatment (T: Control, Calcium, Signum, and Infected), and Their Interaction (C × T)**

| | 'Elsanta' | | | | 'Honeoye' | | | | factors[a] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | mean content ± SE in g/kg FW | | | | mean content ± SE in g/kg FW | | | | | | |
| | control | Signum | calcium | inoculation | control | Signum | calcium | inoculation | C | T | C × T |
| fructose | 33.1 ± 2.2 | 41.0 ± 1.5 | 42.5 ± 2.3 | 50.6 ± 3.0 | 34.9 ± 1.7 | 34.7 ± 1.1 | 37.9 ± 2.8 | 55.0 ± 1.5 | NS | *** | NS |
| glucose | 32.6 ± 1.9 | 38.5 ± 1.6 | 39.2 ± 2.3 | 45.2 ± 1.2 | 32.1 ± 1.8 | 32.1 ± 1.4 | 35.0 ± 2.5 | 47.9 ± 1.8 | NS | *** | NS |
| sucrose | 10.0 ± 1.2 | 9.8 ± 1.3 | 6.7 ± 0.7 | 1.6 ± 0.11 | 9.7 ± 1.3 | 9.3 ± 1.1 | 9.4 ± 1.3 | 5.0 ± 0.26 | NS | *** | NS |
| **total sugars** | **75.7 ± 3.9** | **85.2 ± 5.1** | **88.5 ± 5.0** | **97.5 ± 4.1** | **76.8 ± 4.2** | **76.1 ± 3.5** | **82.3 ± 5.1** | **108.0 ± 2.8** | **NS** | ******* | **NS** |
| citric acid | 6.7 ± 0.3 | 7.0 ± 0.3 | 6.0 ± 0.2 | 4.7 ± 0.6 | 6.6 ± 0.2 | 7.4 ± 0.3 | 6.0 ± 0.2 | 5.4 ± 0.6 | NS | *** | NS |
| malic acid | 2.4 ± 0.17 | 2.6 ± 0.2 | 1.7 ± 0.2 | 1.3 ± 0.06 | 2.1 ± 0.16 | 1.9 ± 0.2 | 1.9 ± 0.18 | 0.8 ± 0.09 | * | *** | NS |
| fumaric acid ($\times 10^{-3}$) | 7.3 ± 0.6 | 7.6 ± 0.5 | 6.4 ± 0.5 | 11.9 ± 1.5 | 7.3 ± 0.4 | 7.0 ± 0.5 | 8.0 ± 0.9 | 12.4 ± 1.6 | NS | *** | NS |
| shikimic acid ($\times 10^{-3}$) | 9.8 ± 0.5 | 9.0 ± 0.6 | 8.5 ± 0.4 | 8.2 ± 0.5 | 9.9 ± 0.4 | 9.0 ± 0.6 | 8.9 ± 0.4 | 10.7 ± 0.2 | NS | NS | NS |
| **total organic acids** | **9.2 ± 0.4** | **9.6 ± 0.5** | **7.3 ± 0.4** | **6.1 ± 0.5** | **8.8 ± 0.3** | **9.3 ± 0.5** | **7.9 ± 0.2** | **6.2 ± 0.7** | **NS** | ******* | **NS** |
| sugars/organic acids ratio | 8.4 ± 0.6 | 8.9 ± 0.8 | 12.3 ± 1.2 | 16.4 ± 1.4 | 8.8 ± 0.5 | 8.2 ± 0.4 | 10.3 ± 0.7 | 18.4 ± 2.1 | NS | *** | NS |

[a]*, statistically significant differences at $P$ value <0.05; **, statistically significant differences at $P$ value <0.01; ***< statistically significant differences at $P$ value <0.001.

infected fruits and runners were recorded in the inoculation treatment. This indicates that the fungus caused a significant increase of infected fruits and a moderate increase of infected runners. However, no statistical differences in the numbers of infected fruit were recorded among control, Signum, and calcium treatments. Due to unfavorable natural conditions for anthracnose infection in 2012 the control plants did not develop visible signs of infection of strawberry fruit. Nevertheless, the infection has been detected on runners, particularly on the 'Elsanta' cultivar, which was less infected if treated with Signum and calcium. The infection rate was also significantly correlated with combined fruit yield: lowest yields were documented in the inoculation treatment for 'Elsanta' cultivar and in inoculation and control treatments for tolerant 'Honeoye' cultivar. Compared to the 'Elsanta' cultivar the latter blooms earlier, and in 2012 late frosts in April damaged many blooms. Long-term strawberry yields are approximately 60% higher compared to total yield in 2012 due to late spring frosts causing flower and fruit deformations.

**Content of Sugars and Organic Acids in Strawberry Fruit.** Previously identified sugars in strawberry fruit mainly belong to mono- and disaccharides (glucose, fructose, and sucrose).[23] Fructose and glucose represent the major sugars in strawberries, and sucrose accounts for only 10% of total sugars (Table 2). *C. nymphaeae* infected fruit contained up to 1.4-fold levels of total sugars compared to the control treatment (noninfected fruit). Several other studies on different plants also report overaccumulation of sugars in infected organs. Namely, when plants are exposed to stress, they commonly react with an increased accumulation of sugars in fruits[24] and leaves.[25] Presumably, carbohydrate consumption is required for production of energy to support the biosynthesis of defensive phenolic compounds induced by wounding.[26] Phytochemical analysis provides strong evidence that some sugars play a key role in the antimicrobial defense system in plants. Naqvi et al.[27] reported that the presence of a significant concentration of sugars is in correlation with antimicrobial compounds produced by plants which possess the capability to kill pathogens or inhibit their growth. It is also likely that, due to damaged cuticle of the infected fruit, the content of total sugars increases as a result of partial desiccation. Interestingly, higher total sugar content was measured in calcium-treated fruits with respect to the control. Calcium increases fruit firmness, altering the composition of cell walls and potentially causing modification in primary metabolite content levels. Lara et al.[28] also determined high sugar levels in fruits of plants subjected to calcium application. Calcium-treated fruits were able to retain more sugars in their cell walls, probably as a consequence of calcium deposition in pectin polysaccharides. This also greatly improves strawberry fruit texture.[28]

The main organic acids determined in strawberry fruit were citric and malic acids. This is in accordance with previous results.[19,29] The share of citric and malic acids in strawberries represented almost 99% total organic acids (Table 2). Although organic acids in strawberries are present in much lower concentration than sugars, their effect on fruit flavor is considerable. *C. nymphaeae* had an opposite effect on total organic acid content levels compared to sugars. Infected fruits of both analyzed cultivars contained lowest levels of organic acids compared to other treatments. This can be explained by the fact that the *Colletotrichum* fungus secretes ammonia and increases the pH level of fruits, which favors pectate lyase enzyme secretion.[30] Similarly, Kamilova et al.[31] reported that the content of organic acids was lower in *Fusarium oxysporum* f. sp. *radicis-lycopersici* infected tomato fruit. The reason for this effect is that pathogenic fungi utilize carbon from organic acids for their growth and development[31] and at the same time secrete cell wall degrading enzymes.[30] Several studies have evaluated the antimicrobial effect of organic acids on the growth of microorganisms in food. Organic acids and their salts alone or in combination have been reported to inhibit the growth of bacteria or fungi.[6,32]

The sugar to acid ratio is defined as the proportion of total sugars compared to the total organic acids in plant sample. The sugar/acid ratio is largely accountable for the taste and flavor of fruits. The calculated ratio is often utilized as an index of sweetness for specific fruits, and those with high sugar to acid ratios are considered to be sweeter than fruits with a low ratio. Fruits that taste sweet do not necessarily have a high sugar content, but they generally contain characteristically low levels of organic acids, especially malic acid.[33] Moreover, individual sugar-to-acid ratios influence the perception of sweetness;

**Table 3. Identification of Phenolic Compounds in Strawberry Fruit in Positive and Negative Ions with HPLC-MS, $MS^2$, and $MS^3$**

| λ (nm) | $[M - H]^-$ (*m*/*z*) | $MS^2$ (*m*/*z*) | $MS^3$ (*m*/*z*) | tentative identification | content expressed as |
|---|---|---|---|---|---|
| 280 | 783 | 481, 301 | 257 | bis-HHDP[a] glucose 1 | ellagic acid |
| | 783 | 481, 301 | 257 | bis-HHDP glucose 2 | ellagic acid |
| | 577 | 425, 407, 289 | | procyanidin dimer 1 | procyanidin B2 |
| | 577 | 425, 407, 289 | | procyanidin dimer 2 | procyanidin B2 |
| | 865 | 577,425 | 407, 289 | procyanidin trimer 1 | procyanidin B2 |
| | 289 | 245 | | epicatechin | epicatechin |
| | 849 | 577, 425, 407 | 425, 407, 289 | procyanidin trimer 2 | procyanidin B2 |
| | 325 | 163 | | *p*-coumaroyl glucoside | *p*-coumaric acid |
| | 633 | 301 | 257 | HHDP-galloyl-glucose | ellagic acid |
| | 849 | 577, 425, 407 | 425, 407, 289 | procyanidin trimer 3 | procyanidin B2 |
| | 935 | 633, 301 | 301 | galloyl-bis-HHDP glucose 1 | ellagic acid |
| | 935 | 633, 301 | 301 | galloyl-bis-HHDP glucose 2 | ellagic acid |
| 350 | 463 | 301 | 257 | ellagic acid hexoside | ellagic acid |
| | 433 | 301 | 257 | ellagic acid pentoside | ellagic acid |
| | 447 | 301 | 257 | ellagic acid deoxyhexoside | ellagic acid |
| | 463 | 301 | 179, 151 | quercetin-3-*O*-glucoside | quercetin-3-*O*-glucoside |
| | 477 | 301 | 179, 151 | quercetin-3-*O*-glucuronide | quercetin-3-*O*-glucuronide |
| | 447 | 285 | | kaempferol-3-*O*-glucoside | kaempferol-3-glucoside |
| | 461 | 285 | | kaempferol-3-glucuronide | kaempferol-3-glucoside |
| | 489 | 285 | | kaempferol-3-*O*-acetylglucoside | kaempferol-3-glucoside |
| | 593 | 285 | | kaempferol-3-*O*-coumaroylglucoside | kaempferol-3-glucoside |
| 530 | 449[b] | 287 | | cyanidin-3-*O*-glucoside | cyanidin-3-*O*-glucoside |
| | 433[b] | 271 | | pelargonidin-3-*O*-glucoside | pelargonidin-3-glucoside |
| | 519[b] | 433/271 | | pelargonidin-3-*O*-malonylglucoside | pelargonidin-3-glucoside |

[a]HHDP, hexahydroxydiphenic acid. [b]$[M + H]^+$ (*m*/*z*) anthocyanins were obtained in the positive ion mode.

however, in strawberry, total sugar and acid content was shown to have a greater effect on fruit flavor.[5] The sugar/acid ratio was statistically highest in infected fruit (values of >16 were recorded) (Table 2). This ratio was approximately 2-fold higher than the sugar/acid ratio of noninfected strawberries and can be correlated to sweeter-tasting fruit. Similarly, calcium-treated fruits were characterized by higher sugar/acid ratios compared to fruits of the control and Signum treatments. Other researchers reported a higher sugar to acid ratio of calcium-treated fruits.[34,35] The present study also indicates that this ratio can be altered by different factors, that is, pathogen infection.

**Content of Individual and Total Phenolics in Strawberry Fruit.** The study of phenolic compounds during the progression of infection is very relevant on the basis of their proposed defensive role in plants. In strawberry fruit, 24 different phenolic compounds (Table 3) have been determined and grouped into the following phenolic classes: ellagic acid derivatives, flavonols, flavan-3-ols, derivatives of hydroxycinnamic acids, and anthocyanins. The greatest share of all identified phenolic compounds (AIP) in strawberry fruit was represented by flavan-3-ols (60% AIP) and anthocyanins (33% AIP) (Table 4).

Ellagic acid conjugates have frequently been identified in *Fragaria* genus. Previous studies on strawberry have reported the presence of several derivatives of ellagic acid in different plant organs, that is, fruits and leaves.[10,11] The content of ellagic acid derivatives also differed between the inoculation treatment and other treatments (Table 4). However, no differences have been determined between calcium, Signum, and control treatments. This suggests that the infection caused the increase of ellagic acid derivatives. Specifically, 'Elsanta' and 'Honeoye' fruits of the inoculation treatment contained significantly higher levels (from 1.6- to 2-fold, respectively) of some individual and total ellagic acid conjugates. Higher content of ellagic acid conjugates can be ascribed to modifications in the metabolic processes within the plant as several studies report increased accumulation of specific compounds as a defense mechanism to stress. With up-regulation of certain metabolic processes, the plant aims to stop or reduce the growth of the pathogen, and ellagic acid and its derivatives are known to possess antimicrobial activity.[36,37]

Flavonols represented approximately 2% AIP in strawberry fruit. Several authors report health-related importance of flavonols, and particularly antimutagenic and anticarcinogenic effects have been studied.[38] The results of our study indicate that infected strawberry fruit contained significantly higher levels of quercetin-3-glucuronide and all glycosides of kaempferol (Table 4). Again, no differences have been determined among other treatments (calcium or Signum application and control). Similar to these findings, higher content levels of several flavonols have been recorded in fruit infected with various pathogens, bacteria, or viruses compared to healthy fruit tissue.[7,39] It has been shown that apple scab infection increased the synthesis of flavonols, for example, rutin and quercetin-3-rhamnoside.[8,40]

Epicatechin was the most abundant flavan-3-ol in strawberry fruit and ranged from 50 to 70% of total analyzed flavan-3-ols (Table 4). The tolerant 'Honeoye' cultivar contained 50% higher levels of epicatechin in healthy fruit compared to the more susceptible 'Elsanta' cultivar. Different authors have also reported higher levels of individual flavan-3-ols in fruits of resistant cultivars.[33,41] Moreover, infected fruits of the 'Honeoye' cultivar were characterized by 1.2−2-fold higher levels of epicatechin compared to other treatments. In contrast, the 'Elsanta' cultivar contained the lowest levels of epicatechin


dx.doi.org/10.1021/jf402105g | *J. Agric. Food Chem.* 2013, 61, 5987−5995

**Table 4. Content of Individual and Total Analyzed Phenolic Compounds and Total Phenolic Content in Strawberry Fruit and Two-Way ANOVA of Cultivar (C), Treatment (T: Control, Calcium, Signum, and Infected), and Their Interaction (C × T)**

| | 'Elsanta' | | | | 'Honeoye' | | | | factors[b] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | mean content[a] ± SE in mg/kg FW | | | | mean content[a] ± SE in mg/kg FW | | | | | | |
| polyphenol[c] | control | Signum | calcium | inoculation | control | Signum | calcium | inoculation | C | T | C × T |
| 1 | 10.1 ± 1.1 | 11.7 ± 2.0 | 9.6 ± 0.7 | 13.7 ± 1.1 | 12.3 ± 0.9 | 15.9 ± 1.1 | 11.4 ± 2.1 | 15.9 ± 2.4 | * | * | NS |
| 2 | 20.1 ± 6.5 ab | 32.1 ± 4.1 a | 10.1 ± 4.7 bc | 0.15 ± 0.01 c | 3.4 ± 0.2 c | 0.28 ± 0.05 c | 2.4 ± 0.2 c | 13.8 ± 1.9 bc | ** | NS | *** |
| 3 | 4.9 ± 0.4 | 4.5 ± 0.6 | 6.1 ± 0.9 | 6.8 ± 0.8 | 4.6 ± 0.3 | 5.0 ± 0.09 | 5.3 ± 0.8 | 6.0 ± 0.7 | NS | * | NS |
| 4 | 0.7 ± 0.06 | 0.7 ± 0.03 | 0.8 ± 0.07 | 1.5 ± 0.2 | 2.1 ± 0.2 | 2.1 ± 0.09 | 2.5 ± 0.3 | 3.2 ± 0.4 | *** | *** | NS |
| 5 | 6.8 ± 0.4 | 7.1 ± 0.5 | 7.5 ± 0.8 | 7.5 ± 0.9 | 7.2 ± 0.5 | 7.3 ± 0.2 | 7.4 ± 0.8 | 5.8 ± 0.4 | NS | NS | NS |
| 6 | 7.3 ± 1.0 | 9.8 ± 1.1 | 6.2 ± 0.8 | 8.9 ± 0.9 | 3.9 ± 0.4 | 4.3 ± 0.5 | 5.5 ± 0.5 | 11.8 ± 1.1 | NS | * | NS |
| 7 | 2.8 ± 0.4 | 2.0 ± 0.2 | 4.8 ± 0.6 | 5.1 ± 0.5 | 2.5 ± 0.4 | 2.8 ± 0.4 | 3.2 ± 0.5 | 5.2 ± 0.8 | NS | *** | NS |
| 8 | 2.3 ± 0.1 cd | 1.3 ± 0.1 d | 2.6 ± 0.4 cd | 7.8 ± 0.9 a | 3.5 ± 0.3 c | 3.9 ± 0.2 c | 4.2 ± 0.6 bc | 6.0 ± 0.5 ab | NS | *** | * |
| **9** | **34.2 ± 2.4** | **30.9 ± 2.1** | **42.1 ± 4.8** | **67.2 ± 7.5** | **39.8 ± 3.3** | **41.8 ± 1.7** | **42.1 ± 5.2** | **64.6 ± 6.5** | **NS** | *** | **NS** |
| 10 | 5.8 ± 1.1 | 4.0 ± 0.6 | 5.2 ± 0.9 | 11.3 ± 0.1 | 9.2 ± 1.0 | 7.4 ± 0.8 | 7.0 ± 0.9 | 14.5 ± 1.8 | * | *** | NS |
| 11 | 2.5 ± 0.1 | 2.5 ± 0.3 | 2.9 ± 0.1 | 4.5 ± 0.5 | 3.7 ± 0.3 | 4.3 ± 0.3 | 4.4 ± 0.3 | 6.6 ± 0.8 | *** | *** | NS |
| 12 | 4.4 ± 0.4 | 4.2 ± 0.7 | 3.5 ± 0.5 | 7.3 ± 0.9 | 5.8 ± 0.7 | 8.8 ± 0.2 | 7.4 ± 0.8 | 9.3 ± 0.8 | *** | *** | NS |
| 13 | 0.2 ± 0.06 | 0.8 ± 0.07 | 0.14 ± 0.02 | 1.8 ± 0.2 | 0.7 ± 0.08 | 0.6 ± 0.09 | 0.9 ± 0.1 | 1.5 ± 0.3 | NS | *** | NS |
| 14 | 1.3 ± 0.17 | 0.9 ± 0.11 | 1.0 ± 0.12 | 3.3 ± 0.3 | 1.0 ± 0.1 | 0.9 ± 0.1 | 1.1 ± 0.11 | 2.9 ± 0.3 | NS | *** | NS |
| 15 | 1.6 ± 0.2 | 1.9 ± 0.25 | 1.2 ± 0.19 | 3.5 ± 0.39 | 1.7 ± 0.09 | 1.9 ± 0.08 | 1.6 ± 0.17 | 3.1 ± 0.33 | NS | *** | NS |
| **16** | **15.8 ± 0.1.9** | **13.5 ± 1.4** | **14.1 ± 1.9** | **31.8 ± 2.8** | **22.2 ± 0.17** | **24.0 ± 0.8** | **22.6 ± 0.87** | **38.0 ± 3.8** | *** | *** | **NS** |
| 17 | 281.5 ± 27.8 cd | 198.0 ± 20.9 d | 315.2 ± 32.4 cd | 554.2 ± 53.7 b | 424.0 ± 45.2 bc | 724.6 ± 67.3 a | 450.1 ± 48.1 bc | 873.0 ± 88.7 a | *** | *** | *** |
| 18 | 36.4 ± 2.4 | 37.9 ± 4.2 | 38.8 ± 4.2 | 57.3 ± 6.3 | 51.3 ± 4.7 | 53.6 ± 6.2 | 53.5 ± 7.4 | 79.4 ± 8.1 | *** | *** | NS |
| 19 | 28.1 ± 3.4 cde | 73.4 ± 8.1 ab | 31.8 ± 4.1 cd | 54.3 ± 5.9 bc | 21.3 ± 2.9 de | 31.4 ± 3.5 cd | 4.2 ± 0.5 e | 95.9 ± 10.2 a | NS | *** | ** |
| 20 | 58.2 ± 6.3 | 55.1 ± 4.4 | 42.1 ± 5.3 | 81.5 ± 9.1 | 43.1 ± 5.1 | 79.0 ± 8.9 | 64.3 ± 8.2 | 104.5 ± 12.0 | NS | *** | NS |
| 21 | 36.1 ± 4.7 | 23.3 ± 3.4 | 41.4 ± 4.7 | 140.1 ± 18.7 | 48.9 ± 6.8 | 37.4 ± 4.8 | 63.5 ± 4.0 | 145.4 ± 17.9 | NS | *** | NS |
| 22 | 41.7 ± 5.8 d | 28.7 ± 4.2 d | 46.5 ± 6.1 cd | 127.8 ± 13.7 b | 52.4 ± 7.8 d | 113.6 ± 12.0 bc | 90.2 ± 10.6 bc | 160.8 ± 15.4 a | *** | *** | * |
| **23** | **478.6 ± 39.0 de** | **416.5 ± 33.1 e** | **515.9 ± 62.0 de** | **1015.3 ± 42.9 b** | **641.1 ± 62.6 cd** | **1039.6 ± 75.0 b** | **722.5 ± 33.1 c** | **1459.1 ± 109.0 a** | *** | *** | ** |
| 24 | 19.6 ± 2.0 | 16.3 ± 2.3 | 15.1 ± 2.1 | 30.4 ± 4.4 | 20.6 ± 1.8 | 27.4 ± 3.4 | 17.6 ± 2.6 | 33.0 ± 4.2 | NS | *** | NS |
| 25 | 210.4 ± 14.0 c | 347.3 ± 15.6 b | 345.1 ± 40.2 b | 186.7 ± 20.4 c | 173.7 ± 11.7 c | 416.8 ± 16.9 a | 414.5 ± 30.6 a | 197.8 ± 20.5 bc | NS | *** | * |
| 26 | 20.9 ± 8.2 c | 72.2 ± 9.6 b | 105.2 ± 2.8 a | 70.6 ± 7.2 b | 61.5 ± 8.3 b | 76.3 ± 4.3 b | 107.5 ± 6.5 a | 66.4 ± 7.3 b | * | *** | * |
| **27** | **269.7 ± 22.3 cd** | **205.3 ± 13.0 d** | **308.8 ± 41.2 cd** | **371.6 ± 47.5 bc** | **477.5 ± 32.7 ab** | **535.2 ± 26.5 a** | **517.4 ± 42.4 a** | **244.6 ± 26.4 d** | *** | **NS** | *** |
| 28 | 5.8 ± 0.5 bc | 3.5 ± 0.4 c | 6.7 ± 0.9 bc | 20.0 ± 3.1 a | 8.9 ± 0.8 b | 10.0 ± 0.5 b | 10.8 ± 1.9 ab | 16.7 ± 1.1 a | * | *** | * |
| 29 | 1299.1 ± 45.0 | 1585.0 ± 59.1 | 1634.8 ± 80.3 | 1822.1 ± 78.4 | 1267.7 ± 49.1 | 1550.3 ± 65.0 | 1543.8 ± 61.1 | 1904.7.0 ± 76.1 | NS | *** | NS |

