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Flavonoid biosynthesis and degradation play a role in early defence responses of bilberry (*Vaccinium myrtillus*) against biotic stress

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Abstract Bilberry (*Vaccinium myrtillus*) represents one of the richest flavonoid sources among plants. Flavonoids play variable, species-dependent roles in plant defences. In bilberry, flavonoid metabolism is activated in response to solar radiation but not against mechanical injury. In this paper, the defence reaction and biosynthesis of phenolic compounds of bilberry was studied after infection by a fungal endophyte (*Paraphaeosphaeria* sp.) and a pathogen (*Botrytis cinerea*). The defence response of bilberry was faster against the endophyte than the pathogen. All flavonoid biosynthesis genes tested were activated by each infection. Biosynthesis and accumulation of phenolic acids, flavan-3-ols and oligomeric proanthocyanidins were clearly elevated in both infected samples. Infection by the pathogen promoted specifically accumulation of epigallocatechin, quercetin-3-glucoside, quercetin-3-O-α-rhamnoside, quercetin-3-O-(4”-HMG)-R-rhamnoside, chlorogenic acid and coumaroyl quinic acid. The endophyte-infected plants had a higher content of quercetin-3-glucuronide and coumaroyl iridoid. Therefore, accumulation of individual phenolic compounds could be specific for each infection. Quantity of insoluble proanthocyanidins was the highest in control plants, suggesting that they might act as storage compounds and become activated by degradation upon infection.

Keywords *Vaccinium myrtillus* · Gene expression · LC-MS · Flavonoid biosynthesis · Proanthocyanidin · Pathogenesis-related

Abbreviations

CHS	chalcone synthase
DFR	dihydroflavonol 4-reductase
ANS	anthocyanidin synthase
ANR	anthocyanidin reductase
PR4	pathogenesis-related protein 4
MEA	malt-extract agar

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Introduction

Flavonoids are known to play a role in plant defence against both abiotic and biotic stresses (Dixon et al. 2002). Flavonoids are one among several factors contributing to plant resistance

(Treutter 2005). Many flavonoid compounds can function as passive or inducible barriers against herbivores or microbial pathogens, and the flavonoid content can increase or the flavonoid composition can change in response to pathogen attack (Dixon and Paiva 1995; Miranda et al. 2007; Carlsen et al. 2008). The involvement of flavonoids in plant defence depends on the species. In annual grasses that have low flavonoid content, flavonoids are less significant in defence (Logemann and Hahlbrock 2002).

Bilberry, or European blueberry (*Vaccinium myrtillus*) is one of the richest sources of flavonoids, having a long history of use in folk medicine (Morazzoni and Bombardelli, 1996). Leaves of bilberry contain high quantities of proanthocyanidins, catechins, and the flavonols quercetin and kaempferol (Jaakola et al. 2004). Flavonoids may play a prominent role in the defence of bilberry. When subjected to solar radiation, the content of anthocyanins, catechins, flavonols and hydroxycinnamic acids increases and proanthocyanidins decrease in the leaves (Jaakola et al. 2004). However, flavonoid biosynthesis is not increased against mechanical injury and therefore flavonoid compounds may represent passive defences against herbivores in bilberry (Jaakola et al. 2008).

Economically, bilberry is one of the most important wild berry species in Northern Europe due to its high flavonoid content. Very little is currently known of the pathogenesis of bilberry. Some studies have concentrated on the ecology of the parasitic fungus *Valdensia heterodoxa* on bilberry with respect to nitrogen fertilisation (Witzell and Shevtsova, 2004). The multihost pathogen *Botrytis cinerea* has been isolated from bilberry (Vlassova et al. 2000) and is one of the most significant pathogens found on cultivated blueberries (Hildebrandt et al. 2001). After infection by *B. cinerea*, leaves turn light-brown and grey mould is sometimes found on the surface. Flowers turn brown and shrivel up, and entire flower clusters can become destroyed (Hildebrandt et al. 2001). In addition to pathogen attack, bilberry has constant interaction with other microbes in the field. Several species of endophytic fungi that cause no symptoms of disease are found inside the aerial parts of *Vaccinium* species (Sauer et al. 2002). This work concentrates on the activation of the flavonoid metabolism of wild bilberry during biotic stress. We studied accumulation of phenolic compounds and the defence reaction of bilberry in response to infection by *B. cinerea* and an endophytic fungus isolated from the bilberry stem.

Materials and methods

Isolation of bilberry endophytes

Stem cuttings of healthy, symptomless bilberry plants were surface-sterilised for 2 min in 70% ethanol and for 20 min in 6% calcium hypochlorite. The cuttings were rinsed three times 10 min with sterile water and aseptically cut into 5 mm-long segments that were further split in half. The stem pieces were placed onto 2% malt extract agar (MEA) plates and grown at room temperature (RT) for up to 4 weeks. The plates were checked every second day for fungal growth and when detected, the fungal cells were removed and transferred to a new plate with a cork borer. The microbes were further subcultured until a pure culture was obtained. The isolates were studied under a light microscope for morphological characteristics, and one representative of each morphologically-distinct form was collected and maintained in 25% glycerol at -70°C for further study.

DNA extraction, PCR, cloning, and sequencing of the fungal 18 S rDNA

One endophyte isolate was selected for the study. For identification, the isolate was transferred onto 2% MEA plate topped with cellophane film and grown at RT for one week. DNA was isolated from the fungus according to Pirttilä et al. (2001). The 18 S ribosomal DNA (rDNA) was amplified from the isolated fungal DNA by PCR (PTC200, MJ Research, Waltham, MA) in a reaction mixture of universal primers NS1 (5'-GTAGTCATATGCTTGTCTC-3', *Saccharomyces cerevisiae* positions 20–38) and NS8 (5'-TCCGCA GGTTCACCTACGGA-3', positions 1788–1769) (White et al. 1990), nucleotides (MBI Fermentas, Vilnius, Lithuania), buffer, and PCR enzyme (Dynazyme, Finnzymes, Espoo, Finland), using the following PCR programme: 94°C 5 min, 3 cycles of 1 min at 94°C, 1 min at 53°C, and 3 min at 72°C, followed by 3 cycles with 51°C, and 30 cycles with 49°C as annealing temperatures, and extension at 72°C for

5 min. The PCR products were cloned into pGEM-T Easy vector with pGEM T-easy vector cloning kit (Promega, Madison, WI), and transformed into *Escherichia coli* DH5α. Plasmid DNA was isolated and sequenced according to the manufacturer's instructions (Abi 3730 DNA Analyser, Abi Prism BigDye Terminator Cycle Sequencing Kit, Applied Biosystems, Warrington, UK). The sequence was aligned with all accessible sequences obtained through the Basic Local Alignment Search Tool (BLAST).

