Plant Physiology and Biochemistry 135 (2019) 174–181

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

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

Research article

Polyphenolic profiling of roots (*Vitis* spp.) under grape phylloxera (*D. vitifoliae* Fitch) attack

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ARTICLE INFO

Keywords:
Constitutive defence
Gall physiology
Host-parasite interaction
Plant resistance
Polyphenol biosynthesis
Root physiology
Vitis

ABSTRACT

Many plants respond to herbivore attacks by the formation of secondary metabolites, such as polyphenols. Grape phylloxera *(Daktulosphaira vitifoliae* FITCH*)* induces organoid root galls on fibrous root tips of tolerant *Vitis* spp. rootstocks. We aim to understand if and how secondary metabolites are involved in the compatible interaction of *D. vitifoliae* and tolerant *Vitis* ssp. rootstocks belowground. We hypothise that *D. vitifoliae* infestation triggers the accumulation of phenolic key compounds in root gall tissue without preventing the compatible host-parasite interaction on two tolerant rootstocks with different genetic background: Teleki 5C (*V. berlandieri x V. riparia*) and Fercal (B.C. n°1B x 31 Richter). Plants and insects are grown in isolated climate chambers to sample root tips of non-infested plants (control) as well as root tips and galls of *D. vitifoliae* infested plants (5–14 dai). HPLC-MS-based analyses of phenolic key compounds are compared with gene expression levels of the biosynthetic phenylpropanoid pathway analysing temporal sequences of *D. vitifoliae* infested root tissue. The results show that the induction of the phenylpropanoid pathway by *D. vitifoliae* infestation plays an important role in the plant response. Concentrations of phenolic key compounds vary significantly among the rootstocks tested. Both rootstocks display an accumulation of flavan-3-ols and stilbenes in infested root gall tissue. Comparing the host responses of the two rootstocks Fercal shows a stronger accumulation of stilbenes locally in infested root galls, whereas Teleki 5C indicates elevated amounts of stilbenes in non-infested root tip tissue.

1. Introduction

Plants respond to herbivore feeding through morphological and molecular mechanisms to encounter/offset the effects of herbivory attacks. The more intimate the insect-plant interaction, the higher the level of co-evolution to avoid each other's defence system (War et al., 2012). Plant defence mechanisms can be classified into either constitutive and/or induced after herbivore attack (Agrawal, 2011) including structural barriers, toxic chemicals and attraction of natural enemies. Because of high metabolic costs most defence mechanisms are induced and enhance plasticity in the host plant's phenotype upon biotic stress in order to limit herbivore's chances for adaptation (War et al., 2012).

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch) is a monophagous and sedentary gall forming phylloxerid that establishes an intimate association with the roots of *Vitis* spp. (Powell et al., 2013; Forneck and Huber, 2009; Granett et al., 2001). The insect forms organoid galls on fibrous root tips (nodosities) and callus tissue on mature roots (tuberosities). Nodosities serve as the insect's nutritional basis and represent the exclusive feeding site required for growth and development (Griesser et al., 2015a; Du et al., 2008; Kellow et al., 2004). The compatibility of the *D. vitifoliae-Vitis* spp. interaction depends in first place on the combination of the phylloxera biotype, the host plant genotype and the feeding tissue leading to different resistance degrees (Forneck

Abbreviations: dai, days after infestation; ESI, electrospray ionization; HAMP, herbivory-associated molecular patterns; HPLC, high-performance liquid chromatography; L1-4, larval stages of *D. vitifoliae*; A, adult life stage of *D. vitifoliae*; MS, mass-spectrometry; MeOH, methanol; NIRT, non-infested root tips of infested plants; qRT-PCR, quantitative real-time PCR; SRM, selected reaction monitoring; PP, photoperiod

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https://doi.org/10.1016/j.plaphy.2018.12.004
Received 16 October 2018; Received in revised form 3 December 2018; Accepted 3 December 2018
Available online 04 December 2018
0981-9428/

et al., 2016; Rispe et al., 2016).

Secondary metabolites have been postulated as host plant defensive and/or parasite inhibiting molecules. Grapevine (*V. vinifera* L.) is known for its high phenolic content and tight spatio-temporal regulation patterns of secondary metabolites derived from the phenylpropanoid biosynthetic pathway (Czemmel et al., 2012). In general, induction of phenolic metabolites along the phenylpropanoid pathway is shown to be a major part of grapevine's defence response to encounter biotic stress conditions (Treutter, 2006; Nabity et al., 2013). A large body of knowledge is available for secondary metabolites in grapevine (*V. vinifera* L.) due to their gustatory and health beneficial properties for the resulting wine, fresh fruit and seeds (Ali et al., 2010; Mikes et al., 2008). Comparably fewer studies focus on polyphenols as plant defensive agents and their effects against abiotic and biotic stresses in fruit, leaf and stem of the vines (e.g. Griesser et al., 2015b). Secondary metabolites are shown to be effective defensive compounds against root-feeding herbivores involved in the plant defence, among them *D. vitifoliae* (Powell et al., 2013). Both constitutively and induced polyphenols play an important role for insect-plant interactions: induced upon parasite attack and constitutively acumulated e.g. as structures to protect plant tissue prior infestation. Studies on the role of phenolic compounds in the *D. vitifoliae* - *Vitis* root interaction are scarce. Early studies show periderm deformation and inclusion of phenols in tolerant host genotypes (e.g. Niklowitz, 1954). Galled tissue, reared under aseptic conditions analysed by histochemical techniques, shows significant changes in the phenolic content and activity of detoxifying enzymes (peroxidase, leucine aminopeptidase, acidic phosphatase) in different root gall compartments (Forneck et al., 2002). A study on the volatile metabolome of roots parasitized by grape phylloxera shows an increase of secondary metabolites (Lawo et al., 2011) without resisting or/and repelling the insect.