[a]Different letters in rows denote significant differences (Duncan's test, $p < 0.05$). [b]*, statistically significant differences at $P$ value <0.05; **, statistically significant differences at $P$ value <0.01; ***, statistically significant differences at $P$ value <0.001. [c]Polyphenol names: 1, bis-HHDP glucose isomer 1; 2, bis-HHDP glucose isomer 2; 3, ellagic acid deoxyhexoside; 4, ellagic acid hexoside; 5, ellagic acid pentoside; 6, galloyl bis-HHDP glucose 1; 7, galloyl bis-HHDP glucose 2; 8, HHDP galloyl glucose; 9, total ellagic acid conjugates; 10, kaempferol-3-glucoside; 11, kaempferol-3-glucuronide; 12, kaempferol-3-acetylglucoside; 13, kaempferol-3-coumaroylglucoside; 14, quercetin-3-glucoside; 15, quercetin-3-glucuronide; 16, total flavonols; 17, epicatechin; 18, procyanidin dimer 1; 19, procyanidin dimer 2; 20, procyanidin trimer 1; 21, procyanidin trimer 2; 22, procyanidin trimer 3; 23, total flavan-3-ols; 24, cyanidin-3-glucoside; 25, pelargonidin-3-glucoside; 26, pelargonidin-3-malonylglucoside; 27, total anthocyanins; 28, *p*-coumaroyl glucoside; 29, total phenolic content (mg gallic acid equiv/kg FW).

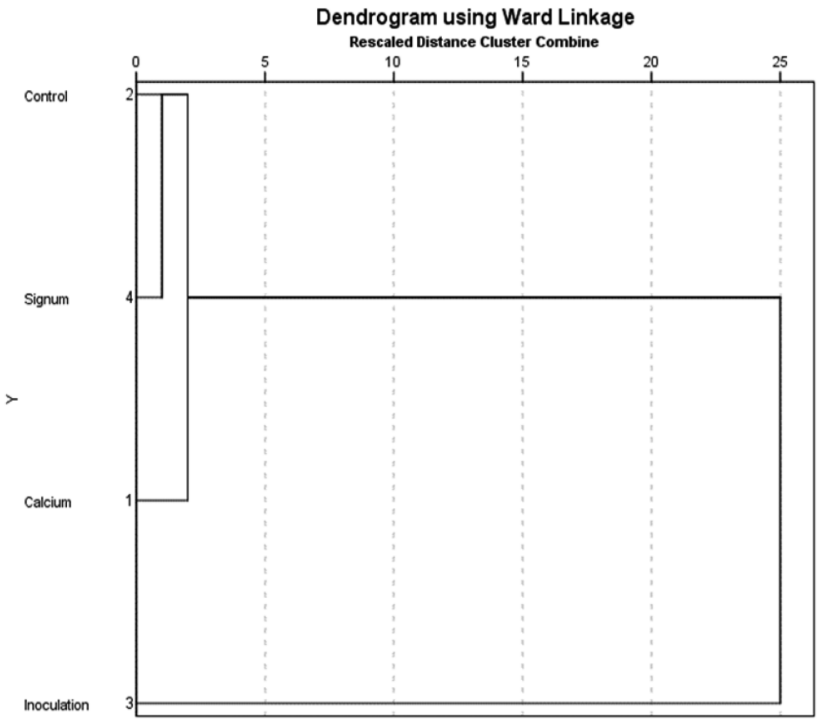


**Figure 1.** Dendrogram combining data on primary and secondary metabolites analyzed in strawberry fruit subjected to different treatments (control, calcium, Signum, and inoculation), using Ward's method based on squared Euclidian distance.

in fruit treated with Signum fungicide and the highest content in inoculated fruits. It seems that the fungus caused increased accumulation of epicatechin in infected tissue. Some researchers[8,40] have also observed a dramatic increase in monomeric flavanols and their polymers in the boundary zones near the infection site, potentially being the reason for its restricted spread. The role of flavan-3-ols in pathogen defense of plants might be their interaction with proteins and the inhibition of enzyme activity secreted by pathogenic fungi.[9] A high diversity of procyanidins has also been determined in strawberry fruit; di-, tri-, and tetramers have been identified. Oligomeric procyanidins were increased up to 4.5-fold as a result of infection with *C. nymphaeae*. Correspondingly, a 36% procyanidin increase has been reported in virus-infected grapevine leaves[39] and a 200% increase in fungus-infected cacao leaves.[42] Higher levels of individual procyanidin forms and epicatechin in infected strawberry fruit have also caused a 2.2−2.4-fold higher levels of total flavan-3-ols compared to other treatments. Previous studies report similar plant reactions in terms of phenolic synthesis as a response to disease or pest attack.[9] It has been suggested that a rapid accumulation of monomeric and polymeric flavanols at the infection site stops further dissemination of the pathogens.

Among hydroxycinamic acid derivatives *p*-coumaroyl glucoside has been identified in the range of 3.51−20.08 mg/kg FW in strawberry fruit (Table 4). The inoculation treatment caused a significant increase of *p*-coumaroyl glucoside, and 1.8−5.7-fold higher levels were measured in infected fruit compared to other treatments. Likewise, *p*-coumaroyl glucoside was increased in strawberry fruit after powdery mildew infection.[43] Our previous investigation also reported that apple scab infection increased specific hydroxycinnamic acid content.[8,40] It has been determined that hydroxycinnamic acid derivatives play a major role in plant resistance and exhibit a fungitoxic effect against different pathogens, because they inhibit the growth and sporulation of fungi.[33,44]

The major anthocyanins identified in strawberry fruit were pelargonidin-3-glucoside and pelargonidin-3-malonylglucoside, which combined represented 85−95% total analyzed anthocyanins (TA) (Table 4). Cyanidin-3-glucoside has also been determined in strawberries and accounted for 5−15% TA. The content of anthocyanins is greatly influenced by fruit maturity level and cultivar. On the other hand, inoculation with fungi, fungicide, or calcium treatment did not cause significant changes in anthocyanin content levels. However, the bordering tissue near the infection site is darker and possibly accumulates more anthocyanins (visual observations). Similarly, Slatnar et al.[45] reported higher content levels of anthocyanins at the infected apple peel site, which also resulted in intensified red coloration.

The phenolic profile (content and abundance of different compounds) is suitable as an indicator for stress conditions that occur in plants. The first step of the defense mechanism in plant involves a rapid accumulation of phenols at the infection site, which inhibit or restrict pathogen growth and development.[46] TPC in strawberry ranged from 1267 to 1904 mg/kg gallic acid equivalents (GAE) (Table 4). TPC is closely related with the levels of analyzed phenolics, and thus higher levels of individual compounds are indicative of a higher TPC in plant tissue. Therefore, similarly to individual phenolic increase, inoculation with *C. nymphaeae* caused from 1.4- to 1.5-fold higher levels of TPC compared to other treatments (healthy fruit). Other papers indicate a significantly higher TPC as a

reaction to fungal, bacterial, and viral infection[47,48] or pest attack.[49]

Multivariate statistical analysis was employed to determine which treatments differentiated the content of primary and secondary metabolites in strawberries, and a dendrogram revealed similarities and differences among treatments in all analyzed parameters (Figure 1). Fruits of the inoculation treatment were characterized by almost 2-fold higher levels of ellagic acid conjugates, flavan-3-ols, and flavanols and a higher content of total sugars compared to other treatments. This is indicative of the involvement of these phenolic groups in plant defense mechanisms against *C. nymphaeae*. Strawberries of the control and Signum treatments were very similar in all analyzed parameters, which could be attributed to similar health status of these fruits. Also, it is an indication that fungicide spraying did not alter the polyphenolic level in plant tissue. Both treatments yielded completely healthy fruit. Likewise, fruits of the calcium treatment were similar to the control and Signum treatments in all analyzed parameters except for sugars. Again, calcium-treated fruits contained somewhat higher levels of specific compounds. Hernandez-Munoz et al.[50] reported that calcium applications reduce fungal decay incidence on strawberries. The authors explain that postharvest treatment with calcium salts is expected to reinforce the cell wall and middle lamella of fruits and vegetables, thus enhancing tissue resistance to fungal enzyme activity.

The accumulation of some phenolic compounds may explain the broad and unspecific prevention of plant diseases such as black spot disease. Our results demonstrate that *C. nymphaeae* infection caused a significant increase in sugars, conjugates of ellagic acid, epicatechin, procyanidins, a derivate of hydroxycinnamic acid, and some flavonols (mostly glycosides of kaempferol) in infected fruits. It has been demonstrated that the runners and fruits of the 'Honeoye' cultivar were less infected compared to the 'Elsanta' cultivar. The cultivars also differed in the content levels of flavonols and flavanols; both phenolic groups were more abundant in fruits of the 'Honeoye' cultivar. Results support the hypothesis that phenolic compounds play an important role in host resistance in infected tissue and that the mechanism of resistance may be influenced by responses linked to the host−pathogen interaction. However, it is obvious that the strong defense reaction is not enough to overcome the disease. In addition to polyphenols, other biochemical compounds are potentially involved in strawberry defense response. Additional studies may help clarify the precise mechanisms, processes, and important resistance indicators within the plants, which take place after the pathogen attack. Specific studies are of great importance to help minimize the negative effects of fungal diseases such as strawberry anthracnose, causing large yield losses due to its fast spread, ineffective chemical treatments, and long withdrawal periods of fungicides. Therefore, the development of strawberry cultivars resistant to *C. nymphaeae* in terms of high accumulation of specific phenolics is promising for controlling anthracnose as a method that is both economical and environmentally acceptable.

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### Funding

The research is part of the program Horticulture P4-0013-0481 and Project J4-4187 funded by the Slovenian Research Agency (ARRS).

### Notes

The authors declare no competing financial interest.

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### 2.1.3 Kopičenje fenolnih snovi v plodovih in pritlikah žlahtnega jagodnjaka (*Fragaria* × *ananassa* Duch.) kot odgovor na okužbo z glivo *Colletotrichum nymphaeae*

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METABOLITE ACCUMULATION IN STRAWBERRY (*Fragaria* × *ananassa* Duch.) FRUIT AND RUNNERS IN RESPONSE TO C*olletotrichum nymphaeae* INFECTION

Physiological and Molecular Plant Pathology, 2015, 92: 119–129

Primerjali smo vsebnost izbranih primarnih in sekundarnih metabolitov zdravih in z glivo *Colletotrichum nymphaeae* okuženih plodov in pritlik žlahtnega jagodnjaka. Poskus smo izvedli na sorti 'Asia', ki jo pridelovalci v našem okolju veliko pridelujejo in imajo, sploh v deževnih letih, težave zaradi okužbe. Želeli smo ugotoviti, ali stopnja zrelosti ploda in okužba vplivata na metabolni odziv žlahtnega jagodnjaka. Plodove smo ločili na zdrave in okužene: belo obarvane (ne zrele), rahlo rdeče obarvane (dozorevanje) in rdeče obarvane (zrele). Ugotovili smo, da je okužba z glivo *C. nymphaeae* v zrelih plodovih povzročila 1,1-kratno povečanje vsebnost sladkorjev, v dozorevajočih plodovih pa značilno 1,4-kratno zmanjšanje vsebnosti sladkorjev. Vsebnost organskih kislin se je po okužbi z glivo 1,7-krat zmanjšala v vseh stopnjah zrelosti. Stopnja zrelosti pa ni imela značilnega vpliva na vsebnost fenolnih snovi v plodovih žlahtnega jagodnjaka. Po analizi vsebnosti fenolov smo ugotovili, da so se derivati elagnih kislin po okužbi 1,9-krat povečali, flavanoli 1,5-krat in flavonoli 5,1-krat v vseh stopnjah zrelosti. Po analizi fenolnih snovi v pritlikah smo ugotovili, da se je zgodilo ravno obratno kot v plodovih, vsebnost fenolnih snovi se je namreč po okužbi značilno zmanjšala.

Physiological and Molecular Plant Pathology 92 (2015) 119–129

Contents lists available at ScienceDirect

Physiological and Molecular Plant Pathology

journal homepage: www.elsevier.com/locate/pmpp

# Metabolite accumulation in strawberry (*Fragaria* × *ananassa* Duch.) fruits and runners in response to *Colletotrichum nymphaeae* infection

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ARTICLE INFO

*Article history:*
Received 24 July 2015
Received in revised form
22 September 2015
Accepted 7 October 2015
Available online xxx

*Keywords:*
*Fragaria* × *ananassa*
Strawberry anthracnose
Ripeness
Primary metabolites
Secondary metabolites

ABSTRACT

The composition of non-infected and *Colletotrichum nymphaeae* infected strawberry fruits (cultivar 'Asia') in three different stages of ripeness were evaluated. Additionally, the effect of infection on strawberry runners was monitored. *C. nymphaeae* infection caused a significant increase in total sugar content in mature fruits (1.1 fold) and a decrease in total sugar content in semi-mature fruits (1.4 fold) and organic acid content in all stages of ripeness (1.7 fold). Ellagic acid derivatives (1.9 fold), flavanols (1.5 fold) and flavonols (5.1 fold) significantly increased during all stages of ripeness after infection. The pattern of phenolic accumulation in strawberry runners was altered by *C. nymphaeae* infection in contrast to strawberry fruits as most of identified phenols decreased as a response to the infection.



## 1. Introduction

Strawberry anthracnose or strawberry black spot is a major disease, infecting all parts of strawberry plants: fruits, runners, crown and roots [7]. Ref. [8] identified *Colletotrichum acutatum* as the most important causal agent of strawberry black spot. The species has been subjected to a thorough taxonomic reassessment and is now suggested to be a species complex, comprising 31 species. *Colletotrichum nymphaeae* (formerly known as *C. acutatum*, molecular group A2) is the most important agent of strawberry anthracnose [6]. Symptoms induced by *C. nymphaeae* and other members of *C. acutatum* species complex include petiole and stolon necroses as well as fruit and crown rot, eventually causing the collapse and death of the entire plant. The infection and colonisation strategy employed by these pathogens is best described as hemibiotrophic, encompassing both biotrophic and necrotrophic phases [28]. However, diverse infection strategies can be employed when colonising different host species and cultivars or different tissues of a specific host. Histological investigations revealed necrotrophic behaviour of the pathogen when it infects vegetative parts of the strawberry plant [5]. The same strategy is employed in strawberry fruits. However, the course of infection differs depending on the ripeness of the fruit. In red fruits, colonisation and infection follow a typical subcuticular intramural necrotrophic pattern, the pathogen forming invasive hyphae that quickly spread and colonise subcuticular tissues both intra- and intercellularly. In unripe fruits, the pathogen enters a period of quiescence, where only melanised appressoria are formed, thus trying to avoid defences in the immature fruits and resuming its growth when the fruit ripens [9].

Plant phenolic compounds are strongly involved in the interaction between the pathogen and the plant. Thus, in the defence against pathogens, phenolics may act as soluble antimicrobial and deterrent compounds or they may cross-link with callose, proteins and polysaccharides in the cell walls, inhibiting fungal penetration and the absorption of nutrients by the invading fungus [27]. The most frequently identified phenolic groups in strawberries are hydroxybenzoic and hydroxycinnamic acids, hydrolysable tannins, flavonols, flavanols and anthocyanins [1]. In addition, phenylpropanoid and flavonoid metabolites, such as catechin, have been shown to be crucial in the induction of fungal quiescence in white fruits [3]. Strawberry fruits undergo an initial phase of growth

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http://dx.doi.org/10.1016/j.pmpp.2015.10.003
0885-5765/

followed by a maturation phase, during which the fruit acquires the capacity to ripen. Ripening itself is defined by a set of physico-chemical changes characteristic of each individual fruit.

[1] reported major differences among 27 different strawberry cultivars and a significant effect of ripeness on anthocyanins and cinnamic acid conjugates. Moreover, Kajdžanoska et al. [10,11], identified phenolic constituents in four different strawberry cultivars and reported interesting differences among the analysed samples, depending on the cultivar and location. Ref. [13] also reported that the stability of strawberry chemical composition in different horticultural systems was mainly correlated to the cultivar.

The aim of the present study was to determine the influence of *C. nymphaeae* infection on different biochemical components in strawberry fruits at different stages of ripeness. Moreover, the effects of fungal infection were also assessed on strawberry runners. More specifically, the objectives were to characterise sugars, organic acids and phenolic compounds in the fruits of strawberries cv. 'Asia' and determine a possible influence by infection of *C. nymphaeae*. Runners represent the basic organs for reproduction of strawberry plants and, to our knowledge, have not yet been closely examined in connection with *C. nymphaeae* infection before. For that reason, we aimed to determine the content of selected phenolic compounds in non-infected and infected runners and potentially link their content to defence against specific fungal pathogens.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

The experiment was performed in cooperation with a local strawberry producer near Ljubljana, where signs of anthracnose regularly occur. An open field trial was conducted in 2012 in Dobrova-Polhov Gradec (latitude, 45° 58′ N; longitude, 15° 32′ E). Frigo plants of cv. 'Asia' were planted in July 2011, on slightly elevated beds covered with black polyethylene. Before planting, leaf petioles were sampled from all plants and tested to be free from latent *Colletotrichum* infection using the method of [19]. Thus, leaf petioles were frozen at −20 °C for 2 h, surface sterilized with 0.05% NaOCl for 1 min and incubated in a moist chamber at 25 °C under continuous light for 6 days. After incubation, the petioles were examined under microscope for the presence of *Colletotrichum* acervuli and conidia. Plants were arranged in double rows, with 0.25 × 0.25 m spacing between the plants and 1 m spacing between the rows. The production field was equipped with a drip irrigation system. Plants were cultivated according to the commercial guidelines for integrated production [25]. They were subjected to natural infection originating from an adjacent, infected strawberry bed. Random samples of non-infected and infected runners and fruits were collected on the 21st of May in different stages of ripeness in five repetitions from a 0.5 ha experimental field.