Molecular phylogenetic analysis

In order to further characterise the fungal isolate, the phylogenetic position was determined. Based on the alignment data, a phylogenetic analysis for the isolate was performed with the 18 S rDNA sequences of close relatives of *Paraphaeosphaeria*, with *Phaeosphaeria nodorum* as an outgroup. The sequences were retrieved from GenBank and aligned by using ClustalW 1.8.2. (Chenna et al. 2003) with the following default parameters: gap opening penalty 15.0, gap extension penalty 6.6, and DNA weight matrix identity. The gap positions were excluded manually. A distance matrix was created with DNADIST of Phylip (Felsenstein 1989), from which the tree topology was built by the neighbour-joining method in the programme NEIGHBOR. The confidence for individual branches of the resulting tree was estimated by performing 1000 bootstrap replicates by using the programmes SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE.

Isolation of pathogenesis-related gene 4 and anthocyanidin reductase gene (*ANR*) from bilberry

The pathogenesis-related protein 4 (PR4) was chosen to characterise the defence responses, because the PR4 proteins are chitinases that specifically inhibit growth of various fungal pathogens. *PR4*-genes of *Vaccinium vitis-idaea* (Pehkonen et al. 2008), *Hordeum vulgare, Triticum monococcum* and *Prunus dulcis* and *ANR*-genes of *Vitis vinifera*, *Camellia sinensis* and *Medicago truncatula* were aligned in the ClustalW programme (Chenna et al. 2003), and the most degenerated sequences in the alignments were selected as primer targets. This way, primers PR4F (5'-AARTATGGATGGACGGCVTT-3') and PR4R (5'-TYVACRAASTGGTAGTTGAC-3') and ANRF (5'-AGCTGAGAAAGCWGCDTGGA-3') and ANRR (5'-TCTTTTGYTVAGGAACTTTGC-3') were obtained and used in a PCR reaction (94°C for 4 min, then 6 times 94°C 1 min 15 s, 70°C 3 min, -0.1°C s^{-1} to 38°C, 72°C 2 min, then 50 times 94°C 1 min, 53°C 2 min, 72°C 2 min, and finally 72°C for 5 min). cDNAs isolated from *B. cinerea*-infected or from healthy control leaves of bilberry plants were used as the template. The products were cloned into pGEM T-Easy vector (Promega), sequenced and submitted to BLAST as described above. Full-length sequences were isolated with SMART™ RACE cDNA Amplification kit (Clontech, Palo Alto, CA, USA).

Infection of bilberry plants with endophyte and pathogen (*Botrytis cinerea*)

Plant material

Micropropagated plants were used for the study because micropropagated plants typically are more susceptible to infection. Micropropagated bilberry clones representing the same origin (Botanical Gardens, University of Oulu, Finland) were grown on ½ MS agar medium supplemented with 2-isopentenyl adenine (5 mg l^{-1}) in sterile conditions for 2 months. Plants were transferred to root-inducing ½ MS medium containing indole-3-butyric acid (0.1 mg l^{-1}) and cultured for 2 months until roots were fully developed (Jaakola et al. 2001a). Finally, plants were maintained in plastic containers containing sterilised soil for 6 weeks (16 h photoperiod, irradiance of 75 µmol $m^{-2}s^{-1}$, temperature 22°C).

Fungal infection

Botrytis cinerea (strain DSM 5145, DSMZ, Braunschweig, Germany) was used in this study as the model pathogen because it is a known pathogen of blueberry (Hildebrandt et al. 2001) and widely used in pathogenesis studies. Pathogen and endophyte spores were diluted in 50 mM HEPES buffer with an estimate of 1,100 spores $µl^{-1}$;. Bilberry plants were infected by *B. cinerea* and the endophyte by pipetting 10 µl of spore suspension on leaf surfaces. Infected plants were covered with film to keep the moisture at an optimal level for fungal growth. Samples were taken 12 h and 24 h after infection. The plant leaves were

cut, frozen in liquid nitrogen and stored subsequently at -70°C.

Success of infection was tested by PCR using primers specific for the fungal 18 S rRNA. Primers BC-F (5'-GGCTAGCTTTGGCTGGTCG-3') and BC-R (5'-GTGGTGTTGCCACCTCCCTAA-3'), were used to verify *B. cinerea* infection. Endophyte infection was confirmed with primers EF-F (5'-TCACTGAGCCATTCAATCGGTAG-3') and EF-R (5'-ACGAACGAGACCTTAACCTGCT-3'). The infected plants were carefully surface-sterilised in a laminar flow hood in 70% ethanol for 1 min and rinsed three times in sterile deionised water prior to DNA isolation. The template DNA was isolated from the infected, surface-sterilised bilberry leaves according to Pirttilä et al. (2001), and PCR reactions were carried out as described above with 55°C as the annealing temperature.

Real-time RT-PCR

The expression of the flavonoid biosynthetic genes chalcone synthase (*CHS*), dihydroflavonol 4-reductase (*DFR*), anthocyanidin synthase (*ANS*) and anthocyanidin reductase (*ANR*) and the *PR4* gene was studied with Real-Time RT-PCR. The primers used to monitor real-time amplification of each gene are represented in Table 1. RNA was isolated from the plants according to Jaakola et al. (2001b) after 12 and 24 h of infection and reverse-transcribed to cDNA (Superscript II, Invitrogen, Carlsbad, CA). The Real-Time PCR analyses were performed using LightCycler instrument (Roche Molecular Biochemicals, Mannheim, Germany) and LightCycler®SYBR Green I Master qPCR kit (Roche Molecular Biochemicals). The thermal cycling conditions were as follows: Initial denaturation at 95°C for 10 min, followed by 45 cycles at 95°C for 10 s (ramp rate 4.4°C s^{-1}), 60°C for 20 s (ramp rate 2.2°C s^{-1}) and 72 for 10 s (ramp rate 4.4°C s^{-1}). Melting curve was measured at 95°C for 0.5 s (ramp rate 4.4°C s^{-1}), 57°C for 15 s (ramp rate 2.2°C s^{-1}) and 98°C for 0 s (ramp rate 0.11°C s^{-1}). Efficiency of the primers was tested with various dilutions of uninfected bilberry leaf cDNA. The uninfected leaf cDNA of the time point 0 h was also used for normalisation in each experiment, to which the expression of the same gene in other samples was compared. PCR products were analysed using melting curves, and agarose gel electrophoresis was used to ensure single-product amplification. Quantification of PCR products was performed via calibration curve procedure using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or actin as an internal standard. The equations were performed with LightCycler software using standard formulae for Real-Time PCR.