Here we report a comparison of the plant response in roots upon *D. vitifoliae* infestation over the course of root gall development from 5 to 14 dai of two *Vitis* spp. rootstocks. In contrast to previous experiments we employed a single *D. vitifoliae* genotype on two *Vitis* rootstocks with different genetic background to avoid potential effects derived from insect populations. Teleki 5C (*V. berlandieri* x *V. riparia*) represents a classical rootstock hybrid between two American *Vitis* spp.. Fercal (B.C. n° 1B x Richter 31) is a rootstock hybrid of American *Vitis* spp. and *V. vinifera* L.. Both rootstocks allow *D. vitifoliae* feeding and reproduction on root tips without causing severe damage or yield decline under field conditions. The experiments are performed to monitor phenolic key metabolites involved in the *Vitis*spp. – *D. vitifoliae* interaction belowground by HPLC-MS and gene expression analyses (Gene Chip and qRT-PCR). The present study investigates the secondary metabolome of insect-induced root galls. Thereby it enhances our general knowledge regarding plant defensive mechanisms against specialized root-feeding insects.

2. Material and methods

2.1. Grape phylloxera

Eggs of a root-galling grape phylloxera (*D. vitifoliae* Fitch) single founder lineage (biotype C) are taken from an established asexually reproducing stock population feeding on Teleki 5C roots (Eitle and Forneck, 2017). The genetic homogeneity of the grape phylloxera strain is confirmed by genetic analyses (Forneck et al., 2017).

2.2. D. vitifoliae – Vitis spp. Interaction

One-node dormant cuttings from the rootstocks Teleki 5C (V. *berlandieri* x *V. riparia*) and Fercal (B.C. n°1B (*V. berlandieri* x *V. vinifera*) x 31Richter (*V. berlandieri* x (*V. riparia* x *V. rupestris* x *V. candicans*)) are dipped in solution of 0.1% indole-3-butyric acid and 0.6% naphtylacetic acid to promote rooting and placed into "Jiffy-7" pots (40 mm; Jiffy Products International AS, Norway). After six weeks, four growth containers are installed with each 12 rooted plants. Each container (38 × 28 × 20 cm) is filled with 17 L of a 1:1 perlite:seramis substrate. Three containers are inoculated with each 200 phylloxera eggs located in an isolated climate chamber (24 ± 2 °C, 45–55 %rH and 16 h PP) from August to October 2009. The plants in the control container are not infested. Sampling is conducted 60 days after infestation (dai) during root gall formation of the second phylloxera generation. Three independent samples, each consisting of 15 pooled root tips/galls, are collected and categorized according to the developmental larval and adult life stages of the attached insect correlated with the gall infestation period: L2 (5–7 dai), L3 (8–14 dai) and L4 + A (> 14 dai). 'A' corresponds to the adult phylloxera life stage (Forneck and Huber, 2009; Kocsis et al., 1999). Non-infested root tips (NIRTs) without an attached grape phylloxera nor visible piercing spots (wounding) are collected from infested plants. Root tips of non-infested plants are considered control tips. The insect and soil particles are carefully removed from the gall/tip and immediately frozen in liquid nitrogen and stored at −80 °C for further analyses.

2.3. Phenol quantification

Frozen plant tissue is homogenized with a ball mill (Retsch MM400, Haan, Germany). HPLC-MS quantification of phenols is done as previously described in (Schoedl et al., 2011). In brief: 500 mg of pulverized root samples are extracted using 5 ml of 0.02% hydrochloric acid (v/v) in aqueous 80% MeOH (v/v) during ultrasonication, followed by centrifugation for 5 min at 3750 rpm (GS-6 Centrifuge, Beckman Coulter, Inc., Brea, USA). The supernatant is collected and two dilutions are injected into the analytical instrument consisting of a QTrap 4000 LC-MS/MS system (Applied Biosystems, Foster City, USA) equipped with a TurboIonSpray electrospray ionization (ESI) source and a 1100 series HPLC system (Agilent, Waldbronn, Germany). Chromatographic separation is performed at 40 °C on a Gemini RP-18 column, 100 × 2 mm inner diameter, 3 µm particle size (Phenomenex, Torrance, USA) protected with a guard column (Phenomenex, Torrance, USA) packed with the same material. The mobile phase consists of (A) 0.5% formic acid in H_2O and (B) 0.5% formic acid in MeOH and the flow rate is set at 0.4 mL min^{-1}. Detection is carried out by ESI-MS/MS in selected reaction monitoring (SRM) mode in negative polarity and for quantification of phenols, external calibration using standards in pure solvent is applied (Appendix A.1). Peaks are assigned by comparing their retention times and mass spectra with those of respective reference compounds.

2.4. Gene expression analysis

Total RNA is isolated from root tips and gall samples (Reid et al., 2006 modified). RNA integrity and purity are confirmed using Nanodrop ND-1000 spectrophotometer (NanoDrop, Wilmington, Germany) and gel electrophoreses (1.5% agarose, 1xTBE DEPC Buffer, 100 V, 30min). Reverse transcription is done with a QuantiTec Reverse Transcription Kit (Qiagen, Hilden, Germany) using 1000 ng of initial RNA. Quantitative RT-PCR measurements are performed using a Rotor-Gene cycler (Qiagen, Hilden, Germany) employing KAPA SYBR FAST qPCR Universal fluorescent dye (Peqlab, Erlangen, Germany) and two reference genes actin (VIT_04s0044g00580) and ubiquitin (VIT_16s0098g01190) for normalization (Lawo et al., 2013). All PCR reactions are performed in technical duplicates. Primer efficiencies are determined prior to analysis by establishing standard curves with four step template dilutions. A list of the analysed *Vitis* genes and employed primer sequences is attached (Appendix A.2). Cycling conditions are set to: one cycle for 4 min at 95 °C, 35 cycles for 5 s at 95 °C, 20 s at 60 °C, 5 s at 72 °C and 10 s at 75 °C, when the fluorescence signal is measured. Quantitative gene expression levels are determined based on $\log_2$ fold changes.

2.5. Microarray chip design

An Agilent SurePrint Custom GE 4x44 microarray (Agilent #G2514F-031062) is designed using a comprehensive set of functionally annotated cDNAs derived from ESTs and other sources of expressed mRNAs as available through the latest release of the TIGR Gene Index hosted at the Dana Farber Cancer Institute (VVGI v7) as presented previously (Griesser et al., 2013).