For every repetition, 500 g of uniform fruit samples and 100 g of 20 cm long runners were collected. Asymptomatic plant parts were tested for latent infections by freezing, surface sterilization and incubation as described by Ref. [19]. No signs of anthracnose were visible on non-infected samples, and on infected samples, 10–20% of the fruit and runner surfaces were covered with black necrotic lesions, which are typical symptoms for infection with *Colletotrichum* species (Fig. 5). Samples of symptomatic strawberries were collected (Fig. 4) and pieces of the necrotic tissue were plated on potato dextrose agar. Single-spore isolates were obtained from outgrowing colonies and identified as *C. nymphaeae* on the basis of morphological characteristics and sequence analysis of ITS and TUB2 according to the method of [6].

Fruit samples were collected in three different maturity stages (Fig. 3): (1) white fruits, (2) semi-mature fruits with developing light red colour and (3) mature red fruits. Infected and non-infected runners were collected from the same plants as the fruits. Fruits and runners were labelled in plastic bags, frozen in liquid nitrogen, transferred to the laboratory and stored at −20 °C for a week before further analysis. Fruit samples used for vitamin C analyses were immediately processed.

### 2.2. Chemicals

The following standards were used for determination of sugars and organic acids: sucrose, fructose and glucose, as well as citric, malic, ascorbic and fumaric acid from Fluka Chemie (Buchs, Switzerland) and shikimic acid from Sigma–Aldrich Chemicals (St. Louis, MO, USA). Standards for phenolic compounds were acquired from Sigma–Aldrich Chemicals (ellagic acid, cyanidin-3-glucoside and pelargonidin-3-glucoside) and Fluka Chemie (quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-glucuronide, kaempferol-3-glucoside, (−)-catechin, *p*-coumaric acid and procyanidin B2). Methanol for the extraction of phenolics was obtained at Sigma–Aldrich Chemicals. The chemicals for the mobile phase were HPLC-MS grade acetonitrile, sulphuric acid and formic acid from Sigma–Aldrich Chemicals. Water for the mobile phase was double distilled and purified with a Milli-Q system (Millipore, Bedford, MA, USA).

### 2.3. Extraction and determination of sugars and organic acids

Fruits were homogenized (2 g) in 10 ml of double distilled water using an Ultra-Turrax T-25 (Ika-Labortechnik, Staufen, Germany) and left for 30 min at room temperature as reported by Ref. [21]. Homogenized fruits were centrifuged (Eppendorf Centrifuge 5810 R) at 18514×*g* for 7 min at 10 °C. The supernatant was filtered through a 0.20 μm mixed cellulose ester filter (Macherey–Nagel, Düren, Germany, ref. number: 729006) and transferred into a vial, and 20 μl of the sample was used for the analysis. The analyses of primary metabolites were carried out using a high-performance liquid chromatography (HPLC) from Thermo Separation Products (San Jose, CA, USA). Sugar separations were carried out using a Rezex RCM-monosaccharide column from Phenomenex (Ca+ 2%, Torrance, CA, USA) operated at 65 °C (300 mm × 7.8 mm). The mobile phase was double distilled water and the flow rate maintained at 0.6 ml/min; the total run time was 30 min, and a refractive index (RI) detector was used to monitor the eluted carbohydrates as described by Ref. [21]. Analyses of organic acids was performed on the same HPLC system, equipped with a UV detector set at 210 nm, using a Rezex ROA-organic acid (H+ [8%]) column from Phenomenex (300 mm × 7.8 mm) as described by Ref. [21]. The column temperature was set at 65 °C. The elution solvent was 4 mM sulphuric acid in double distilled water and the flow rate 0.6 ml/min. The concentrations of sugars and organic acids were calculated with the help of a corresponding external standard and expressed as g/kg fresh weight (FW). Total sugar content and total organic acids content was calculated as a sum of all individual sugars or organic acids, respectively. For vitamin C detection, fresh strawberry fruits were finely chopped and 5 g were extracted with 10 ml of 2% *m*-phosphoric acid as described earlier. Samples were left to extract at room temperature for 30 min, with frequent stirring and centrifuged for 7 min at 12857×*g* (Eppendorf centrifuge 5810R). The supernatant was filtered through a 0.20 μm cellulose ester filter (Macherey–Nagel), poured into a vial and analysed on a high performance liquid chromatography system (HPLC; Thermo Scientific,

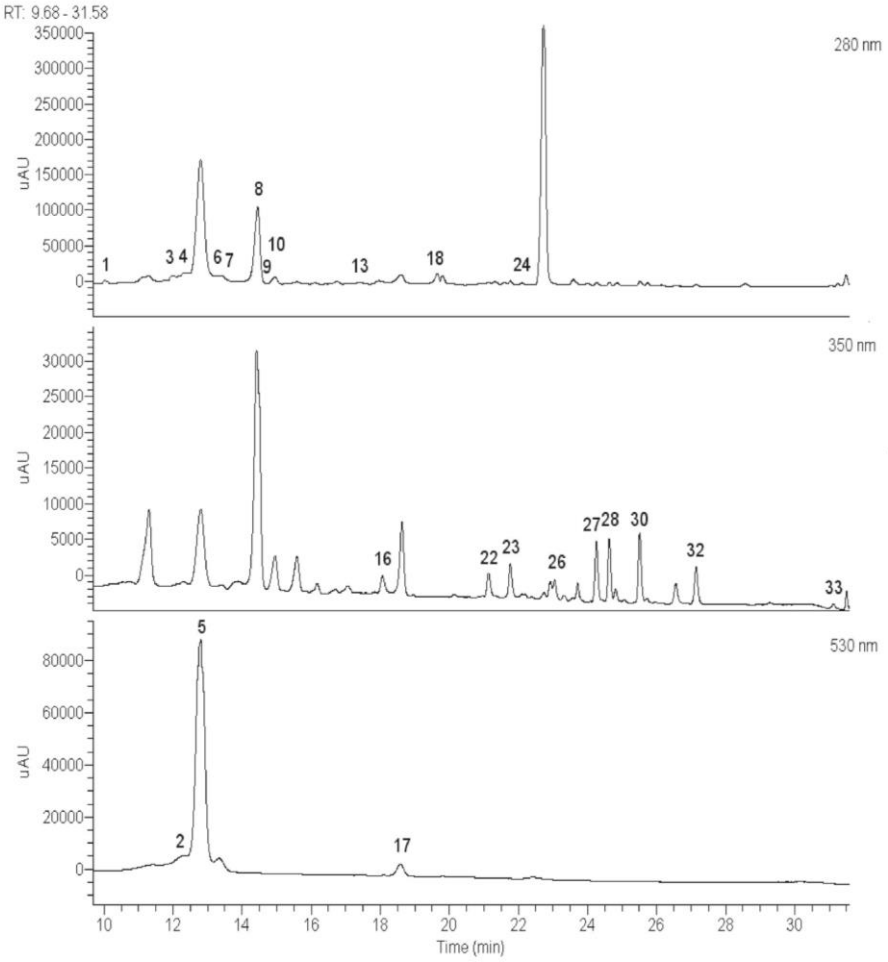


**Fig. 1.** Phenolic chromatogram of strawberry fruits. Peak numbers are explained in Table 1.

Finnigan Spectra System, Waltham, MA, USA). Separation of ascorbic acid was carried out using a Rezex ROA-organic acid H+ (8%) column (300 mm × 7.8 mm) from Phenomenex. The column temperature was set at 20 °C and a 245 nm wavelength UV detector was used for identification. The elution solvent was 4 mM sulphuric acid in double distilled water at a flow rate of 0.6 ml/min. The concentration of ascorbic acid in sample was calculated with the aid of the corresponding external standard and expressed in mg/kg FW.

### 2.4. Extraction of phenolic compounds

Phenolic compounds were analysed in the entire infected or non-infected strawberry fruits and runners. Five replications were carried out (n = 5) per treatment and each replication included several fruits or runners. Fruits (2 g) and runners (1 g) were chopped and extracted with 10 ml methanol containing 3% (v/v) formic acid and 1% (w/v) 2.6-di-*tert*-butyl-4-methylphenol (BHT) in an iced ultrasonic bath for 1 h. BHT was added to samples to prevent oxidation as described by Ref. [21]. After extraction, the extracts were centrifuged for 10 min at 12857×*g*, filtered through a 0.2 μm Chromafil AO-20/25 polyamide filter (Macherey–Nagel) and transferred to a vial prior to injection into the HPLC system.

### 2.5. Determination of individual phenolic compounds using HPLC-DAD-$MS^n$ analysis

Phenolic compounds were analysed on a Thermo Finnigan Accela HPLC system (Thermo Scientific) coupled with a mass spectrometer with a diode array detector at 280 nm (ellagic acid derivatives, flavanols, hydroxycinnamic acids and castalagin), 350 nm (ellagic acid derivatives and flavonols) and 530 nm (anthocyanins) as described by Ref. [21] (Table 1, Figs. 1 and 2). Spectra of the compounds were recorded between 200 and 600 nm. The column was a 150 × 4.6 mm i.d., 3 μm, Gemini $C_{18}$ (Phenomenex) operated at 25 °C. The elution solvents were: (A) aqueous 0.1% formic acid in double distilled water and (B) 0.1% formic acid in acetonitrile. Samples were eluted according to the linear gradient from 5 to 20% B in the first 15 min, followed by a linear gradient from 20 to 30% B for 5 min, then an isocratic mixture for 5 min, followed by a linear gradient from 30 to 90% B for 5 min, and then an isocratic mixture for 15 min before returning to the initial conditions [32]. The injection amount was 20 μl and the flow rate 0.6 ml/min.

All phenolic compounds were identified using a mass with electrospray ionization (ESI) operating in negative ion mode (all phenolic groups except for anthocyanins) and positive ion mode (anthocyanins). The analyses were carried out using full scan data-dependent $MS^n$ scanning from *m/z* 115 to 1500. The injection

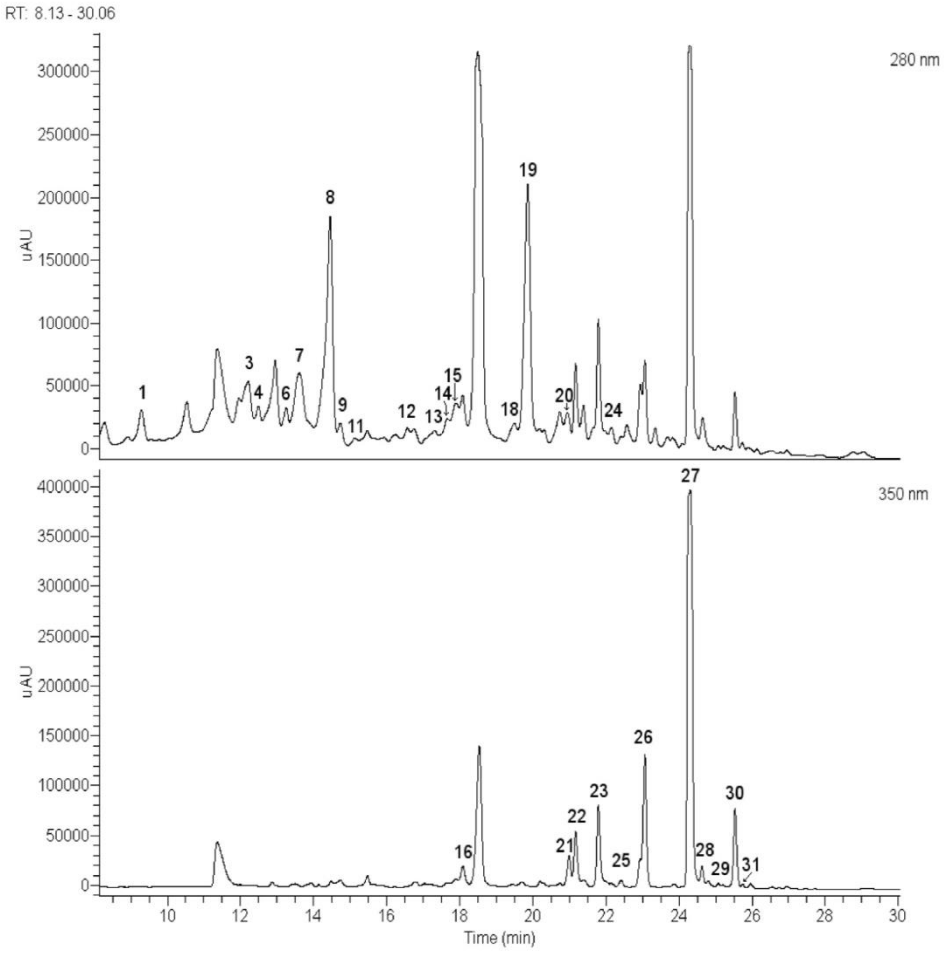


**Fig. 2.** Phenolic chromatogram of strawberry runners. Peak numbers are explained in Table 1.

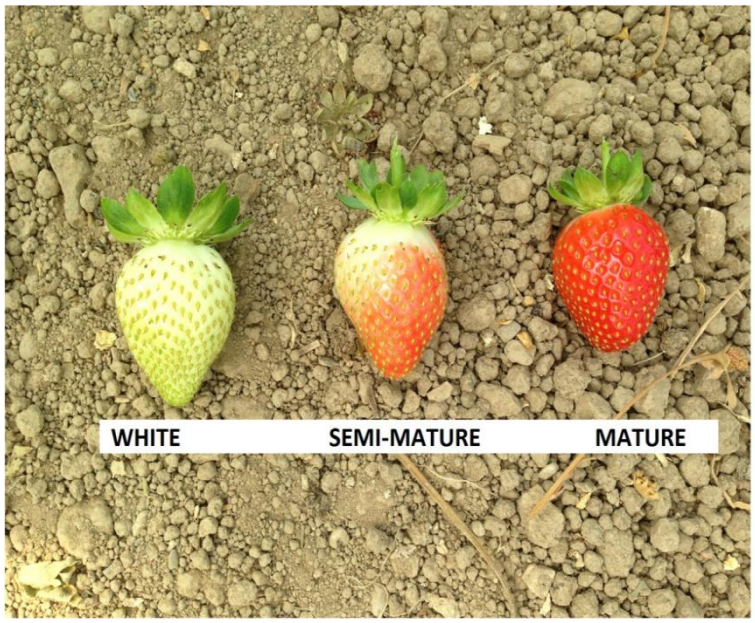


**Fig. 3.** Non-infected strawberry fruits in three stages of ripeness.

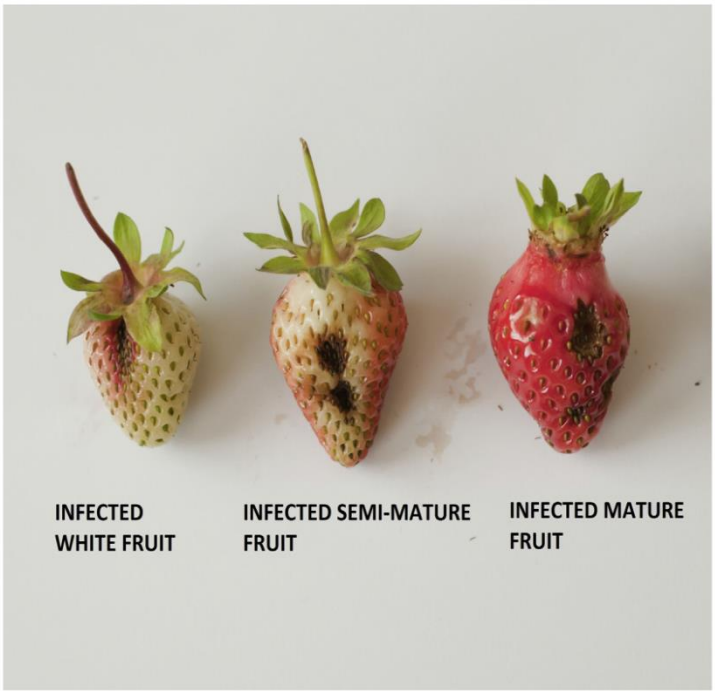


**Fig. 4.** Infected strawberry fruits in three stages of ripeness.

volume was 10 µl, and the flow rate was maintained at 0.6 ml/min. The capillary temperature was 250 °C, the sheath gas and auxiliary gas were 20 and 8 units, respectively, and the source voltage was 4 kV for negative ionization and 0.1 kV for positive ionization. Spectrometric data were elaborated using the Excalibur software (Thermo Scientific). The identification of compounds was confirmed by comparing retention times and their spectra as well as by adding the standard solution to the sample and by fragmentation. Concentrations of phenolic compounds were calculated from peak areas of the sample and the corresponding standards and expressed in mg/kg fresh weight (FW) of strawberry. For compounds lacking standards, quantitation was carried out using similar compounds as standards. Thus, glycosides of kaempferol were quantitated in equivalents of kaempferol-3-glucoside and all procyanidin dimers and trimmers in equivalents of procyanidin B2; *p*-coumaroyl glucoside was quantitated in equivalents of *p*-coumaric acid and pelargonidin-3-malonylglucoside in equivalents of pelargonidin-3glucoside; all ellagic acid derivatives were quantitated in equivalents of ellagic acid.


**Fig. 5.** Infected and non-infected strawberry runners.

**Table 1**
Identification of phenolic compounds in fruits and runners of strawberry cultivar 'Asia' in positive (anthocyanins) and negative (all others) ions with HPLC-MS and $MS^2$.

| Peak no. | $t_R$ (min) | $[M+H]^+$ or $[M-H]$ [a] | $MS^2$ (*m/z*) | Tentative identification | Analysed in[b] |
|---|---|---|---|---|---|
| 1 | 9.29 | 783 | 481, 301 | bis-HHDP glucose 1 | F, R |
| 2 | 12.24 | 449 | 287 | Cyanidin-3-glucoside | F |
| 3 | 12.23 | 783 | 481, 301 | bis-HHDP glucose 2 | F, R |
| 4 | 12.51 | 577 | 425, 407, 289 | Procyanidin dimer 1 | F, R |
| 5 | 12.78 | 433 | 271 | Pelargonidin-3-glucoside | F |
| 6 | 13.34 | 577 | 451, 425, 407, 289 | Procyanidin dimer 2 | F, R |
| 7 | 13.62 | 865 | 577, 425, 407 | Procyanidin trimer 1 | F, R |
| 8 | 14.45 | 289 | 245 | Catechin | F, R |
| 9 | 14.61 | 865 | 577, 425, 407 | Procyanidin trimer 2 | F, R |
| 10 | 14.73 | 325 | 163 | *p*-coumaric acid hexose 1 | F |
| 10 | 14.74 | 633 | 301 | HHDP galloyl glucose | F |
| 11 | 15.21 | 1153 | 865,577,451,425, 289 | Procyanidin tetramer | R |
| 12 | 16.56 | 849 | 723, 577, 289 | Procyanidin trimer 3 | R |
| 13 | 17.33 | 849 | 723, 577, 289 | Procyanidin trimer 4 | F, R |
| 14 | 17.48 | 935 | 451, 631, 301 | Castalagin 1 | R |
| 15 | 17.76 | 865 | 695, 577, 451, 407 | Procyanidin trimer 5 | R |
| 16 | 18.08 | 463 | 301 | Ellagic acid hexoside | F, R |
| 17 | 18.54 | 475 | 271 | Pelargonidin-3-malonylglucoside | F |
| 18 | 19.53 | 935 | 633, 301 | Galloyl bis HHDP glucose 1 | F, R |
| 19 | 19.86 | 325 | 163, 119 | *p*-coumaric acid hexose 2 | R |
| 20 | 20.85 | 933 | 451, 631, 301 | Castalagin 2 | R |
| 21 | 20.99 | 595 | 301 | Quercetin-3-vicianoside | R |
| 22 | 21.13 | 433 | 301 | Ellagic acid pentoside | F, R |
| 23 | 21.77 | 447 | 301 | Ellagic acid deoxyhexoside | F, R |
| 24 | 22.03 | 935 | 633, 301 | Galloyl bis HHDP glucose 2 | F, R |
| 25 | 22.41 | 463 | 301 | Quercetin-3-galactoside | R |
| 26 | 23.06 | 463 | 301 | Quercetin-3-glucoside | F, R |
| 27 | 24.26 | 477 | 301 | Quercetin-3-glucuronide | F, R |
| 28 | 24.63 | 447 | 285 | Kaempferol-3-glucoside | F, R |
| 29 | 25.14 | 461 | 315 | Isorhamnetin-rhamnoside | R |
| 30 | 25.51 | 461 | 285 | Kaempferol-3-glucuronide | F, R |
| 31 | 25.73 | 491 | 315 | Isorhamnetin-3-glucuronide | R |
| 32 | 27.14 | 489 | 285 | Kaempferol-3-acetylglucoside | F |
| 33 | 31.15 | 593 | 285 | Kaempferol-3-coumaroylglucoside | F |

Mean values ± SE are represented. Asterisk represents statistically significant differences between non-infected and infected fruits at P= <0.05 (*), <0.01 (**), <0.001 (***) or NS (not significant).
[a] $M^+$ (*m/z*) anthocyanins were obtained in the positive ion mode, other phenolic in the negative ion mode.
[b] F: fruits, R: runners.