LC-MS analysis of flavonoids and phenolic acids

Sample preparation

Sample preparation was done according to Tolonen and Uusitalo (2004) with minor changes. A total of 5 mg of powdered, freeze-dried bilberry leaves (24 h after infection) were extracted using 500 µl of methanol for 60 min in an ultrasonic bath. After extraction, the samples were centrifuged for 10 min

Table 1 Primers used for Real-Time RT-PCR

Gene	Primers 5'>3'	GenBank No.
Chitinase (*PR4*)	Forward: GATAAGGTGGCCTTGTGCAT	GQ380568
	Reverse: CAAGCTTCTTGTGGCAAGTG	
CHS	Forward: CCAAGGCCATCAAGGAATG	AY123765
	Reverse: TGATACATCATGAGTCGCTTCAC	
DFR	Forward: GAAGTGATCAAGCCGACGAT	AY123767
	Reverse: ATCCAAGTCGCTCCAGTTGT	
ANS	Forward: TCTTCTACGAGGGCAAATGG	AY123768
	Reverse: ACAGCCCATGAAATCCTGAC	
ANR	Forward: GCTGGTGTTTCTCCCACAAT	FJ666338
	Reverse: AAATATATGGGCGCGACAAA	
GAPDH	Forward: CAAACTGTCTTGCCCCACTT	AY123769
	Reverse: CAGGCAACACCTTACCAACA	

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used for the relative quantification of the PCR products

at 12,100*g* and diluted to 1/5 and to 1/100 with 10 % methanol.

Liquid chromatography mass spectrometry

A Waters Acquity ultra-performance liquid chromatographic (UPLC) system (Waters Corporation, Milford, MA, USA) with autosampler, vacuum degasser and column oven was used. The analytical column used was a Waters ACQUITY HSS T3, (2.1 × 50 mm, 1.8 μm, Waters Corporation, Milford, MA, USA). The eluents were 0.1% formic acid (A, pH 2.7) and methanol (B). A linear gradient elution from 10% to 50% B in 12 min was employed, followed by 4 min isocratic elution with 50% B, 4 min isocratic elution with 90% B, and column equilibration for 2.5 min with initial conditions. The flow rate was 0.5 ml min^{-1} and the column oven temperature was 35ºC. The flow was directed to mass spectrometer (MS) via ACQUITY photo diode array detector. UPLC/TOF-MS data were acquired with a Waters LCT Premier XE time-of-flight (TOF) mass spectrometer (Waters Corporation, Milford, MA, USA) using positive (ESI+) ionisation polarity. Leucine enkephaline was used as a lock mass compound ($[M+H]^+$ =m/z 556.2771). Capillary voltage of 2.8 kV was used, while the cone voltage was set to 80 V. Aperture 1 voltages of 5 V and 50 V were used in two parallel data acquisition functions, to obtain only molecular ions with the lower voltage and more in-source fragmentation data with the higher voltage. The mass range of m/z 100 – 1,100 was acquired. The W-mode ion optics and the DRE (dynamic range enhancement) option were used.

Concentration levels of each standard in calibration solutions were 5, 10, 20, 50, 100, 200, 500, 1,000 and 2,000 ng ml^{-1} and each of them were injected in duplicate. Also QC-samples were prepared by spiking methanol to concentrations 400 ng ml^{-1} and 4000 ng ml^{-1} in duplicate and they were treated in the same way as the samples (60 min extraction in ultrasonic bath and 1/5 dilution). Calibration curves for standard compounds were generated by plotting the peak areas of standard compounds as a function of concentration using quadratic fitting and 1/x weighting. Cyanidin was used as external standard for all anthocyanins and anthocyanidins, quercitrin was used as external standard for all flavonols, epicatechin for all flavan-3-ols and *trans*-chlorogenic acid for all phenolic acids. $[M]^+$ ions were used for anthocyanins and anthocyanidins, $[M+H]^+$ ions for all flavan-3-ols and flavonol glycosides, and $[M+Na]^+$ ions were used for phenolic acids. A *m/z* window of ±0.05 amu was used for generating the extracted ion chromatograms for standard compounds and all the detected phenolic compounds in the samples. The detection limits for all standard compounds were <5 ng ml^{-1}. The linear ranges were 5–2,000 ng ml^{-1} for cyanidin, *trans*-chlorogenic acid and epicatechin and 20 – 2,000 ng ml^{-1} for quercitrin (R^2 of the standard curves were >0.997). The back-calculated accuracies were between 94–118% at the limit of quantitation (LoQ) and 94 – 108% above LoQ for all calibration compounds. The precisions (relative standard deviation) were <15% at all standard levels for all calibration compounds. These estimates of accuracy and precision were considered acceptable for the purpose. No disappearance/degradation for standard compounds was detected during the extraction, as the recoveries from QC samples were between 95% – 108% for all standard compounds at both QC-levels.

LC-MS analysis of proanthocyanidins

Sample preparation

Sample preparation was performed according to Määttä et al. (2001) and Jaakola et al. (2004) with minor changes. The bilberry leaf samples (24 h after infection) were ground to a fine powder using mortar and pestle in liquid nitrogen. Powdered leaf samples (0.125 g) were suspended in 5 ml of acidified (0.6 M HCl) methanol and vortexed for 15 min. Samples (1 ml) were then taken for the analysis of extractable proanthocyanidins and filtered through a 0.45 μm syringe filter (GHP Bulk Acrodisc 13; Pall Life Sciences, New York, NY). The remaining suspension was refluxed for 2 h at 60˚C for the analysis of non-extractable proanthocyanidins. Heating in acid hydrolyses proanthocyanidins into cyanidin and delphinidin anthocyanins. Also the previously separated samples were heated at 60˚C in a water bath for 2 h to hydrolyse the extractable proanthocyanidins.

Liquid chromatography mass spectrometry

The HPLC apparatus was a Waters Alliance 2690 instrument (Waters, Milford, MA, USA) and the

separations were done using a Waters Atlantis dC_{18} column (50 × 2.1 mm i.d., 3 μm). A 20-min linear gradient from 5% to 60% MeOH in 1% formic acid was used at flow rate of 0.3 ml min^{-1}. Gradient was followed by 5-min isocratic elution at 60% MeOH and returning to initial conditions in 2 min and re-equilibration of the column for 5 min.