2.6. Data analysis

Statistical data analysis is done using SPSS v21 (IBM). In this study constitutive (preformed) phenols refer to phenolic compounds detected at concentrations above their limit of quantification in control root tips of non-infested plants (Appendix A.3). Sequential changes in phenolic compound concentrations (HPLC-MS) are assessed by comparing control root tips and sequential root gall stages (L2/3/4-A) with each other with Tukey Post Hoc tests with $p < 0.05$. Alterations of gene expression levels (qRT-PCR) are tested with Mann-Whitney U ($p < 0.05$) comparing the $\log_2$FC values of each treatment with the one of the non-infested control tips (Appendix A.4). Systemic effects refer to changes comparing control root tips from non-infested plants with NIRTs (non-infested root tips from infested plants).

3. Results and discussion

Biosynthesis of polyphenols in plants occurs through the shikimic and phenylpropanoid pathways. Phenols are categorized in subgroups depending on their position along the phenylpropanoid pathway sharing similar chemical and functional properties: stilbenes, dihydroflavones, flavonols, flavan-3-ols and anthocyanins (Fig. 1).

3.1. Constitutive phenols in non-infested Vitis spp. roots

Constitutive (preformed) phenolic compounds are biosynthesized and accumulated during growth and development of plant tissue not exposed to biotic nor abiotic stress (Treutter, 2006). According to this definition we consider constitutive phenols as plant physiological compounds accumulated in root tip tissue of untreated (non-infested) plants cultivated under optimized greenhouse conditions and measured above their respective quantification limit. In this study six constitutive phenols with known antimicrobial properties: naringenin, catechin, epicatechin, epicatechin gallate, *trans*-resveratrol glucoside and *cis*-resveratrol glucoside are identified in non-infested root tip tissue (Appendix A.3). Naringenin, reported to be the first stable polyphenol of the phenylpropanoid pathway, is usually detected in low concentrations compared to other polyphenolic compounds (Eftekhari et al., 2017). In line with these findings we detect naringenin above its quantification limit in root tips of both rootstocks demonstrating a constitutively active phenylpropanoid pathway (Fig. 1).

Generally constitutive secondary metabolites are stored in strategically important plant organs such as roots (Németh et al., 2017) and might play roles in signalling or direct plant defences of the specific plant tissue with respect to their specific biological activity (Lattanzio et al., 2006). Furthermore preventive storage of phenolic compounds is considered an efficient response strategy against biotic stresses e.g. acting as feeding deterrents in plant tissue (Constabel et al., 2014).

3.2. Sequential phenol accumulation under D. vitifoliae infestation

In order to assess sequential changes of phenols following root gall formation, a controlled sampling of gall stages as determined by the attached larval and adult life stages of feeding *D. vitifoliae* (Forneck and Huber, 2009) is conducted. Root tips of non-infested plants, sampled at similar ages are considered as controls. In this study nine phenolic metabolites are detected to accumulate in *D. vitifoliae* root galls. Their concentrations increase gradually from the early feeding stages at 5 dai (L2) until the completion of a mature gall at 14 dai (L4 + A). The detected phenols belong to four mayor metabolic groups presented in Fig. 1: Key phenolic compounds of the upper phenylpropanoid pathway (Fig. 2A/B), dihydroflavones (Fig. 2A/B), flavan-3-ols (Fig. 3A/B) and stilbenes (Fig. 4A/B); all of which are described previously with physiological active functions as biotic protecting against insect feeding. The analysed flavonols do not reach detection limits (Appendix A.3). Transcriptional inductions of polyphenol biosynthetic genes are determined by Gene Chip (Griesser et al., 2013) and confirmed by qRT-PCR measurements (Appendix A.4). All 12 analysed (iso-) genes coding for enzymes along of the phenylpropanoid pathway are induced following the infestation time demonstrating that the flavonoid accumulation is due to an induced biosynthesis within the root gall tissue (Fig. 1).

In principal sequential accumulation of secondary metabolites can be achieved by *de-novo* biosynthesis, translocation or induction of constitutive biosynthetic pathways in the respective host tissue. The presented data provides evidence that *D. vitifoliae* feeding promotes and regulates the constitutively active phenylpropanoid pathway in non-stressed plants rather than triggering the *de novo* biosynthesis of the secondary metabolite groups analysed in this study (Fig. 1). This finding is in line with other plant pathogens and herbivores, among them leaf-galling *D. vitifoliae*, shown to trigger the induction of the phenylpropanoid biosynthetic pathway (e.g. Nabity et al., 2013) resulting in elevated concentrations of phenols depending on various factors such as plant and parasite genotypes, host plant tissue and the mode of the host-parasite interaction. *D. vitifoliae* feeds on the parenchymatic cell sap of root galls formed on *Vitis* spp.. Mature *D. vitifoliae* root galls were previously shown to have manipulated host physiological traits (e.g. Griesser et al., 2015a; Forneck et al., 2002; Kellow et al., 2004; Du et al., 2008). This study adds important knowledge about the manipulated host physiological pathways of the *D. vitifoliae-Vitis* spp. interaction.

3.2.1. Upper phenylpropanoid pathway and dihydroflavones

Our results show that *D. vitifoliae* feeding triggers a gradual induction of the phenylpropanoid pathway demonstrated by successively increasing concentrations of selected phenolic key compounds along infestation time: *p*-coumaric acid reaching concentrations up to 0.6 μg/gFW in L4 + A root galls of Teleki 5C and 0.3 μg/gFW in Fercal; naringenin 0.41 μg/gFW in L4 + A root galls of Teleki 5C and 0.21 μg/gFW in Fercal; and eriodictyol 0.7 μg/gFW in L4 + A root galls of Teleki 5C and 0.4 μg/gFW in Fercal (Fig. 2A/B). Significantly induced expression levels of genes coding for biosynthetic key enzymes of the upper phenylpropanoid pathway (Fig. 1) demonstrate that elevated phenol levels are explained by a general induced biosynthetic rate of this pathway in *D. vitifoliae* affected root tissue. Among the induced genes in the upper part of the phenylpropanoid pathway (Fig. 1) chalcone isomerase (*VviCHI,* VIT_13s0067g03820) shows the highest induction compared to the other monitored gene transcripts with 5.1 $\log_2$ FC in L4 + A root galls of Teleki 5C and 4.7 $\log_2$ FC in Fercal, respectively.