### 2.6. Statistical analyses

The data were analysed using the Statgraphics Plus 4.0 program (Manugistics, Inc., Rockville, MD, USA). Significant differences between treatments with respect to the content of individual phenolic compounds, sugars and organic acids were tested using one-way analysis of variance (ANOVA). Hypotheses were rejected at $P < 0.05$.

We conduct multivariate Analyses of Variance (MANOVA) for the phenolic compounds groupings and then the Pillai's Trace was used, for the analyses, the IBM SPSS Statistics 21 program was used (SPSS Inc., H, Chicago, IL, USA).

## 3. Results

### 3.1. The content of sugar and organic acids in strawberry fruits

The contents of individual sugars and organic acids were assessed in infected and non-infected strawberry fruits at three different stages of ripeness. Glucose and fructose were the predominant sugars in all studied samples. Infection caused a decrease in glucose, sucrose and consequently total sugar content in semi mature fruits. In mature fruits, the amount of total sugars was highest in infected fruits compare to non-infected (Table 2). The main identified organic acids in strawberry fruits were citric and malic acids. Together, citric and malic acids constituted almost 99% of the total organic acids in strawberry fruits (Table 2). *C. nymphaeae* infected fruits accumulated lower levels of citric and malic acid and higher levels of fumaric and shikimic acids at all maturity stages. The level of ascorbic acid was higher in infected fruits at all stages of ripeness, except for the mature fruits, where ascorbic acid content in infected and non-infected fruits did not differ.

### 3.2. Phenolic content of strawberry fruits

In strawberry fruits, 25 different phenolic compounds were detected and grouped into the following phenolic classes: ellagic acid conjugates, flavanols, flavonols, anthocyanins and hydroxycinnamic acids (Table 3). The largest share of all identified phenolic compounds in strawberry fruits were flavanols (82% of all identified phenolic compounds) and derivatives of ellagic acids (12% of all identified phenolic compounds). The content of ellagic acid derivatives comprised the third largest polyphenol group.

According to the MANOVA for the phenolic groups, there were no interaction between ripeness and infection, except for anthocyanins (Tables 5 and 6) which were not identified in white fruits. Because of that, we analysed phenolic contents within individual ripening stages according to ANOVA.

The accumulation of ellagic acid derivatives increased significantly (2-fold) after *C. nymphaeae* infection in all three stages of ripeness compared to non-infected fruits (Table 3). Bis-HHDP (hexahydroxydiphenic acid) glucose 1 constituted the largest share (45% in non-infected, semi-mature fruits) of all identified ellagic acid derivatives (a significant 2.1 fold increase in infected, semi-mature compared to non-infected fruits). No significant

**Table 2**
Content of individual and total analysed sugars, organic acids and vitamin C (g/kg FW) in non-infected and infected strawberry fruit at three stages of ripeness.

| | White Mean content ± SE in g/kg FW | | | | Semi-mature Mean content ± SE in g/kg FW | | | | Mature Mean content ± SE in g/kg FW | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Non-infected | Infected | F | *p* | Non-infected | Infected | F | *p* | Non-infected | Infected | F | *p* |
| Fructose | 28.95 ± 2.21 | 35.97 ± 1.81 | 3.08 | NS | 40.71 ± 2.24 | 35.96 ± 0.75 | 4.60 | NS | 35.76 ± 1.44 | 41.18 ± 1.46 | 11.87 | NS |
| Glucose | 26.57 ± 3.72 | 30.09 ± 2.91 | 0.97 | NS | 38.18 ± 1.49 | 30.09 ± 4.16 | 17.32 | ** | 31.61 ± 1.46 | 36.16 ± 3.73 | 8.68 | NS |
| Sucrose | 16.74 ± 2.19 | 10.983 ± 1.74 | 3.79 | NS | 27.82 ± 1.87 | 10.99 ± 1.38 | 61.92 | *** | 18.99 ± 1.39 | 23.11 ± 0.74 | 7.65 | * |
| **Total sugars** | **72.27 ± 7.88** | **84.17 ± 2.18** | **1.40** | **NS** | **106.72 ± 12.82** | **77.06 ± 5.82** | **34.80** | *** | **86.37 ± 2.63** | **100.45 ± 3.29** | **11.99** | ** |
| Citric acid | 9.17 ± 0.08 | 7.15 ± 0.49 | 16.37 | ** | 13.03 ± 1.26 | 7.16 ± 0.35 | 42.23 | *** | 9.81 ± 0.46 | 6.36 ± 0.13 | 50.41 | *** |
| Fumaric | 0.01 ± 0.00 | 0.01 ± 0.00 | 26.16 | *** | 0.01 ± 0.00 | 0.01 ± 0.00 | 16.15 | ** | 0.01 ± 0.00 | 0.03 ± 0.00 | 16.34 | ** |
| Malic acid | 2.39 ± 0.19 | 1.86 ± 0.14 | 6.61 | * | 3.77 ± 0.42 | 2.53 ± 0.09 | 26.52 | *** | 3.02 ± 0.12 | 0.27 ± 0.08 | 15.28 | ** |
| Shikimic acid | 0.01 ± 0.00 | 0.01 ± 0.00 | 37.40 | *** | 0.01 ± 0.00 | 0.01 ± 0.00 | 13.67 | ** | 0.01 ± 0.00 | 0.01 ± 0.00 | 3.17 | NS |
| **Total organic acids** | **11.57 ± 0.11** | **9.05 ± 0.14** | **14.28** | ** | **16.83 ± 0.26** | **9.74 ± 0.66** | **39.90** | *** | **12.86 ± 0.34** | **8.84 ± 0.22** | **48.50** | *** |
| Vitamin C | 0.22 ± 0.02 | 0.33 ± 0.02 | 14.03 | ** | 0.29 ± 0.01 | 0.43 ± 0.03 | 8.78 | * | 0.37 ± 0.01 | 0.35 ± 0.03 | 0.38 | NS |

Mean values ± SE are represented (n = 5). Asterisk represents statistically significant differences between non-infected and infected treatments at $P = <0.05$ (*), <0.01 (**), <0.001 (***) or NS (not significant).

differences in ellagic acid derivative content were seen among different maturity stages of strawberry fruits. Flavonols constituted approximately 5% all identified phenolic compounds in non-infected, mature strawberry fruits and 25% all identified phenolic compounds in infected, mature fruits (Table 3). *C. nymphaeae* infected, white fruits contained up to 5.1-fold higher levels of total flavonols compared to non-infected, white fruits. This indicates that infected strawberry fruits accumulate significantly higher amounts of all identified glycosides of kaempferol and glycosides of quercetin compared to non-infected fruits.

The bright red colour of strawberries is the result of fruit anthocyanin content. Three major anthocyanins were quantified in strawberry fruits: cyanidin-3-glucoside, pelargonidin-3-glucoside and pelargonidin-3-malonylglucoside. No anthocyanins were detected in white fruits. The content of cyanidin-based anthocyanins in strawberry fruits was much lower than that of pelargonidin-based anthocyanins, which constituted 98.6% of total anthocyanins (Table 3). Total anthocyanin content increased with *C. nymphaeae* infection in mature fruits. The content of anthocyanins also increased (5.1 fold in mature non-infected fruits

**Table 3**
Content of individual and total phenolic compounds (mg/kg FW) in non-infected and infected strawberry fruit at three stages of ripeness.

| | White Mean content ± SE in mg/kg FW | | | | Semi-mature Mean content ± SE in mg/kg FW | | | | Mature Mean content ± SE in mg/kg FW | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Non-infected | Infected | F | *p* | Non-infected | Infected | F | *p* | Non-infected | Infected | F | *p* |
| **Total ellagic acid derivatives** | **15.02 ± 1.71** | **28.49 ± 2.83** | **10.48** | * | **13.86 ± 0.37** | **29.68 ± 3.13** | **49.33** | *** | **15.41 ± 1.88** | **30.18 ± 2.01** | **29.65** | *** |
| Bis-HHDP glucose 1 | 5.37 ± 0.55 | 15.15 ± 1.92 | 10.04 | * | 6.05 ± 0.75 | 14.89 ± 2.44 | 42.3 | *** | 4.92 ± 0.29 | 7.47 ± 0.73 | 2.79 | NS |
| Bis-HHDP glucose 2 | 1.84 ± 0.22 | 3.23 ± 0.45 | 0.47 | NS | 3.94 ± 0.38 | 3.26 ± 0.23 | 0.08 | NS | 4.76 ± 0.52 | 7.85 ± 0.85 | 17.71 | ** |
| Galloyl-bis-HHDP-glucose 1 | 0.46 ± 0.01 | 1.53 ± 0.24 | 5.11 | NS | 0.97 ± 0.25 | 2.39 ± 0.16 | 8.70 | * | 1.19 ± 0.00 | 3.59 ± 0.16 | 15.86 | ** |
| Galloyl-bis-HHDP-glucose 2 | 0.51 ± 0.02 | 0.71 ± 0.01 | 0.54 | NS | 0.23 ± 0.02 | 0.67 ± 0.02 | 16.95 | ** | 0.50 ± 0.00 | 1.02 ± 0.01 | 45.48 | ** |
| Ellagic acid deoxyhexoside | 3.22 ± 0.44 | 5.29 ± 0.15 | 12.98 | ** | 3.01 ± 0.31 | 4.85 ± 0.56 | 6.69 | * | 1.52 ± 0.10 | 3.42 ± 0.22 | 71.47 | *** |
| Ellagic acid hexoside | 0.10 ± 0.06 | 0.36 ± 0.01 | 24.27 | ** | 0.83 ± 0.03 | 1.17 ± 0.06 | 1.08 | NS | 2.28 ± 0.23 | 2.56 ± 0.11 | 0.37 | NS |
| Ellagic acid pentoside | 1.75 ± 0.25 | 3.33 ± 0.35 | 48.03 | *** | 1.76 ± 0.23 | 3.34 ± 0.07 | 8.24 | * | 1.29 ± 0.10 | 2.67 ± 0.26 | 11.19 | * |
| HHDP galloyl glucose | 0.19 ± 0.00[a] | 0.46 ± 0.00[a] | 7.40 | * | 0.42 ± 0.03 | 0.58 ± 0.58 | 1.33 | NS | 0.62 ± 0.01 | 2.00 ± 0.00 | 80.31 | *** |
| **Total flavanols** | **90.69 ± 11.84** | **192.43 ± 18.53** | **30.29** | *** | **200.88 ± 21.93** | **287.24 ± 25.45** | **15.51** | ** | **341.89 ± 41.63** | **496.12 ± 41.93** | **7.58** | * |
| Procyanidin dimer 1 | 7.82 ± 0.45 | 53.81 ± 8.97 | 10.9 | ** | 41.21 ± 3.14 | 59.89 ± 0.32 | 11.57 | * | 28.21 ± 3.18 | 31.16 ± 4.11 | 0.44 | NS |
| procyanidin dimer 2 | 18.00 ± 2.53 | 15.81 ± 2.23 | 2.88 | NS | 36.42 ± 1.49 | 76.33 ± 2.22 | 12.94 | ** | 24.84 ± 3.21 | 54.99 ± 1.08 | 7.46 | * |
| Procyanidin trimer 1 | 19.97 ± 2.56 | 45.01 ± 5.36 | 13.75 | ** | 26.17 ± 1.85 | 30.19 ± 2.81 | 0.05 | NS | 6.70 ± 0.92 | 36.64 ± 9.12 | 25.01 | ** |
| Procyanidin trimer 2 | 3.17 ± 0.35 | 11.54 ± 1.01 | 27.60 | *** | 10.25 ± 1.99 | 11.93 ± 1.02 | 0.42 | NS | 14.52 ± 0.82 | 80.71 ± 3.41 | 22.82 | ** |
| Procyanidin trimer 4 | 2.37 ± 0.05 | 14.73 ± 0.73 | 19.53 | ** | 12.72 ± 1.41 | 20.39 ± 2.01 | 6.54 | * | 25.89 ± 2.95 | 54.02 ± 2.07 | 6.10 | * |
| Catechin | 52.15 ± 6.95 | 39.32 ± 6.95 | 4.10 | NS | 89.86 ± 3.98 | 140.24 ± 14.81 | 6.60 | * | 248.89 ± 6.72 | 189.89 ± 7.81 | 2.91 | NS |
| **Total flavonols** | **6.35 ± 1.15** | **34.52 ± 3.16** | **55.76** | *** | **6.55 ± 0.35** | **32.18 ± 4.73** | **39.14** | *** | **8.52 ± 0.42** | **27.17 ± 2.62** | **15.59** | ** |
| Kaempferol-3-glucoside | 1.33 ± 0.28 | 15.73 ± 2.25 | 31.28 | *** | 1.18 ± 0.05 | 11.97 ± 1.48 | 25.07 | *** | 1.64 ± 0.16 | 7.56 ± 0.72 | 8.52 | * |
| Kaempferol-3-glucuronide | 0.81 ± 0.01 | 1.86 ± 0.22 | 47.00 | *** | 0.96 ± 0.03 | 2.61 ± 0.09 | 14.66 | ** | 2.08 ± 0.33 | 3.84 ± 0.25 | 17.18 | ** |
| Kaempferol-3-acetyl-glucoside | 0.77 ± 0.02 | 9.45 ± 0.33 | 29.16 | *** | 1.14 ± 0.05 | 10.07 ± 1.75 | 50.72 | *** | 2.46 ± 0.31 | 10.49 ± 1.42 | 15.1 | ** |
| kaempferol-3-coumaroylglucoside | 0.48 ± 0.02 | 1.05 ± 0.22 | 14.32 | ** | 0.44 ± 0.02 | 1.08 ± 0.32 | 13.59 | ** | 0.14 ± 0.01 | 0.47 ± 0.02 | 9.37 | * |
| Quercetin-3-glucoside | 2.09 ± 0.38 | 3.18 ± 0.46 | 7.49 | * | 1.53 ± 0.14 | 2.74 ± 0.31 | 23.57 | ** | 1.39 ± 0.04 | 2.01 ± 0.04 | 13.95 | ** |
| Quercetin-3-glucuronide | 0.86 ± 0.12 | 5.24 ± 0.42 | 138.35 | *** | 0.86 ± 0.37 | 3.68 ± 0.13 | 22.60 | ** | 0.86 ± 0.02 | 2.69 ± 0.12 | 13.74 | ** |
| **Total anthocyanins** | **ND** | **ND** | **NS** | **NS** | **41.31 ± 7.73** | **44.15 ± 8.05** | **0.22** | **NS** | **197.08 ± 13.11** | **298.96 ± 38.76** | **5.98** | * |
| Cyanidin-3-glucoside | ND | ND | NS | NS | 0.58 ± 0.02 | 6.86 ± 0.32 | 21.48 | ** | 1.69 ± 0.16 | 2.24 ± 0.45 | 2.28 | NS |
| Pelargonidin-3-glucoside | ND | ND | NS | NS | 39.89 ± 4.01 | 33.2 ± 4.19 | 2.60 | NS | 187.99 ± 27.43 | 280.15 ± 20.42 | 6.00 | NS |
| Pelargonidin-3-malonylglucoside | ND | ND | NS | NS | 1.94 ± 0.16 | 1.26 ± 0.13 | 3.28 | NS | 5.55 ± 0.22 | 9.42 ± 0.24 | 7.27 | * |
| **Hidroxycinnamic acid** | | | | | | | | | | | | |
| *p*-coumanoylhexose | 0.57 ± 0.01 | 1.54 ± 0.05 | 8.48 | * | 1.34 ± 0.05 | 1.85 ± 0.08 | 1.33 | NS | 1.99 ± 0.04 | 6.36 ± 0.03 | 80.31 | *** |

Mean values ± SE are represented (n = 5). Asterisks represents statistically significant differences between non-infected fruits at P=<0.05 (*), <0.01 (**), <0.001 (***) or NS (not significant). n = 5.
[a] Exact value is 0.001.

**Table 4**
Content of phenolic compounds (mg/kg FW) in non-infected and infected strawberry runners.

| | Non-infected runners Mean content ± SE in mg/kg FW | Infected runners Mean content ± SE in mg/kg FW | F | *p* |
|---|---|---|---|---|
| **Total ellagic acid derivatives** | **256.53 ± 16.21** | **201.03 ± 7.61** | **48.01** | *** |
| Bis-HHDP glucose isomer 1 | 45.93 ± 5.87 | 39.27 ± 2.17 | 3.05 | NS |
| Bis-HHDP glucose isomer 2 | 55.88 ± 9.44 | 60.75 ± 4.95 | 1.16 | NS |
| Castalagin 1 | 6.94 ± 1.73 | 9.75 ± 1.12 | 10.13 | * |
| Castalagin 2 | 27.18 ± 1.17 | 12.42 ± 1.75 | 8.73 | * |
| Galloyl-bis-HHDP glucose 1 | 8.15 ± 0.99 | 11.77 ± 0.74 | 27.28 | *** |
| Galloyl-bis-HHDP glucose 2 | 4.16 ± 0.49 | 7.21 ± 0.93 | 17.02 | * |
| Ellagic acid deoxyhexoside | 51.44 ± 5.60 | 33.65 ± 1.31 | 49.18 | *** |
| Ellagic acid hexoside | 20.13 ± 1.99 | 11.88 ± 1.63 | 53.06 | *** |
| Ellagic acid pentoside | 36.83 ± 3.65 | 23.79 ± 0.81 | 62.45 | *** |
| **Total flavanols** | **1208.97 ± 130.87** | **1049.81 ± 73.89** | **5.04** | * |
| Procyanidin dimer 1 | 93.19 ± 1.38 | 101.60 ± 2.21 | 0.34 | NS |
| Procyanidin dimer 2 | 225.67 ± 29.17 | 161.90 ± 14.57 | 7.43 | * |
| Procyanidin trimer 1 | 171.78 ± 11.25 | 125.34 ± 10.95 | 3.77 | NS |
| Procyanidin trimer 2 | 18.88 ± 2.13 | 61.22 ± 3.77 | 444.02 | *** |
| Procyanidin trimer 3 | 45.94 ± 2.98 | 46.32 ± 6.12 | 0.02 | NS |
| Procyanidin trimer 4 | 34.16 ± 1.23 | 26.72 ± 1.45 | 0.74 | NS |
| Procyanidin trimer 5 | 49.74 ± 7.47 | 47.26 ± 3.79 | 0.47 | NS |
| Procyanidin tetramer | 30.99 ± 1.13 | 34.23 ± 4.15 | 0.40 | NS |
| Catechin | 535.77 ± 66.97 | 467.68 ± 38.76 | 2.57 | NS |
| **Total flavonols** | **547.54 ± 65.65** | **77.69 ± 1.33** | **256.55** | *** |
| Isorhamnetin-3-glucuronide | 3.29 ± 0.54 | 1.85 ± 0.21 | 29.92 | *** |
| Isorhamnetin-7-rhamnoside | 7.12 ± 0.74 | 2.89 ± 0.21 | 130.31 | *** |
| Kaempferol-3-glucoside | 3.73 ± 0.47 | 1.18 ± 0.09 | 138.54 | *** |
| Kaempferol-3-glucuronide | 32.64 ± 4.19 | 4.14 ± 0.42 | 249.86 | *** |
| Quercetin-3-galactoside | 3.19 ± 0.48 | 0.55 ± 0.00 | 196.77 | *** |
| Quercetin-3-glucoside | 49.23 ± 5.36 | 8.11 ± 0.88 | 210.29 | *** |
| Quercetin-3-glucuronide | 432.52 ± 41.62 | 56.26 ± 1.63 | 265.21 | *** |
| Quercetin-3-vicianoside | 15.64 ± 1.66 | 1.69 ± 0.27 | 263.88 | *** |
| **Hidroxycinnamic acid** | | | | |
| *p*-coumaric acid 4 glucoside | 76.52 ± 8.11 | 114.89 ± 5.34 | 69.80 | *** |

Mean values ± SE are represented (n = 5). Asterisks represents statistically significant differences between non-infected fruits at P=<0.05 (*), <0.01 (**), <0.001 (***) or NS (not significant). n = 5.