The MS instrument used was a Micromass Quattro II triple quadrupole mass spectrometer with ESI Z-spray ion source. MS-detection from 150 to 600 Da was used for identification and quantification of procyanidin and prodelphinidin. Positive ionisation with capillary voltage of 3.5 kV, cone voltage of 47 V, source temperature of 80˚C, desolvation temperature of 350˚C, nebuliser gas flow of 0.3 l min^{-1} and desolvation gas flow of 7 l min^{-1} was used. The quantification was done by integrating the peaks from ion chromatograms, created for cyanidin with molecular ion at m/z 287 (±0.5) and for delphinidin at m/z 303 (±0.5). The cyanidin chloride standard solution concentrations were 0.2, 0.5, 2.0, 5.0, 20, 50 and 100 μg ml^{-1}. Standard curve was generated using linear fitting (R^2 of the standard curve was >0.99).

The proanthocyanidins were quantified by the weight of the phenolic unit of the molecule using the response of cyanidin chloride standard solutions on HPLC/MS. Anthocyanins were not detected in the leaves, so the quantities of procyanidin and prodelphinidin originating from anthocyanins were assumed negligible.

Results

Isolation and identification of bilberry endophytes

Altogether 35 fungal isolates were obtained from bilberry stems, of which 25 morphologically distinct forms were selected and stored at -70°C. For identification, the 18 S rDNA was partially sequenced and aligned with all accessible sequences of the GenBank. The isolate No. 20 (GenBank No. GQ380569) showed the highest similarity to *Paraphaeosphaeria quadriseptata*. In the phylogenetic tree, the isolate grouped with *P. quadriseptata*, *P. filamentosa* and *P. conglomerata* (Fig. 1) and was classified as a *Paraphaeosphaeria* species. Because members of this genus have earlier been found as endophytes (Fukuhara 2002; Ganley and Newcombe, 2006), there are no reports of this fungus as a pathogen of bilberry, and no symptoms of disease were visible after infection; the isolate was therefore chosen for the analysis.

Isolation of pathogenesis-related gene 4 (*PR4*) and anthocyanidin reductase (*ANR*)

The nucleic acid sequence of *PR4* gene (GenBank no. GQ380568) was 76% identical with *PR4b* genes of *Nicotiana tabacum* (X58547.1) and *Prunus persica* (AF362989.1). The protein sequence was 81 – 83% identical and 84 – 87% similar to PR4 proteins of other plant species. For ANR (GenBank no. FJ666338), a high homology between the corresponding genes of other plant species was detected at both amino acid (87–93%) and nucleotide level (73–85%).

Infection of bilberry with *Botrytis cinerea* and endophyte

Plants inoculated with the endophyte, as well as the uninoculated plants, did not show any superficial symptoms of disease during the course of the study. When presence of the endophyte in the bilberry leaves was examined with PCR, a product was obtained with the species-specific primers (data not shown), confirming the infection. Plants infected with *B. cinerea* had necrotic lesions in the young leaves after 12 h. At

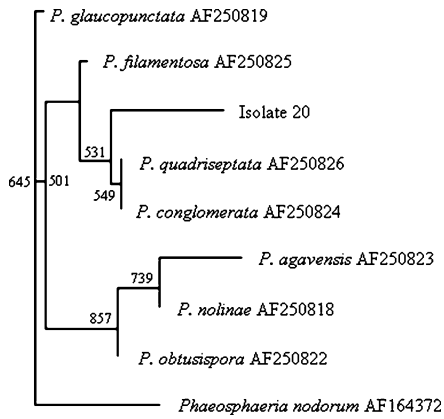

Fig. 1 Phylogenetic position of the endophyte isolate 20 from bilberry stems. The 18 S rDNA sequences of the isolate, *Phaeosphaeria nodorum* (outgroup), and close relatives within the genus *Paraphaeosphaeria*, were analysed with the neighbour-joining method. Values from 1,000 bootstrap repeats are presented if support was >50%. The species names are followed by GenBank accession numbers

the second stage of infection, after 24 h, the lesions had become visible also in the fully-grown leaves. However, the infection was not apparent throughout the whole plant body.

Expression of flavonoid biosynthesis and *PR4* genes of bilberry

Expression of the *PR4* gene and flavonoid biosynthesis genes chalcone synthase (*CHS*), dihydroflavonol 4-reductase (*DFR*), anthocyanidin synthase (*ANS*), and anthocyanidin reductase (*ANR*) was measured in bilberry in response to infection by a pathogen (*B. cinerea*) and the endophyte isolate. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and actin served as the endogenous reference for normalisation. All tested genes showed increased expression in the infected samples. The *PR4* gene of bilberry responded markedly faster against infection by the endophyte than the pathogen (Table 2). All flavonoid biosynthesis genes responded faster against the endophyte infection (Table 2). Expression of the *CHS* gene that begins the flavonoid biosynthesis pathway increased with time in both infected samples (Table 2). The flavonoid biosynthesis genes *DFR* and *ANS* that lead to biosynthesis of leucoanthocyanidins and anthocyanidins, respectively, had a similar expression profile, with the highest expression 12 h after infection (Table 2). The *ANR* gene responsible for biosynthesis of epicatechin, the unit of a proanthocyanidin polymer, had increased expression 24 h after infection by the endophyte but decreased in pathogen-infected samples (Table 2).

Table 2 Relative expression of bilberry genes quantified by Real-Time RT-PCR with SYBR-green as the fluorescent reporter

Gene	Sample	Time (h)		
		0	12	24
	Control	0.88±0.11	0.56±0.34	1.09±0.2
PR4	*B. cinerea*	0.68±0.07	3.95±0.92	2.84±1.13
	Endophyte	0.76±0.01	4.49±0.98	5.66±0.24
	Control	0.78±0.30	0.79±0.41	0.845±0.32
CHS	*B. cinerea*	0.83±0.21	1.38±0.28	2.87±0.66
	Endophyte	0.83±0.16	2.17±0.74	3.73±0.28
	Control	0.73±0.38	0.58±0.11	1.12±0.39
DFR	*B. cinerea*	0.54±0.10	2.13±0.08	1.8±0.91
	Endophyte	0.94±0.04	3.65±0.64	3.06±1.63
	Control	1.01±0.68	0.49±0.25	0.59±0
ANS	*B. cinerea*	0.89±0.46	11.17±2.08	6.31±2.77
	Endophyte	0.88±0.21	18.02±1.8	4.33±0.85
	Control	0.88±0.11	0.55±0.34	1.09±0.2
ANR	*B. cinerea*	0.68±0.07	3.95±0.92	2.84±1.13
	Endophyte	0.76±0.01	4.49±0.98	5.66±0.24