Likewise other insects feeding on *Vitis* spp. (e.g. Cai et al., 2012), we show that *D. vitifoliae* induces the phenylpropanoid pathway and the subsequent accumulation of phenols in the root gall tissue. Accumulated phenols are considered plant defensive compounds protecting host tissues against biotic parasites or associated wound stress damage (Lattanzio et al., 2006, Treutter, 2006). In this study we show that the phenol content in root gall tissue increases significantly with infestation time in both rootstocks tested. However *D. vitifoliae* survival and reproduction are apparently not affected during the compatible interaction. Other factors such as the subcelullar localisation of accumulated phenols might modify anti-herbivore functions and/or activities against invading parasites. Gall tissue-specific, subcellular accumulation of polyphenols was previously shown in cross sections of *D. vitifoliae* root galls (Niklowitz, 1954). In this context further studies are needed to

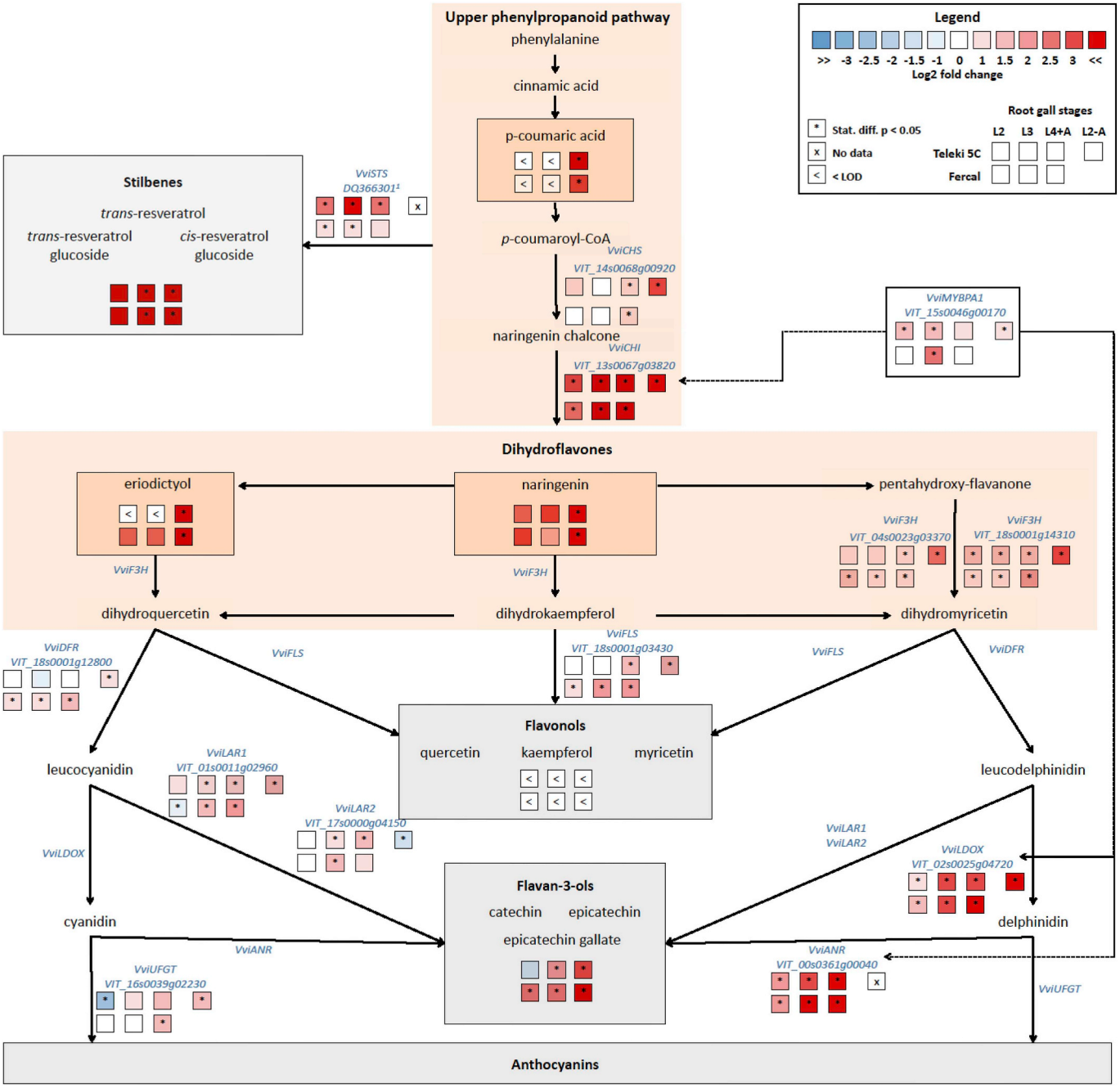

Fig. 1. Simplified phenylpropanoid pathway in *Vitis* spp. root galls modulated by *D. vitifoliae* infestation. Phenolic compounds within boxes and gene expression patterns of *Vvi*-genes marked in blue italics are analysed. Coloured squares indicate alterations of polyphenol concentrations in μg/gFW (HPLC-MS) or gene expression levels in log2 FC (qRT-PCR and Gene Chip). Sequential gall developmental stages categorized by the larval and adult life stages of the attached insect: L2 (5–7 dai); L3 (8–14 dai) and L4 + A (> 14 dai) (Forneck and Huber, 2009) are compared to non-infested control root tips. Gene Chip expression levels (Griesser et al., 2013) are obtained by comparison of control root tips with pooled (L2-A) root galls of Teleki 5C. The symbol " < " indicates compound concentrations below detection limit. Asterisks indicate significant differences obtained by Mann-Whitney U (gene expression) and Tukey Post Hoc (compound quantification) with $p < 0.05$ analysing three independent biological replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

investigate if polyphenol deposition within specific tissues (e.g. cell layers) or cell compartments (e.g. cell wall, cytosol, vacuole) affect the compatible *D. vitifoliae* - *Vitis* spp. interaction in root galls. Apart of their negative impact for insect herbivores phenols are considered to be phytopathogenic compounds (Treutter, 2006). Depositions of anti-microbial phenols in root galls, as shown in this study, might protect the root gall tissue against secondary soil-borne plant pathogen, as they prevent their entry and within plant movement. Thus we confirm and support earlier metabolomic studies showing that *D. vitifoliae* infestation triggers phenol accumulation in resistant and tolerant *Vitis* leaf tissues (Raman et al., 2009).