**Table 5**
Multivariate tests.

| Effect | | F | *p*-value |
|---|---|---|---|
| Intercept | Pillai's Trace | 212.166 | 0.000 |
| Infection | Pillai's Trace | 26.453 | 0.000 |
| Ripness | Pillai's Trace | 7.831 | 0.000 |
| Infection * ripness | Pillai's Trace | 1.483 | 0.191 |

**Table 6**
Tests of between-subjects effects.

| Source | Dependent Variable | F | *p*-value |
|---|---|---|---|
| Corrected model | Totalflavanols | 30.140 | 0.000 |
| | Totalflavonols | 20.415 | 0.000 |
| | Totalellagicacidconjugates | 13.174 | 0.000 |
| | Totalanthocyanins | 33.362 | 0.000 |
| Intercept | Totalflavanols | 653.537 | 0.000 |
| | Totalflavonols | 249.140 | 0.000 |
| | Totalellagicacidconjugates | 592.223 | 0.000 |
| | Totalanthocyanins | 127.734 | 0.000 |
| Infection | Totalflavanols | 29.572 | 0.000 |
| | Totalflavonols | 98.519 | 0.000 |
| | Totalellagicacidconjugates | 65.304 | 0.000 |
| | Totalanthocyanins | 2.292 | 0.143 |
| Ripness | Totalflavanols | 59.605 | 0.000 |
| | Totalflavonols | 0.393 | 0.679 |
| | Totalellagicacidconjugates | 0.143 | 0.867 |
| | Totalanthocyanins | 80.164 | 0.000 |
| Infection * ripness | Totalflavanols | 0.959 | 0.398 |
| | Totalflavonols | 1.385 | 0.270 |
| | Totalellagicacidconjugates | 0.141 | 0.869 |
| | Totalanthocyanins | 2.096 | 0.145 |

compared to semi-mature, non-infected fruits) with the progression of maturation.

Catechin was the most abundant flavanol in all analysed strawberry fruits. Catechin content was increasing with infection (significantly in semi-mature fruits with 1.4 fold and non-significantly in mature fruits). Total content of flavanols increased with infection in all stages of fruit ripeness. The presence of procyanidin dimers and trimers were additionally confirmed in strawberry fruits. Almost all procyanidin forms increased with infection and significant changes were observed for procyanidin dimer 1 and procyanidin trimer 1. Moreover, the accumulation of flavanols also increased with maturity and a 4.2 fold increase of catechin was recorded in non-infected, ripe fruits compared to non-infected, white fruits (Table 3). Among hydroxycinnamic acid derivatives, *p*-coumaric acid hexose 1 was identified. *C. nymphaeae* infection caused a 5.3 fold higher content of *p*-coumaric acid hexose 1 in infected, mature strawberry fruits compared to non-infected fruits at the same stage of ripeness. The content of *p*-coumaric acid hexose 1 also increased during ripening and a 3.5 fold higher content was measured in mature compared to white fruits.

### 3.3. Phenolic content in strawberry runners

In strawberry runners, 27 different phenolic compounds were determined and grouped into the following phenolic classes: derivatives of ellagic acids, flavanols, flavonols and hydroxycinnamic

acids (Table 4). The largest share of all identified phenolic compounds in non-infected strawberry runners were flavanols (60% of all identified phenolic compounds) and flavonols (27% of all identified phenolic compounds). Ten flavanols were quantified in non-infected and infected strawberry runners, catechin being the prevailing flavanol. Infection caused a significant decrease of total flavanols and a 1.2 fold lower level was measured in infected runners. The only exception was procyanidin trimer 2, which showed an increase in infected runners. No significant differences in catechin levels were detected between the infected and non-infected runners. *C. nymphaeae* infection caused a significantly (7.1 fold) lower level of flavonols in infected runners compared to non-infected. The content of all identified flavonols decreased with the infection. Nine ellagic acid derivatives were quantified in strawberry runners and the infection caused a significant (1.3 fold) decrease of total ellagic acid derivatives. From the group of hydroxycinnamic acids, only *p*-coumaric acid hexose 2 was identified in strawberry runners. The content of *p*-coumaric acid hexose 2 increased significantly (1.5 fold) after infection (Table 3).

## 4. Discussion

During the process of fruit ripening, nutrient levels affect fruit suitability for fungal growth. The development of the fungus differs in immature/fully ripe fruits. In immature fruits, the fungus remains quiescent, and further development of the disease is arrested. Consequently, no signs of the disease can be detected on the fruit surfaces. However, necrotic lesions were spotted also on the surface of immature fruits in our present study. It is well known that the development and disease severity largely depends on weather conditions. It is most prominent in the years with frequent precipitation, high relative humidity and moderate temperatures. It can be assumed that the quiescent phase was very short or even absent in our case due to optimal conditions that prevailed during the course of the experiment. This was subsequently reflected in the content of four major phenolic groups (total ellagic acid derivatives, total flavanols, total flavonols and total anthocyanins), which significantly increased after the infection. The pattern of phenolic accumulation in strawberry runners modified by *C. nymphaeae* infection was contrary to strawberry fruits since most identified phenols decreased as a response to the infection. This suggests that infection strategy in green parts of strawberry may be different from subcuticular intramural necrotrophic pattern, recognized as the main infection strategy for the entire strawberry plant. However, histological studies would be necessary to confirm these findings.

### 4.1. Sugar and organic acids content in strawberry fruits

Three main sugars belonging to monosaccharides (glucose and fructose) and disaccharides (sucrose) were identified in fruits of the strawberry cultivar 'Asia'. Infection induces significant changes in sugar content, in semi-mature fruits there was a decrease of glucose, sucrose and total sugar content. In mature fruits, fungal infection stimulated carbohydrate synthesis. Other studies also measured an increased accumulation of sugars in infected strawberry fruits [22]. However [4], reported that differences in carbohydrate accumulation do not always lead to changes in the activity of enzymes involved in the synthesis of phenolic compounds. Total sugar content change as a result of the infection, and a significant decrease in the content of malic and citric acid in strawberry fruits indicates that the fungal infection accelerates the ripening process in the infected tissue.

Citric and malic acid accounted for 99% of total analysed organic acids in fruits of cv. 'Asia'. Lower levels of citric and malic acid and higher levels of fumaric and shikimic acid at all maturity stages were found in infected fruits. Ripeness stage did not significantly affect the content of organic acids. Ref. [26] investigated strawberry quality parameters and determined relatively constant levels of total organic acids during the ripening process. A decrease of major organic acids was measured in 'Asia' fruits after infection. This pattern concurs with the response of other cultivars subjected to pathogen attack [22,23]. Ref. [12] described a similar decrease of organic acids in tomato fruit subjected to *Fusarium oxysporum* f.sp. *radicis-lycopersici* infection. The level of ascorbic acid was higher in infected fruits in all stages of ripeness, with the exception of mature fruits.

### 4.2. Phenolic content of strawberry fruits

Twenty-five different phenolic compounds were identified in strawberry fruits and 27 in runners. Several authors have characterized different phenolic compounds in strawberry fruits in particular [1,2,13]. However, very few phenols are characteristic for all strawberry cultivars. The content and presence of phenolic compounds can be affected by cultivar [1,14], growth conditions [32], bioavailability [16] and also analytical sensitivity.

A significant increase of ellagic acid derivatives was detected as a result of *C. nymphaeae* infection. Especially in mature fruits, almost all identified ellagic acid derivatives increased significantly. Ellagic acid derivatives are known as defence phenolic compounds that plants produce to inhibit the growth of fungus. The biosynthesis of ellagic acid starts by conjugation of gallic acid with carbohydrates to form gallotannins. The coupling of galloyl units in gallotanins produces hexahydroxydiphenic units the components of ellagitannins [29]. These release free ellagic acid after hydrolytic cleavage.

The infection by *C. nymphaeae* and the progression of maturation caused an increase of individual anthocyanins in strawberry fruits. The bright red colour of strawberries is expressed as a result of fruit anthocyanin content with pelargonidin-3-glucoside and cyanidin-3-glucoside being almost exclusively responsible [33] and being formed with the progression of maturation. We found three major anthocyanins in strawberry fruits: cyanidin-3-glucoside, pelargonidin-3-glucoside and pelargonidin-3-malonylglucoside. This is in accordance with [31] which investigated the content of anthocyanin pigment in strawberry fruits of five different cultivars. *C. nymphaeae* infection caused a variable effect on the levels of individual anthocyanins at different ripening stages. The content of cyanidin-3-glucoside was significantly increased in semi-mature fruits, whereas in mature fruits, pelargonidin-3-malonylglucoside increased after infection.

The most abundant flavan-3-ol in fruits of cv. 'Asia' was catechin in all stages of ripeness. In the semi-mature stage, catechin significantly increased with infection. This could be linked to the fact that flavanol metabolites, such as catechin, have been shown to be crucial in the induction of fungal quiescence in unripe fruits [3] and the levels of almost all procyanidin forms increased after the infection.

Interestingly, flavanol levels decreased in fruits of cv. 'Clery' after infection in all stages of infection suggesting a cultivar specific response to the pathogen attack [34]. Ref. [30] reported that the role of flavanols in plant pathogen defence might be explained by their interaction with proteins and the inhibition of enzyme activity secreted by pathogenic fungi.

Among hydroxycinnamic acid derivatives, *p*-coumaroyl hexose was identified. After *C. nymphaeae* infection, a significant increase of *p*-coumaroyl hexose was demonstrated only in white fruits. The synthesis of *p*-coumaric acid was more pronounced in unripe fruits.

### 4.3. Phenolic content in strawberry runners

Fruits of rosaceous plants are known as rich sources of phenolics

[18], but little is known about the phenolic composition of their leaves and runners, which may impact the health status of the plant and participate in disease resistance. In strawberry runners, 27 different phenolic compounds were found. The level of most phenolic compounds increased as a result of *C. nymphaeae* infection in fruits, but in runners, the majority of identified phenols decreased. The only exceptions were procyanidin trimer 2 and *p*-coumaric acid, the content of which was significantly higher in infected runners. Specifically, infected runners contained low levels of all identified flavonols. Quercetin glycosides are precursors for the synthesis of mono- and polymeric flavanols and are used as substrates in the phenylpropanoid pathway [24]. According to histological studies of [5]; *Colletotrichum* species establish a very short biotrophic phase on strawberry petioles and runners before they invade them as subcuticular intramural necrotrophs. They might also establish quiescent infections, but we assume that under the very conductive conditions for disease development prevailing during the course of the experiment in 2012, they did not become quiescent and acted as general necrotrophs.

Ellagitannins are known to inhibit herbivores and act as antimicrobial barriers in woody plants [27]. Correspondingly, as a part of plant defence response, penta-esterified ellagitannins are synthesized in strawberry leaves and potentially induce pathogen resistance [17]. The content of different forms of ellagic acid derivatives depends of the cultivar and also on environmental factors. Ref. [15] demonstrated that strawberry cultivars accumulate different levels of ellagic acid derivatives in analysed tissues (fruits, achenes and leaves). Presumably, and by analogy with other strawberry metabolites, cultivar differences are heritable characteristics, but the accumulation of ellagic acid can also be altered by other environmental factors, such as infection or wounding [22,23]. We found that the levels of ellagic acid derivatives were higher in strawberry runners in comparison to fruits. Similarly [15], measured the highest levels of ellagic acid in strawberry leaves compared to fruits.

Ten flavanols were quantified in non-infected and infected strawberry runners. Previous studies [20] demonstrated that a rapid biosynthesis of flavanols is crucial for enhanced resistance and successful protection against diseases (*Venturia inaequalis* in apples) and pests. Catechin has previously been characterized as an antimicrobial compound in strawberries [35]. We can assume that *Colletotrichum* infection in strawberries does not alter catechin synthesis. The pattern of phenolic accumulation in strawberry runners modified by *C. nymphaeae* infection was in contrast to strawberry fruits since most of identified phenols decreased as a response to the infection. Future studies on phenolic synthesis rate, enzymatic activity and the abundance of phenolic compounds as a response to anthracnose infection should be carried out to further understand the involvement of secondary metabolites in plant defence in the strawberry *C. nymphaeae* interaction.

The current results indicate that *C. nymphaeae* infection causes intense synthesis and accumulation of specific phenolic compounds in strawberry fruits at all stages of ripeness. Among primary metabolites, infection modified the accumulation of sugars and organic acids. Lower levels of citric and malic acid and higher levels of fumaric and shikimic acid in all maturity stages have been determined in infected fruits. Interestingly, the content of ascorbic acid increased after infection in all stages of ripeness except in mature fruits. Metabolic responses of all identified phenolics were similar after *Colletotrichum* infection. The fungus caused an increase of total ellagic acid derivatives, but no differences have been observed among different maturity stages. Total content of flavanols and hydroxycinnamic acids increased after infection and also with maturity. Fruits infected with *C. nymphaeae* contained higher levels of total flavonols and anthocyanins, which also increased with the development of red colour during fruit maturation. Interestingly, an opposite effect of *Colletotrichum* infection has been noted in strawberry runners, in which a significant decrease of almost all identified phenolic groups, except of hydroxycinnamic acid, was determined. The content of the latter increased in infected runners. Flavonols were particularly affected by anthracnose in strawberry runners. Their accumulation was significantly decreased as a result of infection. Future studies on phenolic synthesis rate, enzymatic activity and the abundance of phenolic compounds as a response to anthracnose infection should be carried out to further understand the secondary metabolism-plant defence-*Colletotrichum* interaction.

## Acknowledgements

The research is part of program Horticulture No. P4-0013-0481 and the project No. J4-4187 funded by the Slovenian Research Agency (ARRS).

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## 2.2 OSTALO POVEZOVALNO ZNANSTVENO DELO

### 2.2.1 Vpliv različnih režimov namakanja na kakovost plodov žlahtnega jagodnjaka (*Fragaria* × *ananassa* Duch.)

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INFLUENCE OF DIFFERENT IRRIGATION REGIME ON STRAWBERRY (*Fragaria* × *ananassa* DUCH.) FRUIT QUALITY

Journal of the Science of Food and Agriculture, 2016, v tisku.

Na poskusnem polju smo dve sorti večkrat rodnega žlahtnega jagodnjaka 'Flamenco' in 'Eva's Delight' izpostavili trem različnim režimom namakanja. Količina vode za namakanje je bila določena na podlagi analize tal in spremljana s pomočjo tenziometrov. Ustaljena je bila pri treh različnih tenzijah: -12 kPa (zgornja točka poljske kapacitete), -33 kPa (spodnja točka poljske kapacitete) in -70 kPa (rastlinam težje dostopna voda). Vzorce smo pobirali v treh terminih in spremljali metabolizem rastline z vrednotenjem primarnih in sekundarnih metabolitov ter pridelka. Izkazalo se je, da so imeli plodovi rastlin obeh sort, ki so bile izpostavljene najmanjši količini namakane vode (tenzija -70 kPa) bistveno večje vsebnosti sladkorjev (1,1 do 1,4-krat), organskih kislin (1,1 do 1,2-krat) in vseh analiziranih fenolov (1,2 do 2,4-krat). Pri sorti 'Flamenco' se je izkazalo, da so ob manjših količinah dodane vode (-70 kPa) plodovi imeli bistveno boljše razmerje med sladkorji in organskimi kislinami, zmanjšano namakanje pa ni značilno vplivalo na pridelek. Pod enakimi pogoji se je pri sorti 'Eva's Delight' pokazalo, da namakanje pri tenziji -70 kPa zmanjša pridelek. S primernim namakanjem lahko povečamo vsebnost primarnih in sekundarnih metabolitov in s tem izboljšamo notranjo kakovost plodov.

## NAMEN POSKUSA

V zadnjem poskusu smo želeli ugotoviti, kako različni režimi namakanja vplivajo na pridelek in sintezo primarnih ter sekundarnih metabolitov. Je morda omejeno namakanje eden od možnih ukrepov za izboljšanje notranje kakovosti jagod ali bo le to pomenilo izgubo pridelka. Zanimalo nas je tudi, ali se bosta različni sorti, na določene režime namakanja odzvale enako.

## MATERIALI IN METODE

V rastlinjaku smo na grebene, prekrite s črno polietilensko zastirko, posadili frigo sadike jagod večkrat rodnih sort 'Flamenco' in 'Eva's Delight'. Rastline smo oskrbovali po navodilih za integrirano pridelavo (IP) sadja (Ministrstvo za kmetijstvo, 2014). Na vsakem grebenu so bile posajene rastline v dveh vrstah, vsaka opremljena s svojo namakalno cevjo kapljičnega sistema tipa T-tape. Vsako sorto smo razdelili na tri obravnavanja in jih opremili s tenziometri, ki so bili nastavljeni na globino 25 cm, kjer je bila večina korenin. Po deset rastlin smo izpostavili tenziji: a) -12 kPa (zgornja točka poljske kapacitete), b) -33 kPa (spodnja točka poljske kapacitete) in c) -70 kPa (rastlinam težje dostopna voda). Med poskusom smo vrednotili število cvetov in število ter maso plodov. Vzorce smo pobirali v polni zrelosti, rastline pa so bile omenjenim spremenjenim režimom izpostavljene 2 meseca. Po vzorčenju smo odbrali vzorce za nadaljnje analize primarnih in sekundarnih metabolitov ter izmerili vsebnost suhe snovi. Za določanje primarnih in sekundarnih metabolitov smo uporabili cele plodove, za vsako obravnavanje smo izvedli 5 ponovitev. Ekstrakcijo sladkorjev in organskih kislin ter fenolov smo izvedli enako kot pri prej omenjenih poskusih (Weber in sod., 2013; 2015, Mikulic-Petkovsek in sod., 2013). Prav tako smo primarne in sekundarne metabolite v vzorcih identificirali na sistemu visokoločljivostne tekočinske kromatografije (HPLC) na valovnih dolžinah 280 nm (flavanole in hidroksicimetne kisline), 350 nm (flavonole, flavone) in 530 nm (antociane).

## REZULTATI

Rezultati vsebnosti skupnih sladkorjev in organskih kislin v obravnavanih sortah pri različnih terminih vzorčenja so predstavljeni v preglednici 1. Vsebnosti analiziranih fenolov po posameznih fenolnih skupinah in skupni analizirani fenoli so predstavljeni v preglednicah 2 in 3.