Quantification of PCR products was performed via calibration curve procedure (LightCycler Software Version 4.0, Roche Applied Sciences) using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal standard. Data represent fold differences in expression with the standard deviation from three comparisons

LC-MS analysis of phenolic compounds in bilberry samples

In all bilberry leaf samples the same flavonoid compounds were detected (Table 3). Three simple flavan-3-ols; epicatechin, catechin and epigallocatechin, together with four phenylpropanoid-substituted catechins, cinhonains, were detected. Eight conjugated phenolic acids were detected in the leaf samples, including *trans*- and *cis*-isomers of chlorogenic acid, two different coumaroyl quinic acids, feruloyl quinic acid and caffeoyl shikimic acid, together with two different coumaroyl iridoids. Seven diverse flavonol monoglycosides were identified; quercetin-3-O-glucuronide, quercetin-3-O-galactoside, quercetin-3-O-glucoside, quercetin-3-O-arabinoside, quercetin-3-O-rhamnoside, kaempferol-3-O-glucuronide and quercetin-3-O-[4''-(3-hydroxy-3-methylglutaroyl)]-α-rhamnoside. Also four different procyanidins (PCs) were detected; trimeric PC with two A-type interflavan linkages between (epi)catechin ((E)C) units (A-type PC trimer), trimeric PC with one A-type linkage and one B-type linkage between (E)C units (A/B-type PC trimer), dimeric PC with B-type linkage between (E)C units (B-type PC dimer) and dimeric PC with A-type linkage between (E)C units (A-type PC dimer). Individual procyanidins were not quantitated, but their relative responses were in good agreement with the results obtained for the oligomeric proanthocyanidins (data not shown). Detailed information on the identification of these compounds is described elsewhere (Hokkanen et al., submitted manuscript), except for B-type procyanidin dimer, for which the exact masses of fragment ions in positive ionisation mode and proposed fragmentation reactions are presented in Fig. 2.

Table 3 Flavonoid content of the bilberry leaf samples determined by LC-MS

Compound name	RT [min]	Calibration compound	Content[a] in freeze-dried leaves (mg g^{-1} ±SD[b])		
			Control	*B. cinerea*	Endophyte
Flavan-3-ols					
Epigallocatechin	2.06	epicatechin	0.07±0.00	0.93±0.04	0.10±0.00
Catechin	2.09	epicatechin	0.05±0.00	0.16±0.01	0.13±0.01
Epicatechin	3.43	epicatechin	1.13±0.03	2.28±0.07	1.55±0.05
Cinchonain IIx (isomer 3)	3.58	epicatechin	0.10±0.00	0.29±0.00	0.15±0.00
Cinchonain IIx (isomer 4)	3.72	epicatechin	0.11±0.00	0.40±0.01	0.17±0.00
Cinchonain Ix (isomer 1)	4.41	epicatechin	0.04±0.01	0.26±0.04	0.10±0.02
Cinchonain Ix (isomer 2)	7.88	epicatechin	0.09±0.00	0.02	0.15±0.01
TOTAL			1.58±0.05	4.59±0.19	2.34±0.09
Flavonol monoglycosides					
quercetin-3-glucuronide	6.40	quercitrin	1.32±0.00	1.36±0.00	1.40±0.00
quercetin-3-O-β-galactoside	6.41	quercitrin	0.27±0.01	0.26±0.01	0.21±0.00
quercetin-3-O-glucoside	6.59	quercitrin	0.05±0.00	0.08±0.00	0.06±0.00
quercetin-3-O-α-arabinoside	6.95	quercitrin	0.04±0.00	0.04±0.00	0.03±0.00
kaempferol-3-glucuronide	7.47	quercitrin	0.01±0.00	0.02±0.00	0.02±0.00
quercetin-3-O-α-rhamnoside (quercitrin)	7.54	quercitrin	0.11±0.01	0.16±0.01	0.11±0.01
quercetin-3-O-(4''-HMG)-α-rhamnoside	9.00	quercitrin	0.21±0.01	0.30±0.01	0.24±0.01
TOTAL			2.02±0.02	2.22±0.03	2.07±0.02
Phenolic acids					
trans-chlorogenic acid	2.50	*t*-chlorogenic acid	8.21±0.36	20.29±0.89	9.85±0.43
cis-chlorogenic acid	3.43	*t*-chlorogenic acid	0.75±0.03	1.65±0.07	0.81±0.03
Coumaroyl quinic acid (isomer 2)	3.53	*t*-chlorogenic acid	0.23±0.01	0.47±0.03	0.21±0.01
Caffeoyl shikimic acid	3.98	*t*-chlorogenic acid	0.03±0.00	0.05±0.00	0.03±0.00
Feruloyl quinic acid (isomer 1)	4.00	*t*-chlorogenic acid	0.03±0.00	0.04±0.00	0.02±0.00
Coumaroyl quinic acid (isomer 4)	4.46	*t*-chlorogenic acid	0.09±0.00	0.19±0.01	0.09±0.00
Coumaroyl Iridoid (isomer 1)	5.76	*t*-chlorogenic acid	0.05±0.00	0.06±0.00	0.07±0.00
Coumaroyl Iridoid (isomer 2)	6.21	*t*-chlorogenic acid	0.15±0.00	0.21±0.00	0.34±0.01
TOTAL			9.53±0.42	22.97±1.01	11.42±0.50

[a] The absolute quantities for the compounds different than the standard compounds used (*trans*-chlorogenic acid, quercitrin and epicatechin) may slightly differ from the quantities presented, due to the fact that LC/MS responses for different compounds may vary even though the standard compounds are structurally highly similar. Nevertheless, this has very little effect on the comparison between samples as the difference in the LC/MS response is the same in all samples as long as the quantities are on the linear range of the method.