3.2.2. Flavan-3-ols (catechins)

The concentration of flavan-3-ols increases significantly with root gall development at the L2 (5–7 dai), L3 (8–14 dai) and L4 + A (> 14 dai) stages for both rootstocks (Fig. 3A/B). Catechin is detected to be the predominant metabolite among all analysed phenols, reaching levels up to 139 μg/gFW in Teleki 5C and 321 μg/gFW in Fercal L4 + A

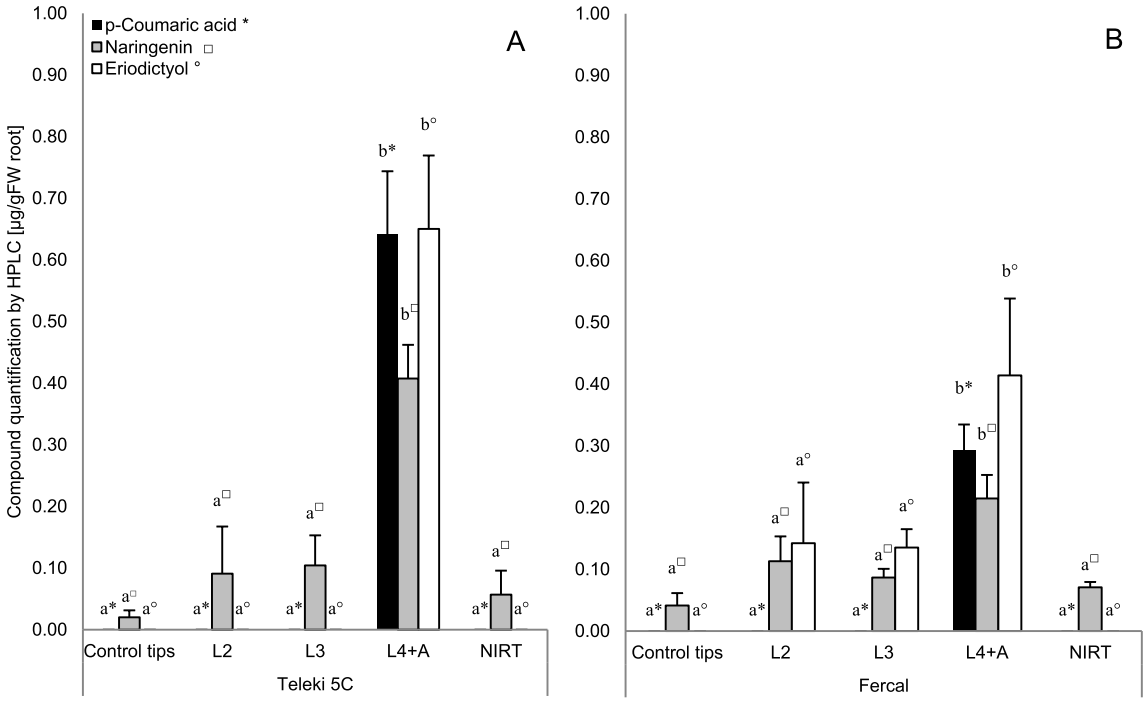

Fig. 2. *p*-coumaric acid and dihydroflavones in *D. vitifoliae* infested root galls. Concentrations of phenols from the upper phenylpropanoid biosynthetic pathway (*p*-coumaric acid, naringenin and eriodictyol) in µg/gFW detected by HPLC-MS measurements in *D. vitifoliae* root galls of the two rootstocks Teleki 5C (A) and Fercal (B). Control tips represent root tips of non-infested plants. Root gall stages are categorized by the larval and adult life stages of the attached insect: L2 (5–7 dai), L3 (8–14) dai and L4 + A (> 14 dai) (Forneck and Huber, 2009). Non-infested root tips of infested plants are named NIRTs. Error bars indicate standard deviations of three independent biological replicates each consisting of pooled 15 root tips or galls. Letters indicate significant differences of the respective metabolite between plant tissues obtained by Tukey Post Hoc testing with $p < 0.05$.