Preglednica 1: Vsebnost skupnih sladkorjev in organskih kislin (g/kg SM) ter njuno razmerje pri sortah 'Flamenco' in 'Eva's Delight' gojenih pri različnih tenzijah (kPa)
Table 1: Content of total sugars and organic acids and their ratio in 'Flamenco' and 'Eva's Delight' strawberry fruits (g/kg FW) grown under various tension (kPa)

| Sorta | Parameter | Tenzija | 1. vzorčenje | 2. vzorčenje | 3. vzorčenje |
|---|---|---|---|---|---|
| 'Flamenco' | Sladkorji | -12 | 53,16 ± 5,02 a* | 65,25 ± 0,98 a | 59,18 ± 1,43 a |
| | | -33 | 58,22 ± 4,49 a | 67,11 ± 1,65 a | 62,36 ± 3,22 a |
| | | -70 | 74,55 ± 7,95 b | 77,14 ± 7,97 b | 75,94 ± 3,03 b |
| | Organske kisline | -12 | 8,29 ± 0,29 a | 11,73 ± 0,53 a | 11,78 ± 0,13 a |
| | | -33 | 9,52 ± 0,43 b | 11,26 ± 0,76 a | 12,06 ± 0,47 a |
| | | -70 | 10,92 ± 0,31 c | 12,96 ± 0,42 b | 13,43 ± 1,05 b |
| | Razmerje sladkorji/organske kisline | -12 | 5,61 ± 0,52 a | 5,34 ± 0,16 a | 5,02 ± 0,12 a |
| | | -33 | 6,12 ± 0,54 ab | 5,98 ± 0,47 b | 5,18 ± 0,31 a |
| | | -70 | 6,65 ± 0,58 b | 5,94 ± 0,52 b | 5,94 ± 0,41 b |
| 'Eva's Delight' | Sladkorji | -12 | 74,73 ± 2,29 a | 82,71 ± 1,52 a | 69,28 ± 2,40 a |
| | | -33 | 88,61 ± 6,59 b | 84,83 ± 3,06 a | 71,53 ± 1,05 a |
| | | -70 | 93,28 ± 9,29 b | 89,37 ± 1,21 b | 78,95 ± 3,62 b |
| | Organske kisline | -12 | 10,02 ± 0,83 a | 10,85 ± 0,93 ns | 10,83 ± 0,44 a |
| | | -33 | 10,39 ± 0,61 a | 11,47 ± 0,79 ns | 12,75 ± 0,48 b |
| | | -70 | 13,78 ± 0,94 b | 10,85 ± 0,36 ns | 13,73 ± 0,42 c |
| | Razmerje sladkorji/organske kisline | -12 | 7,84 ± 1,08 ns | 7,62 ± 0,21 ns | 6,39 ± 0,25 ns |
| | | -33 | 8,57 ± 1,08 ns | 7,76 ± 0,39 ns | 5,61 ± 0,28 ns |
| | | -70 | 6,61 ± 0,86 ns | 8,85 ± 0,74 ns | 5,91 ± 0,48 ns |

* - različne črke označujejo statistično značilne razlike med različnimi tenzijami pri posameznem parametru in posamezni sorti v različnih terminih vzorčenja, ns – ni statistično značilnih razlik

Preglednica 2: Vsebnost fenolov (mg/kg SM) po skupinah pri sorti 'Flamenco' gojeni pri različnih tenzijah (kPa)

Table 2: The content of different phenolic groups in strawberry fruits of cultivar 'Flamenco' (mg/kg FW) grown under various tension (kPa)

| Sorta | Parameter | Tenzija | 1. vzorčenje | 2. vzorčenje | 3. vzorčenje |
|---|---|---|---|---|---|
| 'Flamenco' | Antociani | -12 | 140,93 ± 10,36 b* | 125,83 ± 4,86 a | 95,81 ± 11,72 a |
| | | -33 | 111,44 ± 9,65 a | 130,98 ± 12,24 a | 81,95 ± 7,08 a |
| | | -70 | 158,36 ± 16,21 b | 158,78 ± 8,48 b | 137,23 ± 7,23 b |
| | Derivati elagnih kislin | -12 | 66,03 ± 3,76 a | 76,64 ± 4,79 a | 75,65 ± 5,39 a |
| | | -33 | 57,40 ± 5,19 a | 71,44 ± 6,34 a | 83,62 ± 6,67 a |
| | | -70 | 81,83 ± 6,07 b | 89,62 ± 2,69 b | 123,47 ± 3,27 b |
| | Flavanoli | -12 | 1095,26 ± 78,47 a | 1189,51 ± 22,11 a | 1350,97 ± 127,18 ab |
| | | -33 | 1081,17 ± 88,38 a | 1350,05 ± 22,82 ab | 1233,21 ± 100,99 a |
| | | -70 | 1275,62 ± 74,62 b | 1435,59 ± 74,63 b | 1605,31 ± 160,42 b |
| | Flavonoli | -12 | 8,55 ± 0,76 b | 9,43 ± 0,73 b | 5,97 ± 0,59 a |
| | | -33 | 6,95 ± 0,53 a | 7,81 ± 0,67 a | 6,21 ± 0,11 a |
| | | -70 | 10,59 ± 1,59 c | 10,65 ± 1,29 b | 10,27 ± 1,94 b |
| | Hidroksicimetne kisline | -12 | 92,73 ± 1,16 a | 149,75 ± 12,32 a | 102,72 ± 5,01 a |
| | | -33 | 85,06 ± 6,12 a | 141,67 ± 13,08 a | 96,79 ± 10,39 a |
| | | -70 | 128,92 ± 12,82 b | 178,12 ± 13,31 b | 169,46 ± 17,53 b |
| | Flavoni | -12 | 2,61 ± 0,22 ab | 1,49 ± 0,18 a | 1,70 ± 0,21 a |
| | | -33 | 2,22 ± 0,28 a | 2,82 ± 0,30 b | 1,49 ± 0,13 a |
| | | -70 | 3,37 ± 0,31 b | 1,91 ± 0,18 a | 2,61 ± 0,32 b |
| | Skupni analizirani fenoli | -12 | 1315,66 ± 97,81 a | 1407,95 ± 23,26 a | 1467,33 ± 118,47 a |
| | | -33 | 1261,88 ± 111,62 a | 1570,15 ± 32,86 ab | 1366,47 ± 124,04 a |
| | | -70 | 1532,49 ± 90,05 b | 1696,61 ± 74,82 b | 1817,62 ± 205,91 b |

* - različne črke označujejo statistično značilne razlike med različnimi tenzijami pri posameznem parametru in posamezni sorti v različnih terminih vzorčenja; ns – ni statistično značilnih razlik

Preglednica 3: Vsebnost fenolov (mg/kg SM) po skupinah pri sorti 'Eva's Delight' gojeni pri različnih tenzijah (kPa)

Table 3: The content of different phenolic groups in strawberry fruits of cultivar 'Eva's Delight' (mg/kg FW) grown under various tension (kPa)

| Sorta | Parameter | Tenzija | 1. vzorčenje | 2. vzorčenje | 3. vzorčenje |
|---|---|---|---|---|---|
| 'Eva's Delight' | Antociani | -12 | 125,87 ± 4,14 b | 162,68 ± 3,14 a | 130,23 ± 2,28 a |
| | | -33 | 116,04 ± 2,25 a | 185,55 ± 2,33 b | 151,23 ± 4,60 b |
| | | -70 | 124,79 ± 1,90 b | 206,97 ± 11,08 c | 161,97 ± 2,16 c |
| | Derivati elagnih kislin | -12 | 39,25 ± 0,41 a | 46,19 ± 2,52 ns | 56,51 ± 3,30 a |
| | | -33 | 36,71 ± 0,68 a | 46,24 ± 3,27 ns | 77,88 ± 1,31 b |
| | | -70 | 45,75 ± 2,24 b | 42,08 ± 2,21 ns | 97,51 ± 4,70 c |
| | Flavanoli | -12 | 392,24 ± 25,18 b | 523,79 ± 18,67 b | 486,07 ± 16,90 a |
| | | -33 | 336,79 ± 10,64 a | 508,23 ± 12,83 a | 757,92 ± 19,56 b |
| | | -70 | 427,14 ± 16,83 b | 581,48 ± 22,55 b | 856,57 ± 47,51 c |
| | Flavonoli | -12 | 16,46 ± 0,27 b | 12,04 ± 0,56 a | 9,77 ± 0,65 a |
| | | -33 | 11,38 ± 1,62 a | 15,45 ± 0,38 b | 13,04 ± 1,39 b |
| | | -70 | 12,83 ± 1,21 a | 21,35 ± 1,67 c | 13,16 ± 1,62 b |
| | Hidroksicimetne kisline | -12 | 17,44 ± 1,41 a | 14,61 ± 1,73 a | 14,24 ± 2,27 a |
| | | -33 | 30,12 ± 0,98 b | 25,13 ± 2,32 b | 26,86 ± 5,12 b |
| | | -70 | 28,78 ± 0,97 b | 22,90 ± 2,25 b | 34,77 ± 5,09 b |
| | Flavoni | -12 | 3,68 ± 0,43 b | 3,81 ± 0,32 ns | 3,42 ± 0,33 ns |
| | | -33 | 2,61 ± 0,21 a | 4,57 ± 0,13 ns | 3,79 ± 0,27 ns |
| | | -70 | 2,86 ± 0,34 a | 3,88 ± 0,21 ns | 3,86 ± 0,29 ns |
| | Skupni analizirani fenoli | -12 | 620,13 ± 34,53 ns | 795,92 ± 43,42 a | 720,95 ± 26,71 a |
| | | -33 | 551,36 ± 18,46 ns | 811,67 ± 21,93 a | 1054,53 ± 33,20 b |
| | | -70 | 666,37 ± 24,60 ns | 906,82 ± 36,61 b | 1194,52 ± 62,59 c |

* - različne črke označujejo statistično značilne razlike med različnimi tenzijami pri posameznem parametru in posamezni sorti v različnih terminih vzorčenja; ns – ni statistično značilnih razlik

# 3 RAZPRAVA IN SKLEPI

## 3.1 RAZPRAVA

Čeprav rastline nimajo zgrajenega imunskega sistema, kot ga imajo živali in ljudje, so precej tolerantne, nekatere celo odporne proti različnim boleznim, ki jih povzročajo glive, bakterije ali nematode, ki so prisotne v okolju (Taiz in Zeiger, 2010). Rastline so sposobne izgraditi tako imenovano sistemsko pridobljeno odpornost. Med različnimi načini boja proti patogenu smo z našimi raziskavami poskušali razložiti metabolni odziv žlahtnega jagodnjaka na glivično okužbo in različne režime namakanja.

Nekateri fenoli imajo dokazano protimikrobno delovanje in so lahko v rastlinah prisotni kot aktivne snovi ali pa kot neaktivni prekurzorji. V aktivno obliko jih nato pretvorijo encimi, ki jih rastlina aktivira kot odgovor na vdor patogena ali kakršen koli drug stresni dejavnik (Agrios, 1997). Fenolna sestava je specifična za točno določeno filogenetsko skupino (Fridman in Pichersky, 2005).

Črna pegavost je druga gospodarsko najpomembnejša glivična bolezen žlahtnega jagodnjaka, ki jo povzroča gliva *Colletotrichum nymphaeae*. Zato smo zastavili različne poskuse, da bi proučili vpliv okužbe z omenjeno glivo z vidika odziva primarnega in sekundarnega metabolizma žlahtnega jagodnjaka. V prvem poskusu smo tehnološko zrele plodove sorte 'Clery' ločili na tri različne stopnje okužbe: zdrave plodove, plodove s prvimi vidnimi znaki okužbe (začetek okužbe) in okužene plodove, kjer je pega prekrivala večjo površino ploda. S tem smo spremljali odziv rastline v prvem stadiju okužbe in ga primerjali z zdravimi ter močno okuženimi plodovi. Ker omenjena sorta velja za občutljivejšo na okužbo z omenjeno glivo, smo postavili dodatni poskus, kjer smo primerjali vpliv okužbe na dveh različnih sortah, tolerantnejši 'Honeoye' in občutljivejši 'Elsanti'. Poleg okužbe smo spremljali tudi vpliv različnih pripravkov, ki jih pridelovalci uporabljajo za povečanje naravne odpornosti rastlin in kot kurativno varstvo proti črni pegavosti. Želeli smo ugotoviti, kako kalcijev pripravek Stopit (YARA) in splošno uporabljen fungicid za zatiranje črne pegavosti Signum (BASF) vplivata na sintezo fenolov v rastlinskih celicah in ali so razlike v odzivu med dvema opazovanima sortama. Opazovali smo tudi vpliv na pridelek in spremljali število okuženih plodov in pritlik. Pri prvem in drugem poskus smo analizirali le popolnoma zrele plodove žlahtnega jagodnjaka, zato nas je v tretjem poskusu zanimalo, kako se rastlina na okužbo odzove v drugih stadijih zrelosti plodov. Tokrat je do spontane okužbe prišlo na sorti 'Asia', ki je v evropskem prostoru precej zastopana zaradi okusnih in velikih plodov. Analizirali smo zdrave in okužene plodove v treh različnih stopnjah zrelosti: bele plodove, delno dozorele in popolnoma dozorele plodove. Ker smo v literaturi zasledili, da se fenolni odziv na okužbo vegetativnih delov žlahtnega jagodnjaka odzove podobno kot ne-zreli plodovi, smo analizirali tudi okužene in zdrave pritlike.

Nedozorele plodove gliva okužuje drugače kot dozorele. Kadar pride v stik z nedozorelim plodom, gliva vstopi v fazo mirovanja in ne razvije vidnih znakov okužbe. Pri vzorčenju poskusa smo ugotovili, da je napad glive tako močan, da so bile vidne značilne vdrte črne pege na plodovih v vseh stopnjah zrelosti, ne le na popolnoma zrelih. Pogoji za razvoj bolezni so bili v letu 2012 na izbrani lokaciji izredno ugodni, visoka relativna zračna vlaga, pogoste padavine in temperature med 20 in 25 °C, za katere poročajo kot ugodne za razvoj okužbe s *C. nymphaeae* (Wharton in Dieguez-Uribeondo, 2004). Predvidevamo, da je bila zaradi ugodnih razmer za okužbo na nedozorelih plodovih faza mirovanja izredno kratka ali do nje celo ni prišlo.

Iz skupine primarnih metabolitov smo v izbranih sortah identificirali tri sladkorje (saharozo, fruktozo in glukozo) in štiri organske kisline (citronsko, jabolčno, fumarno in šikimsko). V prvem poskusu smo ugotovili, da okužba z glivo *C. simmondsii* že ob prvih vidnih znakih povzroči 3,4-kratno zmanjšanje vsebnosti saharoze (Weber in sod., 2013), kar pojasnjuje, da je to glavna transportna oblika sladkorja iz gostiteljske celice v glivno celico (Agrios, 1997). V popolnoma okuženih plodovih je bila vsebnost saharoze v vseh treh poskusih manjša kar za 2,7 do 6,3-krat v primerjavi z zdravimi plodovi (Weber in sod., 2013, 2015; Mikulic-Petkovsek in sod. 2013). S tem smo potrdili tudi znanstvena dela drugih avtorjev (Palama in sod., 2012; Broeckling in sod., 2005). Glede na to, da saharozo encim invertaza pretvori v enostavna sladkorja glukozo in fruktozo, bi lahko bil to razlog, zakaj je prišlo do zmanjšanja saharoze in povečanja monosaharidov (Cordenunsi in sod., 2003). A vendar bi bilo za tako trditev treba dokazati povečano delovanje omenjenega encima. Vsebnost fruktoze in glukoze se je v plodovih sorte 'Clery' že ob prvih vidnih znakih okužbe značilno povečala za 1,5-krat, enak trend povečanja pa smo opazili tudi pri nadaljnjih poskusih pri ostalih obravnavanih sortah popolnoma okuženih plodov (Mikulic-Petkovsek in sod., 2013; Weber in sod., 2015) ter potrdili raziskave drugih znanstvenikov (Wang in sod. 1997).

Okužba z glivo *C. nymphaeae* je posledično povzročila 1,2 do 1,4-kratno povečanje skupnih sladkorjev, razen pri tretjem poskusu, kjer se je vsebnost skupnih sladkorjev v delno dozorelih plodovih po okužbi zmanjšala. Poleg okužbe pa je na vsebnost skupnih sladkorjev vplivalo tudi tretiranje s fungicidom in kalcijem. Pri sorti 'Elsanta' sta pripravka značilno povečala vsebnost skupnih sladkorjev, kar je skladno z rezultati, ki jih navajajo Lara in sod. (2004). Drugi raziskovalci kot posledico večje vsebnosti skupnih sladkorjev po nanosu kalcija pripisujejo boljši vezavi pektina s kalcijevimi ioni v celičnih stenah, kar izboljša trdoto plodov (Lara in sod., 2004). Rastlina je torej imela na voljo več energije za obrambo pred patogeni, le ti pa so težje prodrli skozi močnejšo celično steno. Pri tolerantnejši sorti 'Honeoye' sta se vsebnost skupnih sladkorjev in posledično tudi razmerje med sladkorji in organskimi kislinami povečali celo bolj kot pri sorti 'Elsanta'. Rezultati vsebnosti primarnih metabolitov dokazujejo, da je med obravnavanima sortama prišlo do različnega odziva. Fungicid je povečal vsebnost skupnih sladkorjev pri sorti 'Elsanta', kalcij in okužba pa sta pri obeh sortah podobno vplivala na vsebnost primarnih metabolitov v plodovih. Tudi druge študije vpliva napada patogena kažejo na to, da

okužba s *C. simmondsii* poveča sintezo ogljikovih hidratov v različnih organih žlahtnega jagodnjaka (Crespo in sod., 2010; Basson in sod., 2010). Broeckling in sod. (2005) so povečane vrednosti sladkorjev v okuženih plodovih razlagali z dejstvom, da rastlina za nadaljnjo obrambo proti patogenu potrebuje več energije za sintezo obrambnih snovi, fenolov. Stopnja sladkorjev pa se spreminja tudi z dozorevanjem (Sturm in sod., 2003), tako je bila v belih plodovih manjša vsebnost sladkorjev v primerjavi z zrelejšimi. Okužba je vplivala na vsebnost sladkorjev šele v delno dozorelih plodovih, kjer je povzročila zmanjšanje le-teh, najverjetneje zaradi povečanih potreb po energiji za obrambo pred glivo, v dozorelih plodovih pa je okužba povzročila povečanje vsebnosti skupnih sladkorjev.

Ravno nasprotni vpliv pa je imela gliva *C. nymphaeae* na vsebnost organskih kislin, ki so ne glede na to, da so v plodovih zastopane v bistveno manjših koncentracijah kot sladkorji, pomembne za dober okus jagod. Med organskimi kislinami je v plodovih žlahtnega jagodnjaka največ citronske in jabolčne kisline. Okužba z glivo je povzročila zmanjšanje vsebnosti obeh omenjenih kislin v popolnoma okuženih plodovih žlahtnega jagodnjaka v vseh treh poskusih (Weber in sod., 2013, 2015; Mikulic-Petkovsek in sod., 2013). Podoben vpliv pa je imela tudi aplikacija kalcija. V začetnih stadijih okužbe nismo zaznali značilne razlike v vsebnosti organskih kislin, razen pri vsebnosti fumarne kisline, ki se je 1,5-krat povečala takoj po prvih vidnih znakih okužbe. Vsebnost fumarne in šikimske kisline v plodovih se je značilno povečala po okužbi tudi v naših nadaljnjih raziskavah, neodvisno od stopnje zrelosti (Weber in sod., 2013). Vsebnost organskih kislin je prav tako kot vsebnost sladkorjev odvisna od stopnje zrelosti (Sturm in sod., 2003). V delno dozorelih plodovih smo tako dokazali največjo vsebnost skupnih organskih kislin, le-ta pa se je neodvisno od stopnje zrelosti po okužbi zmanjšala. Glive za svoj razvoj porabljajo ogljik, ki ga pridobijo iz organskih kislin (Kamilova in sod., 2006), zato je zmanjšanje vsebnosti skupnih organskih kislin razumljivo. Prav tako je okužba povečala vsebnost vitamina C v belih in delno dozorelih plodovih, največje vsebnosti vitamina C pa imajo dozoreli plodovi žlahtnega jagodnjaka.

Razmerje med sladkorji in organskimi kislinami je bilo v okuženih plodovih pri vseh poskusih za 1,3 do 2-krat večje kot v zdravih rastlinah. Podobno večje razmerje smo zaznali v plodovih žlahtnega jagodnjaka, ki smo jih tretirali s kalcijem, kar pomeni slajši okus. Dognanje se ujema z rezultati Sammi in sod. (2009) in ga gre najverjetneje pripisat večjim vsebnostim sladkorjev.

Iz skupine sekundarnih metabolitov smo v plodovih 4 različnih sort žlahtnega jagodnjaka uspešno identificirali 31 različnih fenolov. Posamezne fenole smo razdelili v glavne fenolne skupine: derivate elagnih kislin, flavanole, flavonole, hidroksicimetne kisline in antociane. Tudi drugi raziskovalci so v svojih študijah identificirali posamezne fenole v plodovih žlahtnega jagodnjaka (Aaby in sod., 2012), večina le teh je bila prisotna tudi v plodovih naših izbranih sort. Prisotnost in vsebnosti posameznih fenolov je odvisna od mnogih dejavnikov, razlikujejo se med sortami (Aaby in sod., 2012), spreminjajo jih

rastne razmere in okolje (Wang in sod., 1996; Manach in sod., 2005) ter tudi tehnologija, s katero jih določamo. Poleg fenolne sestave plodov smo spremljali tudi vsebnost in prisotnost fenolov v pritlikah, kjer smo uspešno identificirali 36 fenolov.