[b] SD=standard deviation of three replicates

The isomer numbers shown in parenthesis refer to those given in Hokkanen et al., submitted manuscript

When content of phenolic compounds of the infected samples was analysed, an increase was observed 24 h after infection (Table 3). Both infected samples had increased content of flavan-3-ols, specifically catechin and epicatechin. The content of epigallocatechin increased over ten-fold in pathogen-infected plants whereas no significant increase was observed in endophyte-infected plants (Table 3). The quantities of quercetin-3-glucoside, quercetin-3-O-α-rhamnoside and quercetin-3-O-(4''-HMG)-R-rhamnoside increased in pathogen-infected samples, and the content of quercetin-3-glucuronide was increased specifically in endophyte-infected plants (Table 3). The content of conjugated phenolic acids, specifically chlorogenic

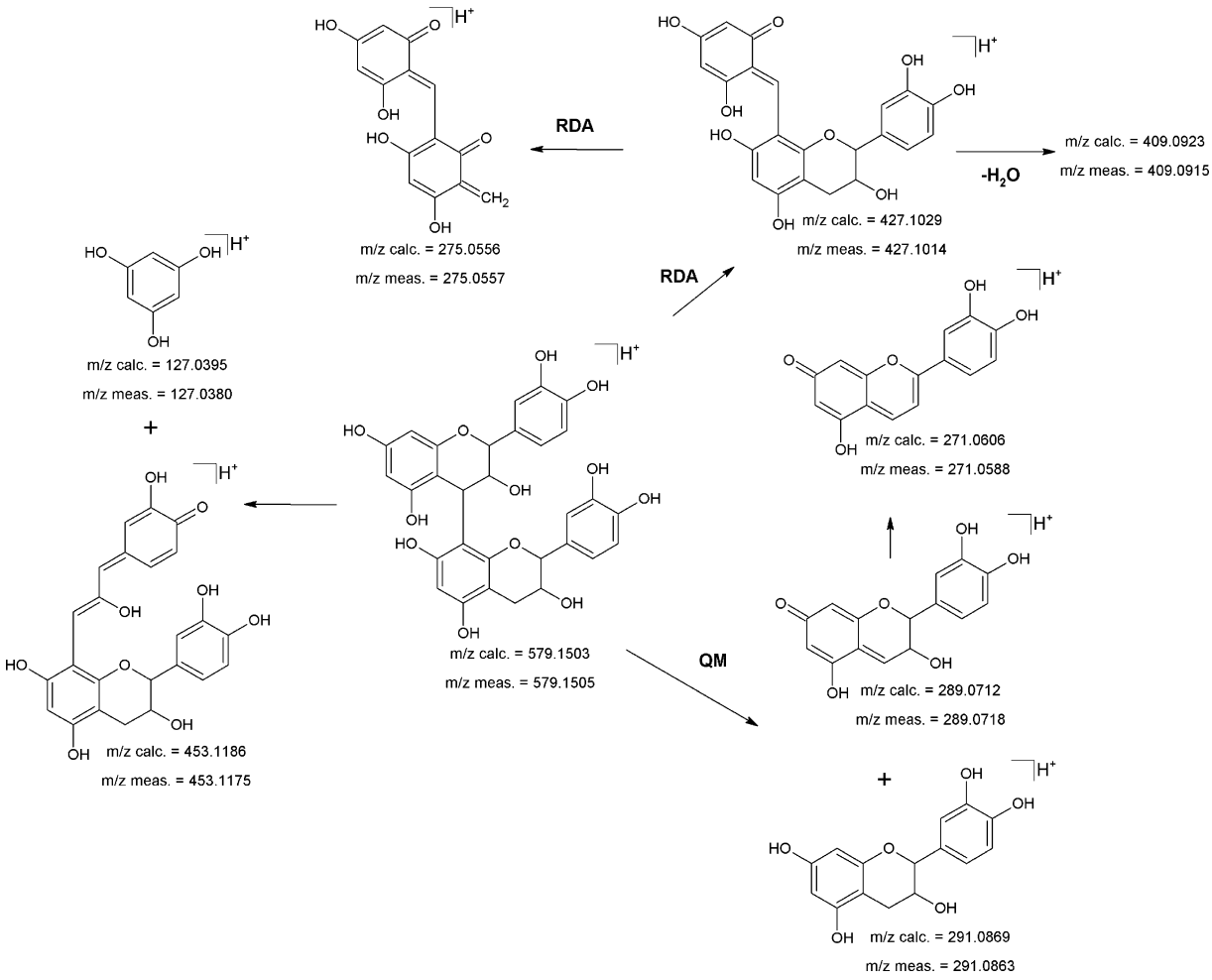

Fig. 2 Detected fragment ions with exact masses together with proposed fragmentation pathway for B-type procyanidin dimer. The retro Diels-Alder (RDA) fragmentation together with quinone-methide (QM) cleavage of the interflavan linkage are shown. Both of these fragmentation reactions are typical of B-type proanthocyanidins

acid and coumaroyl quinic acid, was doubled in the pathogen-infected samples (Table 3). The content of coumaroyl iridoid was doubled in the endophyte-infected plants (Table 3).

The LC-MS analysis showed that both pathogen- and endophyte-infected bilberry leaf samples had almost no change in the quantity of total proanthocyanidins (Table 4). When the proanthocyanidin pools

Table 4 Proanthocyanidin content of the bilberry leaf samples determined by LC-MS

Compound name		Content in freeze-dried leaves (mg g^{-1} ±SD)		
		Control	*B. cinerea*	Endophyte
Insoluble proanthocyanidins	Procyanidin	0.33±0.02	0.20±0.01	0.08±0.004
	Prodelphinidin	0.02±0.001	0.01±0.001	0.001±0.0001
Extractable proanthocyanidins	Procyanidin	0.10±0.005	0.33±0.02	0.40±0.02
	Prodelphinidin	0.02±0.001	0.05±0.002	0.04±0.004
TOTAL	Procyanidin	0.43±0.02	0.53±0.03	0.48±0.02
	Prodelphinidin	0.04±0.002	0.06±0.003	0.04±0.002

were studied further, the quantity of insoluble proanthocyanidins was clearly the highest in the uninfected control samples and the lowest in the infected samples, especially in the endophyte-infected sample (Table 4). In contrast, the quantity of oligomeric proanthocyanidins was the highest in both infected bilberry samples and the lowest in the uninfected sample (Table 4). A similar trend was detected among both procyanidins and prodelphinidins (Table 4).

Discussion

Previously we found that flavonoid metabolism is not activated in response to wounding in bilberry, suggesting that flavonoids represent mainly passive defences against mechanical injury (Jaakola et al. 2008). The current study concentrated on the biosynthesis of phenolic compounds of bilberry in response to biotic stress. Based on both the analysis of gene expression and quantification of compounds, the endophyte-infected and pathogen-infected bilberry plants had increased biosynthesis of phenolic compounds.