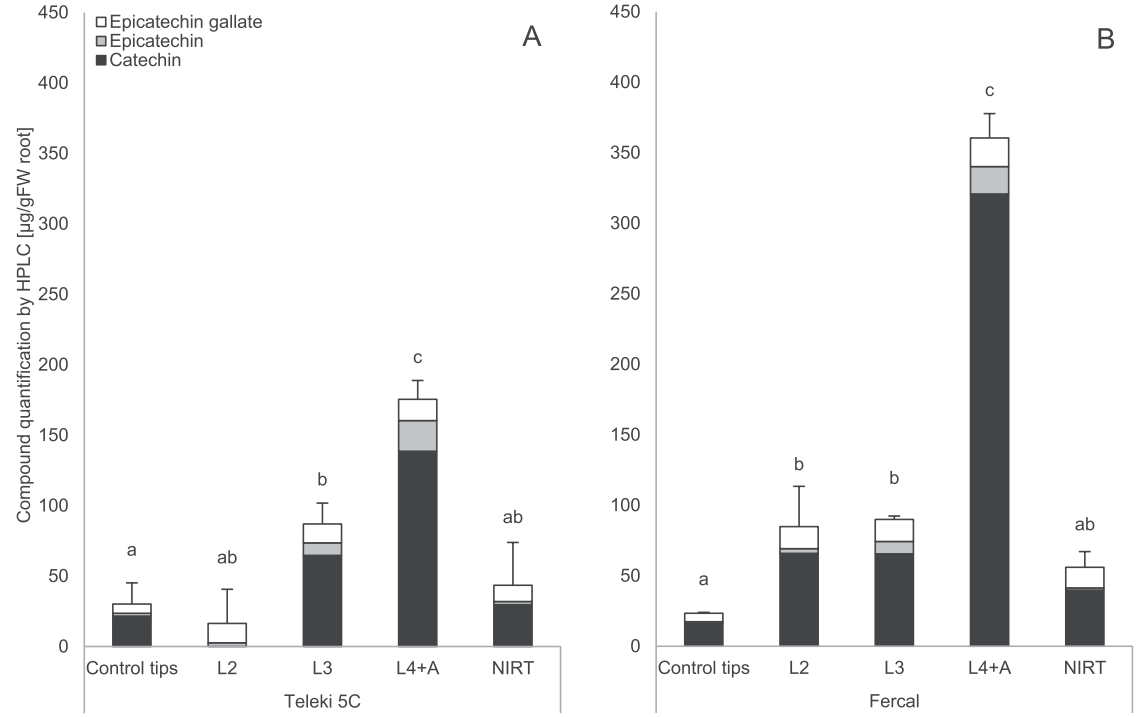

Fig. 3. Flavan-3-ol accumulation in *D. vitifoliae* infested root galls. Concentrations of flavan-3-ols (catechin, epicatechin and epicatechin gallate) in µg/gFW detected by HPLC-MS measurements in *D. vitifoliae* root galls of the two rootstocks Teleki 5C (A) and Fercal (B). Control tips represent root tips of non-infested plants. Root gall stages are categorized by the larval and adult life stages of the attached insect: L2 (5–7 dai), L3 (8–14 dai) and L4 + A (> 14 dai) (Forneck and Huber, 2009). Non-infested root tips of infested plants are named NIRTs. Error bars indicate standard deviations of three independent - biological replicates each consisting of 15 pooled root tips or galls. Minor letters indicate significant differences of the respective metabolite between plant tissues obtained by Tukey Post Hoc testing with $p < 0.05$.

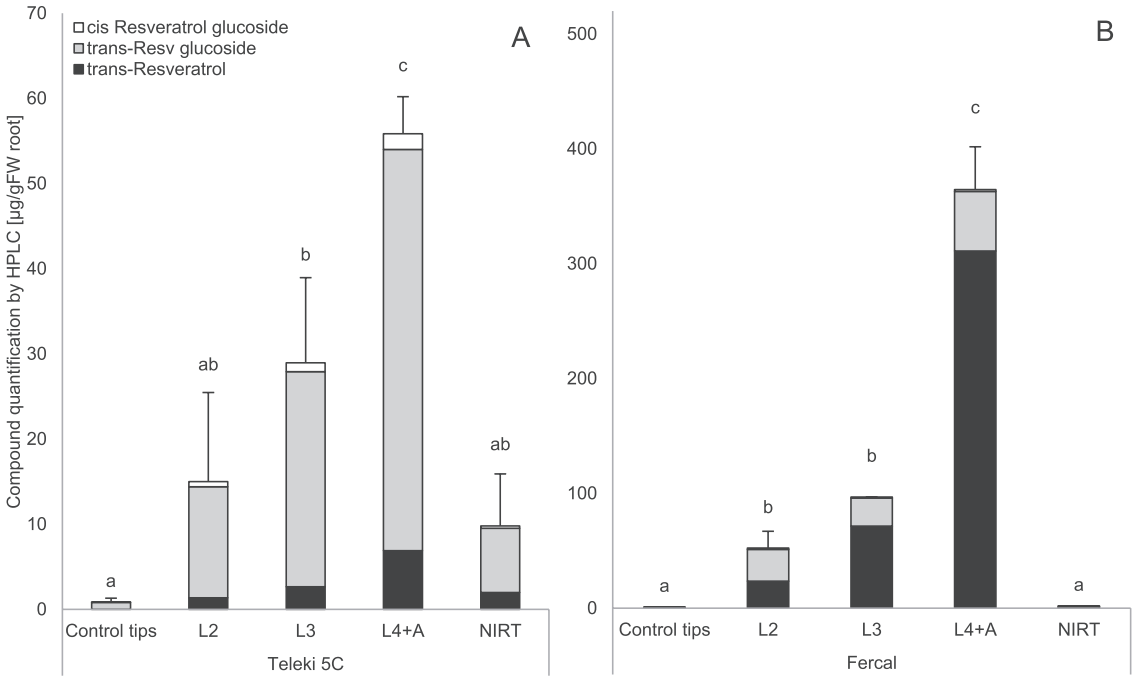

Fig. 4. Stilbene accumulation in *D. vitifoliae* infested root galls. Concentrations of stilbenes (*trans*-resveratrol, *trans*-resveratrol glucoside and *cis*-resveratrol glucoside) in µg/gFW detected by HPLC-MS measurements in *D. vitifoliae* root galls of the two rootstocks Teleki 5C (A) and Fercal (B). Control tips represent root tips of non-infested plants. Root gall stages are categorized by the larval and adult life stages of the attached insect: L2 (5–7 dai), L3 (8–14 dai) and L4 + A (> 14 dai) (Forneck and Huber, 2009). Non-infested root tips of infested plants are named NIRTs. Error bars indicate standard deviations of three independent biological replicates each consisting of 15 pooled root tips/galls. Letters indicate significant differences of the respective metabolite between plant tissues obtained by Tukey Post Hoc testing with $p < 0.05$.

root gall tissue. Levels of epicatechin reach 22 µg/gFW in Teleki 5C and 19 µg/gFW in Fercal L4 + A root gall tissue. Epicatechin gallate is detected up to 15 µg/gFW in Teleki 5C and 20 µg/gFW in Fercal L4 + A root galls (> 14 dai) (Fig. 3A/B). Although the flavan-3-ols, measured in the different types of root gall tissue, show a similar accumulation behaviour among the two tested rootstock genotypes. Fercal displays a stronger host response by accumulating two and a half times more catechin compared to Teleki 5C at the latest sampling stage (L4 + A). Gradually induced expression patterns of the genes coding for flavan-3-ol biosynthetic enzymes confirm that *D. vitifoliae* infestation triggers the local formation of polyphenols within affected host tissue (Fig. 1). The expression levels in L4 + A gall stages (> 14 dai) of the following genes are significantly induced compared to control root tips: leucoanthocyanidin reductase 1 (*VviLAR1,* VIT_01s0011g02960) 1.7/1.6; leucoanthocyanidin reductase 2 (*VviLAR2,* VIT_17s0000g0415) 1.0/0.4; leucoanthocyanidin dioxygenase (*VviLDOX*, VIT_02s0025g04720) 2.5/4.0 and anthocyanidin reductase (*VviANR*, VIT_00s0361g00040) 3.5/4.3 [$\log_2$ FC] in L4 + A root galls of Teleki 5C and Fercal respectively (Fig. 1, Appendix A.4). Induced gene expression patterns are confirmed by the Gene Chip data of pooled (L2-A) Teleki 5C galls (Fig. 1, Appendix A.4).