Značilna rdeča barva, v katero se obarvajo plodovi žlahtnega jagodnjaka, ko dozorijo, je posledica sinteze antocianov, ki so prisotni v plodu, zato jih v belih plodovih nismo zaznali (Weber in sod., 2015). Vsebnost antocianov se torej povečuje z zorenjem (Aaby in sod., 2012) in že na videz so plodovi nekaterih sort temneje rdeče obarvani kot drugi, zato so med sortami velike razlike v vsebnosti antocianov (Aaby in sod., 2012). Če primerjamo vsebnosti antocianov v sortah, ki smo jih odbrali za izvedbo naših poskusov, lahko z gotovostjo potrdimo, da so razlike med njimi. Sorta 'Clery' je vsebovala kar 5-krat več skupnih antocianov kot sorta 'Asia', prav tako pa je tolerantna sorta 'Honeoye' vsebovala več skupnih antocianov kot občutljivejša 'Elsanta'. Pri proučevanju vpliva okužbe z glivo *C. simmondsii*, smo ugotovili, da je-le ta povzročila povečanje antocianov v plodovih, kjer so bili vidni prvi znaki okužbe (Weber in sod., 2013). Prav tako smo v ostalih dveh poskusih potrdili povečane vsebnosti antocianov v okuženih plodovih (Mikulic-Petkovsek in sod., 2013; Weber in sod., 2015). V delno dozorelih okuženih plodovih se je najbolj povečala vsebnost cianidin glukozida, v zrelih plodovih pa smo po okužbi zaznali večje vsebnosti pelargonidin malonil glukozida (Weber in sod., 2015). Mejno tkivo ob črnih pegah je postalo temnejše rdeče obarvano (vidna opazovanja). Tam se je najverjetneje kopičilo več antocianov. Podobno se je tkivo rdeče obarvalo ob pegi, ki je bila posledica glivične okužbe (jablanov škrlup) na kožici jabolk (Slatnar in sod., 2012).

Žlahtni jagodnjak je ena redkih sadnih vrst, ki je bogata z elagitanini. Derivati elagne kisline so se v naših poskusih izkazali za izredno občutljive na okužbo s patogenom, saj so se vsebnosti povečale po okužbi tako v plodovih kot pritlikah. Vsebnost pentozida elagne kisline se je v prvem poskusu povečala za 5,2-krat v popolnoma okuženem plodu v primerjavi z zdravimi (Weber in sod., 2013). Pri drugem poskusu smo potrdili rezultate prvega, saj je okužba s *C. nymphaeae* povzročila povečanje vsebnosti derivatov elagne kisline, vpliva tretiranja s kalcijem in fungicidom pa nismo dokazali. Razlik v vsebnosti derivatov elagne kisline med občutljivo in tolerantno sorto nismo opazili (Mikulic-Petkovsek in sod., 2013), prav tako pa na njeno vsebnost ni imela vpliva stopnja zrelosti plodov (Weber in sod., 2015). Tudi drugi znanstveniki so poročali o povečanih vrednostih elagne kisline in njenih derivatov po napadu patogena (Zhou in sod., 2007; Quave in sod., 2012), zato jim mnogi pripisujejo protiglivično delovanje v vseh rastlinskih organih. Saucedo in sod. (2007) so dokazali, da različne vsebnosti elagne kisline uspešno podaljšajo polično kakovost avokada, ki so ga predhodno okužili z glivo iz rodu *Colletotrichum*. Rastlina s sintezo elagnih kislin poskuša preprečiti nadaljnji razvoj patogena, saj tvorijo elagne kisline z beljakovinami in polisaharidi močne komplekse, ki preprečijo oziroma otežijo vdor glive (Haslam, 1996). Ekstrakti iz rastlin, ki vsebujejo veliko elagne kisline in njenih derivatov, so dokazano zavirali razvoj gliv in bakterij (Nascimento in sod., 2000). Elagitanini delujejo kot antimikrobne ovire v

lesnatih rastlinah (Ossipov in sod., 2001), v pritlikah in listih žlahtnega jagodnjaka pa so sintetizirani z namenom potencialne obrambe pred napadi patogenov (Mamani in sod., 2012). Čeprav se je v naši prvi in tretji raziskavi izkazalo, da se je večina derivatov elagne kisline v pritlikah zmanjšala, to težko pojasnimo. Pritlike so izredno tanko tkivo in okužba hitro uniči celice na mestu, kjer se pojavi, zato je lahko razlog za manjše vrednosti derivatov elagne upočasnjen metabolni odziv.

V plodovih in pritlikah žlahtnega jagodnjaka so flavonoli najmanj zastopana skupina vseh analiziranih fenolnih snovi. Sem spadajo izoramnetin, kvercetin in kempferol. Okužba z glivo *C. nymphaeae* je povzročila povečanje vsebnosti večine flavonolov v drugem in tretjem poskusu. Izjema je bil prvi poskus, kjer se je vsebnost kvarcetin glukozida in izoramnetina v okuženih plodovih zmanjšala, izoramnetin pa smo uspešno identificirali le v dotičnem poskusu v plodovih sorte 'Clery'. Stopnja okužbe, ki smo jo opazovali v prvem poskusu, ni bistveno vplivala na vsebnost flavonolov. Na vsebnost flavonolov pa je poleg okužbe z glivo *C. nymhaeae* vplivala tudi sorta. Tolerantnejša sorta 'Honeoye' je vsebovala več flavonolov kot sorta občutljivejša 'Elsanta', po okužbi pa se je pri obeh sortah vsebnost flavonolov značilno povečala (Mikulic-Petkovsek in sod., 2013). Sorta 'Elsanta' je v okuženih plodovih vsebovala 2-krat večje vsebnosti skupnih flavanolov kot v zdravih plodovih, pri sorti 'Honeoye' pa je okužba povzročila 1,7-kratno povečanje skupnih flavonolov. Do podobnih rezultatov so prišli v predhodnih raziskavah, kjer so dokazali, da je v listih žlahtnega jagodnjaka, ki je dovzeten na okužbe s pepelasto plesnijo, značilno manjša vsebnost flavonolov v primerjavi s tolerantnejšimi sortami, kjer so prisotne večje vsebnosti le-teh (Hanhineva in sod., 2009). Glede na dobljene rezultate drugega poskusa, tretiranje s kalcijem in fungicidom ni imelo bistvenega vpliva na sintezo flavonolov v plodovih žlahtnega jagodnjaka. V tretjem poskusu smo v okuženih plodovih zaznali povečane vsebnosti flavonolov v vseh treh stopnjah zrelosti, medtem ko sama stopnja zrelosti ni imela značilnega vpliva na vsebnost skupnih flavonolov. V okuženih belih plodovih je bila vsebnost flavonolov največja, kar za 5,6-krat večja kot v zdravih plodovih iste stopnje zrelosti. Pri sorti 'Clery' se je vsebnost nekaterih flavonolov z okužbo zmanjšala, pri sortah 'Asia', ' Elsanta' in 'Honeoye' pa povečala. Morda gre v tem primeru za sortno značilnost in bi bilo potrebno pri različnih sortah raziskati ekspresijo gena TA9432_57918, ki je odgovoren za kodiranje flavonol sintaze (Guidarelli in sod., 2011). V pritlikah sorte 'Clery' se je v prvem poskusu vsebnost flavonolov 2,1-krat povečala po okužbi z glivo *C. simmondsii*, medtem ko se je pri sorti 'Asia' kar za 7,1-krat zmanjšala. Jia in sod. (2010) so v svoji raziskavi ugotovili, da se je v okuženih listih repnjaka, ki so jih tretirali s kvarcetini, značilno povečala vsebnost vodikovega peroksida ($H_2O_2$) in je posledično prišlo do povečane stopnje polimerizacije flavonolov. Nastali polimeri se nato kopičijo v celičnih stenah in otežijo razvoj in vdor patogena (Zhao in sod., 2008).

Največji delež vseh fenolov v plodovih žlahtnega jagodnjaka, ki smo jih analizirali v poskusu, spada v skupino flavanolov. V plodovih sorte 'Clery' smo identificirali le procianidine, v sortah 'Elsanta' in 'Honeoye' smo poleg procianidinov uspešno potrdili še

epikatehin ter v sorti 'Asia' katehin. Ob prvih vidnih znakih okužbe na plodovih sorte 'Clery' smo v naši raziskavi dokazali povečano vsebnost flavanolov, statistično značilno dveh procianidinov, vsebnost procinidin dimera 3 pa se je povečala šele v popolnoma okuženih plodovih. Za to skupino fenolov so že Ardi in sod. (1998) dokazali, da imajo pomembno vlogo pri obrambi rastlin pred patogeni. Zanimivo je, da se je vsebnost flavanolov najbolj povečala ob začetnih znakih okužbe z glivo *C. simmondsii* in ob popolni okužbi zopet malo zmanjšala. Mnogi znanstveniki so dokazali, da je sinteza flavanolov v prvih fazah okužbe z glivo odločilna za uspešno obrambo celotne rastline (Michalek in sod., 1999). Rastlina je torej ob prvih znakih okužbe sintetizirala flavanole in tako povečala obrambo pred patogenom. Na vsebnost skupnih flavanolov sta vplivala tako sorta kot tretiranje s fungicidom in kalcijem. Sorta 'Honeoye' je vsebovala kar 50 % več epikatehina v zdravih plodovih kot sorta 'Elsanta', kar je potrdilo dognanje drugih znanstvenikov, da je vsebnost epikatehina večja v tolerantnejših sortah (Ardi in sod., 1998; Mikulic-Petkovsek in sod., 2007). Poleg tega so okuženi in s fungicidom tretirani plodovi sorte 'Honeoye' vsebovali 1,2 do 2-krat večje vsebnosti epikatehina v primerjavi s kontrolo ali tretiranjem s kalcijem. Pri sorti 'Elsanta' je okužba z glivo *C. nymphaeae* povzročila večjo sintezo epikatehina in skoraj za 2-krat povečala vsebnost skupnih flavanolov v primerjavi z zdravimi. Večje vsebnosti flavanolov po okužbi lahko potrdimo z raziskavo Chavez in Gianfagna (2007), kjer poročajo o 2-kratnem povečanju procianidinov po glivični okužbi kakavovca. Vsebnost katehina se je v okuženih delno dozorelih plodovih značilno povečala za 1,5-krat, kar je skladno z dognanji Ardi in sod. (1998), ki so v svojih študijah dokazali, da je vsebnost katehina v ključni povezavi z mirovanjem glive v nezrelih plodovih. Vsebnost flavanolov se je v okuženih plodovih značilno povečala v vseh treh stopnjah zrelosti, a najbolj v belih plodovih, kjer se je vsebnost procianidin trimer 4 povečala za več kot 6-krat, skupnih flavanolov pa za 2,1-krat (Weber in sod., 2015). Obrambna vloga flavanolov v rastlinah nastopi zaradi interakcije flavanolov z beljakovinami, zaradi česar je preprečena encimska aktivnost glive in ob manjšem napadu ne pride do okužbe (Vasco in sod., 2009). Flavanoli imajo spodobnost kelirati kovine – funkcionalna skupina tako predstavlja ligand, ki se poveže z osrednjim kovinskim kationom in tako tvori kelat. Na ta način se rastlina poskuša braniti pred patogeni, saj so kovinski ioni pomembni kofaktorji, ki so vezani na encime (Scalbert, 1991). Flavanoli so torej snovi, ki dokazano ščitijo rastlino pred napadom gliv, mikrobov, insektov in drugih škodljivih organizmov (Khanbabaee in sod., 2001).

Analizirali smo tudi okužene in zdrave pritlike sorte 'Clery' in 'Asia' ter ugotovili, da je odziv posameznih flavanolov na okužbo zelo različen. Vsebnost nekaterih se je po okužbi povečala, drugih zmanjšala. Glive iz rodu *Colletotrichum* na pritlikah in listnih pecljih po okužbi najprej vzpostavijo kratko biotropično fazo in šele nato prodrejo pod povrhnjico in okužujejo kot nekrotropi (Curry in sod., 2002). Manjše vsebnosti flavanolov lahko razložimo z vremenskimi razmerami, ki so bile v letu 2012 idealne za razvoj okužbe in je gliva najverjetneje delovala kot nekrotrop, tako je bilo že tako tanko tkivo pritlik ob razvoju znakov okužbe tako poškodovano, da so bili upočasnjeni vsi metabolni procesi.

V drugem in tretjem poskusu smo v sortah 'Elsanta', 'Honeoye' in 'Asia' identificirali tudi *p*-kumarno kislino in ugotovili, da je bila vsebnost le-te po okužbi z glivo *C. nymphaeae* povečana za 1,3 do 6,6-krat v primerjavi z ostalimi tretiranji oziroma zdravim tkivom v plodovih in pritlikah žlahtnega jagodnjaka. Ugotovili smo, da so razlike tudi med sortami. Tolerantnejša sorta 'Honeoye' je v zdravih plodovih vsebovala večje vsebnosti *p*-kumarne kisline kot občutljivejša sorta 'Elsanta' (Mikulic-Petkovsek in sod., 2013). V okuženih plodovih občutljivejše sorte pa se je vsebnost hidroksicimetne kisline močneje povečala kot v tolerantnejši. Vsebnost *p*-kumarne kisline je bila največja v dozorelih plodovih sorte 'Asia', le-ta pa se je značilno povečala po okužbi tako v belih kot dozorelih plodovih (Weber in sod., 2015). O večji vsebnosti hidroksicimetnih kislin po napadu patogena poročajo mnogi znanstveniki (Hukkanen in sod., 2007; Mikulic-Petkovsek in sod., 2009; Slatnar in sod., 2010). Dokazano je bilo, da imajo derivati hidroksicimetnih kislin pomembno funkcijo pri obrambi rastlin pred patogeni, saj zavirajo sporulacijo glive (Mikulic-Petkovsek in sod., 2007).

Okužba je bila torej glavni razlog za povečano vsebnost fenolov v plodovih žlahtnega jagodnjaka. Tako lahko na podlagi rezultatov vseh poskusov potrdimo, da so fenoli intenzivno vključeni v obrambo rastline pred *C. nymphaeae*. Rastline, ki smo jih tretirali s fungicidom, so bile glede na rezultate vsebnosti fenolov v zelo podobnem stanju kot netretirane rastline. Fungicid torej ni spremenil sinteze fenolov v plodovih. Kalcij statistično gledano ni imel vpliva na sintezo fenolov, a se je vsebnost nekaterih fenolov po aplikaciji kalcija vseeno približala vsebnosti v okuženih plodovih. Mnoge raziskave poročajo o pozitivnih učinkih kalcija na zmanjšanje gnilobe jagod pri skladiščenju, saj kalcij okrepi celične stene (Hernandez-Munoz in sod., 2006). Tolerantnejša sorta 'Honeoye' je imela manjši odstotek okuženih plodov in pritlik kot sorta 'Elsanta'. Prav tako sta se sorti razlikovali med seboj v vsebnosti flavanolov in flavonolov, saj je sorta 'Honeoye' vsebovala bistveno večjo vsebnost le-teh. Ugotovili smo, da imata tako sorta kot tretiranje vpliv na pridelek jagod. Pokazalo se je, da je sorta 'Elsanta' imela značilno manjši tržni pridelek na rastlinah, ki smo jih okužili z glivo *C. nymphaeae*. Zanimivo je, da je bil pridelek pri kontroli in okuženih rastlinah sorte 'Honeoye' manjši, a še vedno enak pridelku na umetno okuženi 'Elsanti', kar nakazuje na izboljšane lastnosti novejših sort. Tretiranje s fungicidom je pri sorti 'Honeoye' značilno povečalo pridelek. Sorta 'Elsanta' se je izkazala za občutljivo na črno pegavost, saj se je pridelek pri okuženih rastlinah zmanjšal kar za 1,7-krat. V letu 2012 niso bili ustrezni pogoji za razvoj črne pegavosti, zato na kontrolnih rastlinah ni prišlo do spontane okužbe plodov, okužba se je razvila le na pritlikah. Predvsem pri sorti 'Elsanta' sta tretiranje s fungicidom in kalcijem bistveno zmanjšala število okuženih pritlik, le-te pa so bile pri sorti 'Honeoye' manj okužene kot pri sorti 'Elsanta'. Pridelek žlahtnega jagodnjaka je bil v letu 2012 v našem poskusu zelo slab, za kar 60 % manjši od povprečnega, zaradi pozne spomladanske zmrzali, zaradi katere je propadlo mnogo cvetov.

V četrtem poskusu nas je zanimalo ali lahko z določenim režimom namakanja vplivamo na kakovost plodov žlahtnega jagodnjaka, kakšen je vpliv na količino pridelka in ali je

določen režim optimalen za obe sorti. Na ta vprašanja smo poskušali odgovoriti z analizo primarnih in sekundarnih metabolitov, ki so za rastline eden od pokazateljev stresa. V poskus smo vključili sorti 'Flamenco' in 'Eva's Delight'. Odločili smo se za večkrat rodni sorti, ker nas je zanimalo, kako dolgo morajo biti rastline izpostavljene različnim režimom namakanja, da se le-to odrazi na vsebnosti merjenih parametrov. Količina vode za namakanje je bila spremljana s pomočjo tenziometrov in ustaljena pri -12 kPa (zgornja točka poljske kapacitete), -33 kPa (spodnja točka poljske kapacitete) in -70 kPa (rastlinam težje dostopna voda). Vzorce smo pobirali v treh terminih in tako spremljali metabolizem rastline z vrednotenjem primarnih in sekundarnih metabolitov, pridelek ter število cvetov in plodov.

Izkazalo se je, da sta obravnavani sorti pod enakimi pogoji različno odreagirali. Predvsem so bile bistvene razlike v pridelku, razmerju med sladkorji in organskimi kislinami ter vsebnosti nekaterih fenolnih snovi. Pri sorti 'Flamenco' se je izkazalo, da namakanje do te mere, da je rastlinam voda še komaj dostopna (-70 kPa) ni povzročilo zmanjšanja pridelka. Pri sorti 'Eva's Delight' pa je enaka količina dodane vode že vplivala na pridelek, ki je bil zaradi tega za 33 % manjši kot pri namakanju do zgornje točke poljske kapacitete. Tako lahko na podlagi dobljenih rezultatov trdimo, da je odziv žlahtnega jagodnjaka na količino dodane vode med obravnavanima sortama različen. S tem smo potrdili predhodne raziskave, v katerih so spremljali odziv različnih sort žlahtnega jagodnjaka na manjše količine dodane vode (Gine-Bordonaba in Terry, 2010; Grant in sod., 2010). Pri obeh sortah se je z zmanjševanjem dodane vode povečeval odstotek suhe snovi, a vendar šele pri drugem terminu vzorčenja, ko so bile rastline spremenjenim pogojem izpostavljene približno mesec dni. Plodovi sorte 'Flamenco' so vsebovali največ suhe snovi (28 %) pri najmanj namakanih rastlinah, a so v primerjavi z optimalnim namakanjem (22 %) razlike relativno majhne. Medtem ko so razlike pri sorti 'Eva's Delight' bistveno večje. Pri najmanj dodane vode (-70 kPa) so plodovi sorte 'Eva's Delight' vsebovali kar 38 % suhe snovi, pri optimalnem namakanju pa 19 %. O povečanju količine suhe snovi v plodovih, izpostavljenih manjšim količinam namakane vode, poročajo tudi drugi raziskovalci (Gine-Bordonaba in Terry, 2010). Smo pa med poskusom ugotovili, da je sorta 'Flamenco' tvorila več listne mase in več pritlik, zato lahko sklepamo, da bi to lahko bilo povezano z večjo fotosintezo in posledično boljšo preskrbo rastlin.

Rastline, ki smo jih namakali do meje komaj dostopne vode (-70 kPa) so imele značilno večje vsebnosti sladkorjev in organskih kislin, če jih preračunamo na svežo snov. Ko pa preračunamo vsebnost sladkorjev in organskih kislin glede na vsebnost suhe snovi vidimo, da ima sorta 'Eva's Delight' bistveno večje vsebnosti primarnih metabolitov v rastlinah, ki so bile optimalno namakane. Najverjetneje je povečana vsebnost sladkorjev in organskih kislin v plodovih najmanj namakane sorte 'Flamenco' posledica stresa, ki ga je rastlina doživela ob manjših količinah vode, sorta 'Eva's Delight' pa je verjetno optimalno dodano vodo izkoristila za boljšo sintezo primarnih metabolitov. Naši rezultati so podprti z drugimi objavami, tako so tudi Terry in sod. (2007) večje vsebnosti

sladkorjev v plodovih žlahtnega jagodnjaka izpostavljenim suši pripisali vplivu razredčitve. Optimalno namakanje se je pri sorti 'Flamenco' odrazilo z večjim razmerjem med sladkorji in organskimi kislinami, kar pomeni slajši okus jagod, do enakih rezultatov pa so prišli tudi Terry in sod. (2007). Za sorto 'Eva's Delight' je značilen slajši okus in z našim poskusom smo to tudi potrdili, saj je imela večje razmerje med sladkorji in organskimi kislinami in različni režimi namakanja na to niso imeli značilnega vpliva.