There are no earlier studies on biosynthesis of phenolic compounds in response to endophyte infection, although infection by rhizobia and mycorrhiza increase flavonoid biosynthesis moderately and locally, and can be highly species-specific (McKhann et al. 1997; Larose et al. 2002; Carlsen et al. 2008). Infection of plant tissue by rhizobia and mycorrhiza can induce production of chitinases and defence proteins specific for the interaction (Pozo et al. 1999; Salzer et al. 2004). PR genes are produced equally, or to a less degree towards rhizobia and mycorrhiza than promoted by a pathogen (Mohr et al. 1998). Endophytes typically promote stronger and faster defence responses than pathogens, characterised by production of H_2O_2, and elevated activity of the phenylalanine ammonia lyase (PAL) enzyme (Schulz et al. 1999; Laukkanen et al. 2000). Based on these earlier findings, the faster activation of the *PR4* gene by endophyte infection than by pathogen infection was expected in bilberry.

The content of phenolic compounds increased in both infected samples. Infection by the pathogen promoted specifically accumulation of epigallocatechin, quercetin-3-glucoside, quercetin-3-O-α-rhamnoside, quercetin-3-O-(4''-HMG)-R-rhamnoside, chlorogenic acid and coumaroyl quinic acid. The endophyte-infected plants had a higher content of quercetin-3-glucuronide and coumaroyl iridoid. Therefore, accumulation of individual phenolic compounds could be specific for each infection. Another interesting detail was discovered in the proanthocyanidin pools of the test samples. The uninfected samples contained high quantities of insoluble proanthocyanidins, whereas the infected samples had an abundance of less polymerised, oligomeric proanthocyanidins. Meanwhile, the content of insoluble proanthocyanidins had dropped.

Our results suggest that flavan-3-ols, oligomeric proanthocyanidins and phenolic acids are needed for the defence of bilberry, and that the proanthocyanidins might be stored in a polymerised form that becomes activated by degradation. We obtained similar results in our earlier experiments on bilberry plants responding to stress caused by solar radiation (Jaakola et al. 2004). Proanthocyanidin degradation in response to biotic stress has not been reported to date in other plant species, but polymerisation of catechins to oligomeric proanthocyanidins and 2,3-*cis* isomerisation occurs in tea (*Camellia sinensis*) during infection by the fungal pathogen *Exobasidium vexans* (Punyasiri et al. 2004).

Whereas many phenolic acids fail to exhibit direct antifungal activity, they can contribute to plant resistance via secondary mechanisms (Lee & Bostock 2007; Muthuswamy & Vasantha 2007; Pandey et al. 2007). In contrast, previous studies have shown that flavan-3-ols, such as catechin and epicatechin, and oligomeric proanthocyanidins have inhibitory and antifungal properties towards *B. cinerea* (Goetz et al. 1999; Hébert et al. 2002) and other fungi (de Colmenares et al. 1998; Veluri et al. 2004). Proanthocyanidin oligomers specifically inhibit fungal enzymes such as protein kinase and stillbene oxidase (Polya and Foo 1994; Goetz et al. 1999). Due to their potent antifungal properties, flavan-3-ols and oligomeric proanthocyanidins might function as an important growth-limiting factor towards endophytic and pathogenic fungi, and play an important role in plant defence against fungal pathogens.

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References

Carlsen, S. C. K., Understrup, A., Fomsgaard, I. S., Mortensen, A. G., & Ravnskov, S. (2008). Flavonoids in roots of white clover: interaction of arbuscular mycorrhizal fungi and a pathogenic fungus. *Plant Soil, 302*, 33–43.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G., et al. (2003). Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Research, 31*, 497–500.

de Colmenares, N. G., Ramirez-Martinez, J. R., Aldana, J. O., Ramos-Nino, M. E., Clifford, M. N., Pekerar, S., et al. (1998). Isolation, characterisation and determination of biological activity of coffee proanthocyanidins. *Journal of the Science of Food and Agriculture, 77*, 368–372.

Dixon, R. A., & Paiva, N. L. (1995). Stress-Induced Phenylpropanoid Metabolism. *Plant Cell, 7*, 1085–1097.

Dixon, R. A., Achnine, L., Kota, P., Liu, C.-J., Reddy, M. S. S., & Wang, L. (2002). The phenylpropanoid pathway and plant defence—a genomics perspective. *Molecular Plant Pathology, 3*, 371–390.

Felsenstein, J. (1989). PHYLIP — Phylogeny Inference Package (Version 3.2). *Cladistics, 5*, 164–166.

Fukuhara, M. (2002). Three *Phaeosphaeria* species and *Paraphaeosphaeria michotii* isolated from *Phragmites* leaves in Osaka, Japan. *Mycoscience, 43*, 375–382.

Ganley, R. J., & Newcombe, G. (2006). Fungal endophytes in seeds and needles of *Pinus monticola*. *Mycological Research, 110*, 318–327.

Goetz, G., Fkyerat, A., Metais, N., Kunz, M., Tabacchi, R., Pezet, R., et al. (1999). Resistance factors to grey mould in grape berries: identification of some phenolics inhibitors of *Botrytis cinerea* stilbene oxidase. *Phytochemistry, 52*, 759–767.

Hébert, C., Charles, M. T., Gauthier, L., Willemot, C., Khanizadeh, S., & Cousineau, J. (2002). Strawberry proanthocyanidins: Biochemical markers for *Botrytis cinerea* resistance and shelf-life predictability. *Acta Horticulturae, 567*, 659–662.

Hildebrandt, P. D., McRae, K. B., & Lu, X. (2001). Factors affecting flower infection and disease severity of lowbush blueberry by *Botrytis cinerea*. *Canadian Journal of Plant Pathology, 23*, 364–370.

Jaakola, L., Tolvanen, A., Laine, K., & Hohtola, A. (2001a). Effect of N^6-isopentenyladenine concentration on growth initiation *in vitro* and rooting of bilberry and lingonberry microshoots. *Plant Cell Tissue and Organ Culture, 66*, 73–77.

Jaakola, L., Pirttilä, A. M., & Hohtola, A. (2001b). Isolation of RNA from bilberry (*Vaccinium myrtillus*). *Molecular Biotechnology, 19*, 201–203.

Jaakola, L., Määttä-Riihinen, K., Kärenlampi, S., & Hohtola, A. (2004). Activation of flavonoid biosynthesis by solar radiation in bilberry (*Vaccinium myrtillus*) leaves. *Planta, 218*, 721–728.

Jaakola, L., Koskimäki, J. J., Riihinen, K., Tolvanen, A., & Hohtola, A. (2008). Effect of wounding on chalcone synthase and pathogenesis related *PR10* gene expression and content of phenolic compounds in bilberry leaves. *Biologia Plantarum, 52*, 391–395.