Spatio-temporal modifications of secondary metabolites in plant tissue, particularly grapevine *V. vinifera* L., are known to be fine-tuned by MYB transcription factors regulating transcriptional expression patterns of key genes along the phenylpropanoid pathway (e.g. Höll et al., 2013). Here we detect induced gene expression patterns of the transcription factor *VviMYBPA1* up to 1.0 $\log_2$ FC and 1.9 $\log_2$ FC in L3 root galls of Teleki 5C and Fercal (Fig. 1). This transcription factor was previously shown to trigger gene expression levels of *VviCHI*, *VviLDOX* and *VviANR* resulting in the biosynthesis and accumulation of flavan-3-ols in *V. vinifera* L. (Czemmel et al., 2012) suggesting an involvement of the *MYBPA*1 in the *Vitis* spp. - *D. vitifoliae* interaction belowground.

Flavan-3-ols as well as their polymeric forms, the proanthocyanidins, are known to be synthesized as response to biotic stress possessing effective antimicrobial and anti-insecticidal properties (Reichling, 2018; Constabel et al., 2014). Their impact on insect herbivore fitness is complex and affected by several factors, e.g. host-parasite interaction on genotype and developmental level, phenolic compound(s), dosage and exposure time (Ayres et al., 1997). Flavan-3-ols are shown to act as feeding deterrents in host plants due to their astringent flavours and protein complexation properties, resulting in reduced feed digestibility as well as enzymatic inactivation (Lattanzio et al., 2006). However specific insect species evolved mechanisms to encounter high levels of flavan-3-ols e.g. by modifying parts of their internal digestive system (Bernays and Chamberlain, 1980). To date little is known about the digestive features of the root parenchymal sap of *Vitis* spp. as the primary food source of *D. vitifoliae*. Forneck and Wöhrle (2001) developed an artificial diet system for *D. vitifoliae* showing that insect probing and surivival was highest when *D. vitifoliae* larva of different stages fed from a 4.5. pH sucrose solution (5%) with an added amino acid mixture. So far an increasing activity of polyphenol oxidase was observed along with root gall development (Qing et al., 2011) as a potential attempt of feeding *D. vitifoliae* larvae to neutralize polyphenols after their biosynthesis and accumulation in root gall tissue.

3.2.3. Stilbenes

The stilbene content in *D. vitifoliae* root galls is significantly increased along with infestation time. Total stilbene levels increase from comparably low concentrations in control root tips of non-infested plants: 0.9 µg/gFW Teleki 5C and Fercal; to significant higher levels in

later root gall stages: 56 μg/gFW in Teleki 5C and 365 μg/gFW in Fercal. The highest stilbene levels are detected in L4 + A root gall stages (> 14 dai): *trans*-resveratrol 7 μg/gFW in Teleki 5C and 311 μg/gFW in Fercal; *trans*-resveratrol glucoside 47 μg/gFW in Teleki 5C and 52 μg/gFW in Fercal; *cis*-resveratrol glucoside 1.8 μg/gFW in Teleki 5C and 1.7 μg/gFW in Fercal (Fig. 4A/B). Stilbene synthase, genetically encoded by *VviSTS*, is the central biosynthetic enzyme transforming *p*-coumaryl-CoA and malonyl-CoA from the core phenylpropanoid pathway to *cis*- and *trans*-isomeres of stilbenes and their respective glucosides (Chong et al., 2009). The annotated genome of *V. vinifera* L. contains 57 genes annotated as stilbene synthases (Grimplet et al., 2012). To meet this condition a qRT-PCR primer pair is employed to cover multiple stilbene synthases *VviSTS* (Le Henanff et al., 2011). Expression patterns of stilbene synthases (*VviSTS*) are detected to be significantly induced up to 3.3 $\log_2$ FC L2/3 root galls of Teleki 5C and 0.5 $\log_2$FC in Fercal confirming the accumulation of stilbenes due to local biosynthesis in the root gall tissue (Fig. 1). The high accumulation of stilbenes in L4 + A root galls in Fercal does not necessarily correlate with the rather moderate induction of *VviSTS* detected in this study. Due to the high number of genes annotated as stilbene synthases, we suggest the involvement of additional isogenes that are not covered by the employed primer pair in Fercal. However other reasons cannot be excluded.

Stilbenes belong to the phytoalexins defined as plant defensive compounds readily synthesized upon biotic or abiotic stress. Stilbenes are well studied compounds known for their antimicrobial activity in host plant tissue. Absolute concentrations of stilbenes in plant tissues depend on various factors (genotype, plant tissue, cultivation system and period, treatment, etc.). So far few studies detect stilbenes in non-stressed plants as part of the constitutive host defence (e.g. Chong et al., 2009). References document pathogen infections resulting in an increased biosynthesis of stilbenes in *V. vinifera* L. tissues (e.g. Bavaresco et al., 1997). At first it seems to be not surprising that stilbenes, as plant defensive compounds, accumulate in root tissue attacked *by D. vitifoliae*. However our results show that these remarkably high levels of stilbenes do not prevent *D. vitifoliae* feeding nor gall formation. Similar to the detected flavan-3-ols, further research is needed to investigate how *D. vitifoliae* can cope with these multi-fold increased amounts of polyphenols and if there might be a beneficial (protective) effect on the insect's feeding tissue.