Glede na to, da so se vsebnosti vseh analiziranih fenolov značilno povečale v plodovih rastlin, ki smo jih namakali do točke rastlinam komaj dostopne vode (-70 kPa), lahko na podlagi tega trdimo, da so bile rastline žlahtnega jagodnjaka v stresu. Fenoli se v rastlinah sintetizirajo kot posledica neugodnih razmer in zaščita pred stresom (Bhattacharya in sod., 2010). S tem smo potrdili trditev Terry in sod. (2007), ki je v svoji študiji na žlahtnem jagodnjaku dokazal značilno povečanje vsote vseh fenolnih snovi v plodovih, ki so bili izpostavljeni pomanjkanju vode. Obravnavani sorti imata različne potrebe po vodi in posledično zanju stres nastopi na različni točki. Za 'Eva's Delight' je že namakanje pri spodnji točki poljske kapacitete povzročilo zmanjšanje pridelka in povečanje vsebnosti nekaterih fenolnih snovi.

Vsebnost antocianov je bila zelo različna med tremi obravnavanji in prav tako med obravnavanima sortama. Že drugi znanstveniki so dokazali, da se ob pomanjkanju vode poveča ekspresija genov, ki so odgovorni za sintezo antocianov (Castellarin in sod., 2007; Deluc in sod., 2009). Do podobnih ugotovitev o povečanih vsebnostih antocianov so prišli tudi Rahmati in sod. (2015), ki so različnim režimom namakanja izpostavili breskve. V breskvah se večina antocianov nahaja v kožici, zato so povečanje le-teh pripisali morfološkim dejavnikom – manjšim plodovom, ki imajo večji odstotek kožice v primerjavi z mesom (Rahmati in sod., 2015).

Vsebnost derivatov elagnih kislin se je bistveno povečala v rastlinah, ki so bile namakane do spodnje točke poljske kapacitete (-33 kPa) in še komaj dostopne vode (-70 kPa), tako lahko trdimo, da zmanjšano namakanje poveča sintezo elagne kisline in njenih derivatov. Naši rezultati so skladni z rezultati Dodds in sod. (2007), ki je proučeval dva različna režima namakanja jagod in njun vpliv na vsebnost elagne kisline.

Prav tako je bila vsebnost flavanolov spremenjena v različnih režimih namakanja. Pri sorti 'Flamenco' se je izkazalo, da je namakanje do točke komaj dostopne vode povzročilo povečanje flavanolov v prvem terminu, v tretjem terminu pa je tudi namakanje pri zgornji točki poljske kapacitete (-12 kPa) povzročilo večje vsebnosti flavanolov. Enako se je na namakanje pri zgornji točki poljske kapacitete odzvala sorta 'Eva's Delight' v prvih dveh terminih. Glede na to, da različni režimi namakanja vplivajo na biosintezno pot flavanolov (Genebra in sod., 2014) predvidevamo, da je namakanje do zgornje točke poljske kapacitete pomenilo prevelike količine vode in prav tako predstavljalo stres za rastline.

Rastline, izpostavljene okoljskemu stresu, sintetizirajo večje količine flavonolov, s ciljem zmanjševanja oksidativnega stresa, ki nastopi kot posledica (Baratto in sod., 2003). Vsebnost flavonolov v plodovih sorte 'Flamenco' je bila v prvih dveh terminih vzorčenja značilno povečana pri tenziji -12 kPa in -70 kPa, v tretjem terminu pa pri tenziji -70 kPa. Za sorto 'Eva's Delight' je v prvem terminu namakanje na zgornji točki poljske kapacitete povzročilo povečano sintezo flavonolov, v naslednjih terminih pa sta povečanje stimulirala druga dva režima namakanja. Klamkovski in Treder (2008) sta dokazala, da sorta 'Elsanta', ki velja za tolerantnejšo na pomanjkanje vode, sintetizira več flavonolov kot občutljivejše sorte. Do podobne ugotovitve so prišli Sanchez-Rodriguez in sod. (2010) pri občutljivih in tolerantnih sortah paradižnika.

Vsebnosti hidroksicimetnih kislin so se pri sorti 'Flamenco' povečale v rastlinah, ki smo jih namakali do komaj dostopne vode, medtem ko je pri sorti 'Eva's Delight' namakanje na spodnji točki poljske kapacitete (-33 kPa) povzročilo povečanje vsebnosti hidroksicimetnih kislin. Do podobnih rezultatov so prišli tudi Tattini in sod. (2004), ki poročajo o povečanju hidroksicimetnih kislin v *Ligustrum vulgare*, ki so ga izpostavili pomanjkanju vode. Ayaz in sod. (2000) sklepajo, da povečanje fenolnih kislin vodi k povečani biosintezi lignina in posledično pride do manjših izgub vode, poleg tega pa *p*-kumarna kislina deluje kot osmoprotektor.

Flavoni so skupina fenolov, ki so v žlahtnem jagodnjaku najmanj zastopani, a je namakanje prav tako vplivalo na njihovo vsebnost. Rezultati Mandour in sod. (1979), ki so ugotavljali vpliv različnih režimov namakanja na vsebnost flavonov v plodovih hibiskusa, so skladni z našimi rezultati, različni režimi namakanja so namreč vplivali na vsebnost flavonov. Spremembe niso bile tako značilne kot pri ostalih skupinah fenolov.

Dokazali smo, da različni režimi namakanja spremenijo fenolno sestavo plodov žlahtnega jagodnjaka. Namakanje do tenzije -70 kPa, kjer je voda rastlinam še komaj dostopna, se je odrazilo v povečani vsebnosti večine analiziranih fenolnih skupin. Prav tako pa se je pri tenzij -12 kPa povečala vsebnost nekaterih fenolov. Glede na rezultate se poraja dvom ali je namakanje pri tenziji -12 kPa res optimalno. Pri sorti 'Flamenco' lahko na podlagi rezultatov trdimo, da vsebuje veliko fenolnih snovi, saj je bila vsebnost vseh identificiranih kar do 2,7-krat večja od sorte 'Eva's Delight' in morda je ravno v tem razlog, zakaj pri namakanju na meji še dostopne vode ni prišlo do zmanjšanja pridelka. Trend pri prodaji jagod gre vedno bolj v smer okusnih in zdravih plodov, kot pa velikih in prijaznim le očem. Premišljeno namakanje je lahko eden od tehnoloških ukrepov, ki izboljša notranjo kakovost jagod. Ko se pridelovalci odločijo, katero sorto bodo pridelovali, je zelo pomembno, da spremljajo rastline in dobro poznajo lastnosti tal, v katerih jih gojijo.

## 3.2 SKLEPI

Glede na dobljene rezultate lahko podamo naslednje sklepe:

1. hipotezo smo delno potrdili, rastlina se je na okužbo z glivo *C. nymphaeae* odzvala s povečano vsebnostjo nekaterih fenolnih snovi in zmanjšano vsebnostjo drugih, tako v plodovih kot pritlikah, a vendar na podlagi dobljenih rezultatov lahko sklepamo, da se na okužbo ne odzovejo vse obravnavane sorte enako. Odziv je odvisen od:

   - V prvem poskusu na sorti 'Clery' smo ugotovili, da se fenoli v različnih stopnjah okužbe plodov različno odzivajo. Vsebnost večine derivatov elagne kisline se je povečala šele v popolnoma okuženih plodovih, vsebnost flavanolov in antocianov že ob prvih znakih okužbe, medtem ko se je vsebnost nekaterih flavonolov po okužbi zmanjšala.

   - Primerjali smo odziv na okužbo pri tolerantni sorti 'Honeoye' in občutljivi sorti 'Elsanta', in ugotovili, da je tolerantnejša sorta sintetizirala več flavonolov, flavanolov in antocianov kot občutljivejša.

2. hipotezo smo potrdili, saj smo v raziskavi, kjer smo spremljali vpliv stopnje zrelosti plodov sorte 'Asia' na vsebnost fenolnih snovi po okužbi z glivo *C. nymphaeae*, ugotovili, da ima okužba bistveno večji vpliv na sintezo fenolov kot stopnja zrelosti. Izkazalo se je, da je vsebnost skupnih fenolov največja v zrelih plodovih, predvsem zaradi velikih vrednosti katehina in antocianov.

3. hipotezo smo delno potrdili, okužba z glivo *C. nymphaeae* je povzročila povečanje fruktoze in glukoze v plodovih žlahtnega jagodnjaka pri vseh obravnavanih sortah. V plodovih, ki so bili v fazi dozorevanja, pa se je vsebnost sladkorjev po okužbi zmanjšala. Znotraj hipoteze smo ovrgli trditev, da bo okužba povečala vsebnost vseh analiziranih sladkorjev, saj se je vsebnost saharoze po okužbi zmanjšala. Vsebnost organskih kislin, jabolčne in citronske, ki sta v plodovih najbolj zastopani, se je po okužbi z glivo *C. nymphaeae* zmanjšala.

4. hipotezo smo ovrgli, saj smo ugotovili, da so se pri dveh različnih sortah fenoli v pritlikah drugače odzvali. V prvem poskusu so okužene pritlike sorte 'Clery' vsebovale več flavonolov, prav tako so se značilno povečali nekateri derivati elagnih kislin, medtem ko so se drugi zmanjšali, večina flavanolov se je po okužbi zmanjšala. Pri sorti 'Asia' se je vsebnost skupnih derivatov elagne kisline, flavanolov in flavonolov po okužbi zmanjšala, vsebnost hidroksicimetne kisline pa povečala.

5.

6. hipotezo smo ovrgli, vsebnost skupnih fenolov in posameznih fenolnih skupin je bila značilno večja v plodovih okuženih z glivo *C. nymphaeae*, nanos fungicida in kalcija pa ni vplival na fenolno sestavo. Nanos kalcija je povečal razmerje med sladkorji in organskimi kislinami.

7. hipotezo smo potrdili, različni režimi namakanja, katere smo vzpostavili pri večkrat rodnima sortama 'Flamenco' in 'Eva's Delight', so vplivali tako na morfološke kot fiziološke parametre žlahtnega jagodnjaka. Sorta 'Flamenco' se na namakanje na meji še dostopne vode ni odzvala z zmanjšanim pridelkom, medtem ko se je pridelek sorte 'Eva's Delight' ob istem režimu značilno zmanjšal.

   - Plodovi obeh sort so pri namakanju na meji še dostopne vode vsebovali večje vsebnosti sladkorjev in organskih kislin, sorta 'Flamenco' pa posledično tudi večje razmerje med sladkorji in organskimi kislinami.

   - Ob izpostavitvi trem različnim režimom namakanja smo ugotovili, da je vsebnost skupnih fenolnih snovi večja v plodovih, ki smo jih pobrali iz rastlin, ki so bile namakane z najmanjšo količino vode.

   - Pri obeh sortah se je izkazalo, da je vsebnost določenih fenolnih skupin večja tudi pri predpostavljenem optimalnem namakanju na zgornji meji poljske kapacitete. Predvidevamo, da se določeni fenoli povečajo tudi ob prevelikem namakanju.

Z zastavljenimi poskusi smo pridobili odgovore na vse zastavljene hipoteze in potrdili velik vpliv okužbe na vsebnosti primarnih in sekundarnih metabolitov, tako v plodovih kot pritlikah žlahtnega jagodnjaka. Rezultati so v veliko pomoč pri razumevanju povezave med rastlino in patogenom in potrjujejo, da obramba rastline ob večjem napadu patogena ni dovolj. Ugotovili smo, da se fenolna sestava razlikuje med sortami in je njena vsebnost pri tolerantnejših sortah večja. Razvoj novih sort, tolerantnejših na okužbo z glivo *C. nymphaeae*, v smeri večje akumulacije specifičnih fenolov je torej en izmed pomembnejših ukrepov pri omejevanju razvoja črne pegavosti žlahtnega jagodnjaka. Z ustreznim namakanjem in sortami, ki so tolerantnejše na napad patogena, bi lahko tako gospodarsko kot okoljevarstveno izboljšali pridelavo jagod. Naši rezultati so pomembno prispevali k poznavanju fizioloških procesov v rastlini in boljšemu razumevanju odnosa med rastlino in patogenom.

# 4 POVZETEK (SUMMARY)

## 4.1 POVZETEK

Interakcija med rastlino, patogenom in vplivom tehnoloških ukrepov je izrednega pomena, obrambni procesi v rastlini, izpostavljeni določenemu stresu, pa še vedno niso popolnoma razumljivi. Zato smo v štirih ločenih poskusih spremljali biokemijske procese, ki jih vzpostavi žlahtni jagodnjak po napadu patogena in ob treh različnih režimih namakanja. Poskusi so bili zasnovani kot nadgradnja predhodnih raziskav, predvsem smo se osredotočili na primarni in sekundarni metabolizem plodov in pritlik.

Okužba z glivo *C. nymphaeae* je povzročila zmanjšanje vsebnosti saharoze ter povečanje fruktoze in glukoze v plodovih žlahtnega jagodnjaka na vseh obravnavanih sortah. Ravno obratno se je zgodilo z vsebnostjo jabolčne in citronske kisline, ki sta v plodovih najbolj zastopani, vsebnost le-teh se je po okužbi z glivo *C. nymphaeae* zmanjšala pri vseh obravnavanih sortah. Izkazalo se je, da so vse obravnavane sorte pri različnih poskusih imele enak odziv primarnega metabolizma na okužbo z omenjeno glivo.

Znano je, da je sinteza fenolnih snovi v različnih stopnjah okužbe plodov različna, zato smo pričakovali, da bo vsebnost fenolov največja ob začetnih znakih okužbe. Izkazalo se je, da se je večina derivatov elagne kisline povečala šele v okuženih plodovih, vsebnost flavanolov in antocianov pa že ob prvih znakih okužbe, medtem ko se je vsebnost nekaterih flavonolov v prvem poskusu po okužbi celo zmanjšala.

Tolerantnejše sorte vsebujejo dokazano večje količine sekundarnih metabolitov, ki imajo pomembno vlogo pri obrambi rastline pred napadi patogena. Vsebnost nekaterih fenolnih snovi v plodovih in pritlikah je bila pri tolerantnejši sorti 'Honeoye' večja že v zdravih plodovih, po okužbi pa je sintetizirala značilno več flavonolov, flavanolov in antocianov kot občutljivejša sorta 'Elsanta'. Na podlagi dobljenih rezultatov lahko sklepamo, da ima genotip velik vpliv na obrambo pred okužbo.

Prav tako je znano, da gliva *C. nymphaeae* izbere različno strategijo okužbe glede na tkivo, ki ga okužuje. V nezrelih plodovih in vegetativnih delih okužba najprej miruje in se razvije šele v zrelejšem stadiju. Glede na dobljene rezultate lahko zaključimo, da je okužba imela v vseh stopnjah zrelosti podoben vpliv na vsebnost primarnih in sekundarnih metabolitov. Pokazalo se je, da je vsebnost skupnih fenolov največja v zrelih plodovih, predvsem zaradi velikih vsebnosti katehina in antocianov.

Mnoge raziskave so potrdile, da nanos kalcija in fungicida vplivata na fenolno sestavo, v našem poskusu pa bistvenega vpliva nismo zaznali. Opazili smo le, da je nanos kalcija povečala razmerje med sladkorji in organskimi kislinami. Nanos fungicida Signum pa je povečala tržni pridelek pri sorti 'Honeoye'.

Različni režimi namakanja, katere smo vzpostavili pri večkrat rodnih sortah 'Flamenco' in 'Eva's Delight', so vplivali tako na morfološke kot fiziološke parametre žlahtnega jagodnjaka. Sorta 'Flamenco' se na namakanje na meji še dostopne vode ni odzvala s spremenjenim pridelkom, medtem, ko se je pridelek sorte 'Eva's Delight' ob istem režimu značilno zmanjšal. Plodovi obeh sort so pri namakanju na meji še dostopne vode vsebovali večje vsebnosti sladkorjev in organskih kislin, sorta 'Flamenco' pa posledično tudi večje razmerje med sladkorji in organskimi kislinami.

Ob izpostavitvi trem različnim režimom namakanja smo ugotovili, da je vsebnost skupnih fenolnih snovi večja v plodovih iz rastlin, ki so bile namakane na meji še dostopne vode. Pri obeh sortah se je pokazalo, da je vsebnost določenih fenolnih skupin večja tudi pri predpostavljenem optimalnem namakanju na zgornji meji poljske kapacitete. Predvidevamo, da je namakanje pri zgornji poljski kapaciteti pomenilo prevelike količine dodane vode in tako povečalo sintezo določenih fenolov.

## 4.2 SUMMARY

The complex interaction between plants, pathogens and technological procedures is very important and still not fully understood. Plant defense strategy is altered when exposed to certain stress conditions. Four different experiments were conducted in order to clarify the biochemical processes in infected and differently irrigated strawberry plants. Experiments were set to upgrade previous research and were focused on the content of primary and secondary metabolites before and after the infection with fungus from the *Colletotrichum* genus and under three different irrigation regimes.

*C. nymphaeae* infection caused a decrease in sucrose and an increase in fructose and glucose content in strawberry fruits. Oppositely, the content of major organic acids mostly decreased after the infection. Primary metabolism of strawberry fruits responded to the infection in a similar way in all three experiments and in all cultivars analyzed.

It is well known, that the synthesis of phenolic compounds greatly depends on the stage of infection. A higher content of identified phenolics was expected at the initial stages of *C. simmondsii* infection. The content of most ellagic acid derivatives did not increase until the fruits were fully infected, most flavanols and anthocyanins increased already at the beginning of the infection and some flavonols even decreased after the infection in our first experiment. Previous reports show that tolerant cultivars contain higher levels of phenolic compounds compared to susceptible cultivars. The content of several phenolic compounds was higher in tolerant 'Honeoye' non-infected strawberry fruits and runners. Moreover, after the infection developed, this cultivar strongly reacted in defense response with an increased phenolic synthesis. The content of flavonols, flavanols and anthocyanins was significantly higher in the tolerant ‘Honeoye’ cultivar compared to the

susceptible ‘Elsanta’ cultivar. Based on the results it can be concluded, that cultivar significantly influences the rate of plants defense against specific pathogens.

Diverse infection strategies can be employed when colonizing different tissues of a specific host. *C. nymphaeae* infection differs depending on the ripeness of the fruit and therefore, the metabolic response of strawberry fruit to infection in different ripening stages was investigated. However, in our experiments fungal infection similarly influenced primary and secondary metabolism at all stages of strawberry ripeness.

Several papers indicate that fungicide and calcium sprayings alter polyphenolic levels in plant cells, but other studies suggest that no modification occurs. And in our second experiment, we confirmed, that there was no major influence of fungicide and calcium spraying on strawberry phenolic content. Calcium sprayings altered the sugar and organic acid ratio and fungicide Signum increased strawberry yield of the tolerant 'Honeoye' cultivar.

Three different irrigation regimes had a great influence on strawberry yield and content of primary and secondary metabolites in ever-bearing ‘Flamenco’ and ‘Eva’s Delight’ cultivars. Deficit irrigation demonstrated significantly increased content of sugars in both analyzed cultivars, and their ratio at cv. ‘Flamenco’. The level of all identified anthocyanins, flavanols, flavonols, flavones, flavanone, ellagic acid derivatives and hydroxycinnamic acid derivatives also increased under deficit irrigation. Deficit irrigation had an adverse impact on cv. ‘Eva’s Delight’ yield, but on contrary, no significant influence on the yield of cv. ‘Flamenco’.

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## ZAHVALA

Največjo zahvalo bi na tem mestu namenila svojemu mentorju, prof. dr. Franciju ŠTAMPARJU, za vse nasvete, priporočila, prijazne besede in razumevanje na vseh področjih. Hvala vam za zaupanje, in da ste verjeli vame tudi takrat, ko mi sami to ni najbolje uspevalo.

Posebna zahvala gre prof. dr. Robertu VEBERIČU za vse nasvete in veliko pomoč že med raziskovanjem, pisanjem člankov ter za vse kritike in opombe pri pregledovanju mojega zaključnega dela.

Prav tako gre zahvala članicama komisije za oceno in zagovor, doc. dr. Andreji URBANEK in doc. dr. Heleni ŠIRCELJ za zavzeto branje in vse koristne pripombe, ki so doktorsko disertacijo izpopolnile.

Velika zahvala gre tudi mojim staršem, brez vas danes ne bi bila tukaj, kjer sem, hvala za vse življenjske in strokovne izkušnje. Hvala tudi Borisu za podporo in pomoč.

Svoje delo posvečam svoji hčerki Mii, brez tebe bi bilo vse v življenju zaman, rada te imam.

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PRILOGA B

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**Title:** Alteration of the Content of Primary and Secondary Metabolites in Strawberry Fruit by Colletotrichum nymphaeae Infection

**Author:** Maja Mikulic-Petkovsek, Valentina Schmitzer, Ana Slatnar, et al

**Publication:** Journal of Agricultural and Food Chemistry

**Publisher:** American Chemical Society

**Date:** Jun 1, 2013



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