Larose, G., Chenevert, R., Moutoglis, P., Gagne, S., Piche, Y., & Vierheilig, H. (2002). Flavonoid levels in roots of *Medicago sativa* are modulated by the developmental stage of the symbiosis and the root colonizing arbuscular mycorrhizal fungus. *Journal of Plant Physiology, 159*, 1329–1339.

Laukkanen, H., Soini, H., Kontunen-Soppela, S., Hohtola, A., & Viljanen, M. (2000). A mycobacterium isolated from tissue cultures of mature *Pinus sylvestris* interferes with growth of Scots pine seedlings. *Tree Physiology, 20*, 915–920.

Lee, M. H., & Bostock, R. M. (2007). Fruit exocarp phenols in relation to quiescence and development of *Monilinia fructicola* infections in *Prunus* spp.: A role for cellular redox? *Phytopathology, 97*, 269–277.

Logemann, E., & Hahlbrock, K. (2002). Crosstalk among stress responses in plants: Pathogen defence overrides UV protection through an inversely regulated ACE/ACE type of light-responsive gene promoter unit. *Proceedings of the National Academy of Sciences, 99*, 2428–2432.

Määttä, K., Kamal-Eldin, A., & Törrönen, R. (2001). Phenolic compounds in berries of black, red, green, and white currants (*Ribes* sp.). *Antioxidants & Redox Signaling, 3*, 981–993.

McKhann, H. I., Paiva, N. L., Dixon, R. A., & Hirsch, A. M. (1997). Chalcone synthase transcripts are detected in alfalfa root hairs following inoculation with wild-type *Rhizobium meliloti*. *Molecular Plant-Microbe Interactions, 10*, 50–58.

Miranda, M., Ralph, S. G., Mellway, R., White, R., Heath, M. C., Bohlmann, J., et al. (2007). The transcriptional response of hybrid poplar (*Populus trichocarpa* x *P-deltoides*) to infection by *Melampsora medusae* leaf rust involves induction of flavonoid pathway genes leading to the accumulation of proanthocyanidins. *Molecular Plant-Microbe Interactions, 20*, 816–831.

Mohr, U., Lange, J., Boller, T., Wiemken, A., & Vogeli-Lange, R. (1998). Plant defence genes are induced in the pathogenic interaction between bean roots and *Fusarium solani*, but not in the symbiotic interaction with the arbuscular mycorrhizal fungus *Glomus mosseae*. *New Phytologist, 138*, 589–598.

Morazzoni, P., & Bombardelli, E. (1996). *Vaccinium myrtillus* L. *Fitoterapia, 67*, 3–29.

Muthuswamy, S., & Vasantha, R. H. P. (2007). Fruit phenolics as natural antimicrobial agents : Selective antimicrobial activity of catechin, chlorogenic acid and phloridzin. *International Journal of Food, Agriculture and Environment, 5*, 81–85.

Pandey, M. K., Sarma, B. K., Singh, D. P., & Singh, U. P. (2007). Biochemical investigations of sclerotial exudates of *Sclerotium rolfsii* and their antifungal activity. *Journal of Phytopathology, 155*, 84–89.

Pehkonen, T., Koskimäki, J. J., Riihinen, K., Pirttilä, A. M., Hohtola, A., Jaakola, L., et al. (2008). Artificial infection of *Vaccinium vitis-idaea* L. and defence responses to *Exobasidium* species. *Physiological and Molecular Plant Pathology, 12*, 146–150.

Pirttilä, A. M., Kämäräinen, T., Hirsikorpi, M., Jaakola, L., & Hohtola, A. (2001). DNA isolation methods for medicinal and aromatic plants. *Plant Molecular Biology Reporter, 19*, 273a–f.

Polya, G. M., & Foo, L. Y. (1994). Inhibition of eukaryote signal-regulated protein kinases by plant-derived catechin-related compounds. *Phytochemistry, 35*, 1399–1405.

Pozo, M. J., Azcón-Aguilar, C., Dumas-Gaudot, E., & Barea, J. M. (1999). β-1, 3-Glucanase activities in tomato roots inoculated with arbuscular mycorrhizal fungi and/or *Phytophthora parasitica* and their possible involvement in bioprotection. *Plant Science, 141*, 149–157.

Punyasiri, P. A. N., Tanner, G. J., Abeysinghe, S. B., Kumar, V., Campbell, P. M., & Pradeepa, N. H. L. (2004). *Exobasidium vexans* infection of *Camellia sinensis* increased 2, 3-cis isomerization and gallate esterification of proanthocyanidins. *Phytochemistry, 65*, 2987–2994.

Salzer, P., Feddermann, N., Wiemken, A., Boller, T., & Staehelin, C. (2004). *Sinorhizobium meliloti*-induced chitinase gene expression in *Medicago truncatula* ecotype R108-1: a comparison between symbiosis-specific class V and defence-related class IV chitinases. *Planta, 219*, 626–638.

Sauer, M., Lu, P., Sangar, R., Kennedy, S., Polishook, J., Bills, G., et al. (2002). Estimating polyketide metaholic potential among non-sporulating fungal endophytes of *Vaccinium macrocarpon*. *Mycological Research, 106*, 460–470.

Schulz, B., Römmert, A.-K., Dammann, U., Aust, H.-J., & Strack, D. (1999). The endophyte-host interaction: a balanced antagonism? *Mycological Research, 103*, 1275–1283.

Tolonen, A., & Uusitalo, J. (2004). Fast screening method for the analysis of total flavonoid content in plants and foodstuffs by high-performance liquid chromatography/electrospray ionization time-of-flight mass spectrometry with polarity switching. *Rapid Communications in Mass Spectrometry, 18*, 3113–3122.

Treutter, D. (2005). Significance of flavonoids in plant resistance and enhancement of their biosynthesis. *Plant Biology, 7*, 581–591.

Veluri, R., Weir, T. L., Bais, H. P., Termitz, F. R. S., & Ivanco, J. M. V. (2004). Phytotoxic and antimicrobial activities of catechin derivatives. *Journal of Agricultural and Food Chemistry, 52*, 1077–1082.

Vlassova, T., Likhachev, A. & Blintsov, A. (2000). Accumulation of abscisic acid in culture filtrates of selected species of genus *Botrytis*. (Paper presented at the XIIth International *Botrytis* symposium (p. 23) Reims, France)

White, T. J., Burns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White (Eds.), *PCR Protocols* (pp. 315–322). San Diego: Academic.

Witzell, J., & Shevtsova, A. (2004). Nitrogen-induced changes in phenolics of *Vaccinium myrtillus* - implications for interaction with a parasitic fungus. *Journal of Chemical Ecology, 30*, 1937–1956.