3.3. Host response differences

Both rootstocks employed in this study are commonly propagated in commercial vineyards. Teleki 5C represents an example for an American rootstock hybrid between *V. berlandieri* x *V. riparia*. Fercal (B.C. n° 1B x Richter 31) is a rootstock hybrid of American *Vitis* spp. and *V. vinifera* L. Assessing their tolerance against *D. vitifoliae* is rather complex, because *D. vitifoliae*-rootstock interactions in the field depend on genotype levels and various ecophysiological factors (Powell et al., 2013). Both rootstocks support grape phylloxera gall formation without showing severe canopy damage symptoms or yield decline of the grafted scion cultivar. Although the tolerance levels of the two tested rootstock genotypes against *D. vitifoliae* are not quantitatively differentiated in this study, our data indicates two distinct host response reactions activated upon *D. vitifoliae* infestation based on polyphenol concentration, composition and distribution.

The presented data provides evidence for a stronger host response of Fercal reflected by higher levels of antimicrobial effective catechin 321 μg/gFW (Fig. 3) and *trans*-resveratrol 311 μg/gFW (Fig. 4) in L4 + A root galls. Although insect species are reported to be sensitive against increasing polyphenol levels (Lattanzio et al., 2006; Ayres et al., 1997), our results show that the employed *D. vitifoliae* strain (biotype C) is able to form suitable host tissue and complete its life cycle despite of increasing polyphenol levels in root gall tissue of Teleki 5C. This raises the question whether flavan-3-ol and stilbene accumulations in root galls, do not prevent the compatible *Vitis* spp. - *D. vitifoliae* interaction, but controversially contribute to establish and maintain *D. vitifoliae* root gall tissue in the soil. The mechanisms of stilbene toxicity towards fungal cells are not completely understood, but assumed to interfere with conidial respiration and the difficulties in the penetration of host lipophilic membranes (Pezet and Pont, 1995). Root inoculation experiments with the necrotrophic soil fungi *Fusarium solani* and *Phytium ultimum* confirm their negative effect on the *Vitis* spp. – *D. vitifoliae* interaction (Omer et al., 1995) and underline exemplarily the need for *D. vitifoliae* to protect the host plant's root system against secondary infections.

The results for the rootstock Teleki 5C indicate an elevated defensive status of the root system reflected by *trans*-resveratrol accumulation in both root galls and NIRTs (Fig. 4). Total stilbene levels are increased in NIRT tissue of Teleki 5C 10 μg/gFW compared to 1.6 μg/gFW in Fercal. Furthermore the ratio between *trans*-resveratrol glucoside and *trans*-resveratrol is inversely increased in all analysed Teleki 5C gall stages compared to Fercal (Fig. 4). Glycosylated stilbenes are reported to function as plant physiological storage or transport molecules (Chong et al., 2009). Based on this findings, stilbenes might be transported from *D. vitifoliae* infested root galls towards non-infested root tips in Teleki 5C. Alternatively other physiological messenger molecules may induce plant defence mechanisms in organs distal to the attacked tissue. Their systemic translocation is reported to occur within plant structures (e.g. plasmodesmata, vascular system) and/or as airborne volatiles, with MeSA being the most prominent volatile signal (Kumar, 2014). Indeed MeSA is detected in the volatile metabolome of Teleki 5C galls infested by *D. vitifoliae* (Lawo et al., 2011). Its reception by NIRT tissue could play a role for the induction of *VviSTS* detected in Teleki 5C with 2.3 $\log_2$ FC (Appendix A.4) and consequentially contribute to the systemic stilbene accumulation. Targeted experiments are needed to investigate whether there is an active stilbene transport pathway within the root system upon *D. vitifoliae* infestation and/or a volatile signal triggering systemic stilbene biosynthesis. Besides the reason(s) explaining the stilbene accumulation in NIRTs, their antimicrobial properties likely protect the residual (non-infested) root tips on *D. vitifoliae* infested plants against secondary infections. The enhanced immune status of the infested Teleki 5C root system, concluded by the detected stilbene accumulation in root galls and non-infested part of the root system, could be part of a strategy to maintain overall host fitness and ensure the nutrient and water uptake of the host plant.

4. Conclusion

Taken together the present study reveals that flavan-3-ols and stilbenes predominate the secondary metabolite profile of non-infested *Vitis spp.* root tips, indicating their potential involvement in constitutive defence and root growth. *D. vitifoliae* feeding triggers the accumulation of flavan-3-ols and stilbenes via the induction of the phenylpropanoid pathway without preventing root gall formation raising the question whether *D. vitifoliae* might profit from polyphenol accumulation in the feeding tissue by e.g. suppressing microorganisms in the rhizosphere. Fercal provides a stronger host response locally at the feeding tissue than Teleki 5C. Our data indicates that Teleki 5C reacts to *D. vitifoliae* infestation with a general increased immune status of the root system, marked by accumulation of stilbenes in infested and non-infested parts of the root system. Whether this accumulation reflects an insect strategy to promote overall host plant fitness by protection of the remaining root tips needs to be addressed by further experiments.

Funding

This work was supported by a DOC research fellowship of the Austrian Academy of Sciences (ÖAW).

CRediT authorship contribution statement

Markus W. Eitle: Data curation, Data curation, Formal analysis. **Julia Loacker:** Data curation. **Jacqueline Meng-Reiterer:** Data curation. **Rainer Schuhmacher:** Conceptualization, Project administration, Funding acquisition. **Michaela Griesser:** Project administration, Data curation. **Astrid Forneck:** Conceptualization, Project administration, Funding acquisition.

Acknowledgements

This work partly contributes to the thesis of J. Loacker (M.sc.) and M. W. Eitle (PhD.). We appreciate the technical assistance in the lab of S. Hao, V. Dockner, R. Prinz-Mammerler, S. Hensler and for fruitful scientific discussion with Dr. S. Savoi (SupAgro Montpellier), Dr. J.C. Herrera (BOKU Vienna), Dr. H. Halbwirth and Prof. Dr. K. Stich (TU Vienna).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2018.12.004.

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