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Exobasidium vexans Infection of *Camellia sinensis* Increased 2,3-*cis* Isomerisation and Gallate Esterification of Proanthocyanidins

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ABSTRACT

Infection of leaves of tea [*Camellia sinensis* (Kuntze) L, cv TRI 2025] which was susceptible to blister blight *(Exobasidium vexans* Massee), resulted in a shift of the proanthocyanidin stereochemistry away from *2,3 trans* (e.g. catechin and gallocatechin) and towards *2,3-cis* (e.g. epicatechin and epigallocatechin). Infection also resulted in increased gallic acid esterification of the initiating subunits of proanthocyanidins. This was shown by both mass spectroscopy and phloroglucinolysis.

Proanthocyanidins isolated from healthy tissue had a predominantly *2,3- trans* stereochemistry which accounted for 53% and 61% of the total initiating and extension units of pro ant hocyani din, respectively. Conversely in infected tissue, proanthocyanidin subunits with a *2,3-trans* stereochemistry accounted for 26% and 40% of the total initiating and extension units, respectively.

Infection had little impact on the hydroxylation state of the Brings of proanthocyanidins. The products of acid hydrolysis under oxidative conditions had a slight excess of di-hydroxylated B-rings with cyanidin accounting for 58.3 ± 0.05% and 60.4 ± 0.2% of the total anthocyanidin recovered following hydrolysis of proanthocyanidin isolated from infected and healthy leaves, respectively. Sim-ilar results were obtained by phloroglucinolysis.

Keywords: *Camellia sinensis;* Tea; *Exobasidium vexans;* Proanthocyanidin stereochemistry; Gallic acid esters

INTRODUCTION

Blister blight is a leaf disease of tea [*Camellia sinensis* (Kuntze) L.] caused by the fungus *Exobasidium vexans* Massee, an obligatory parasite spread by windborne basidiospores. *E. vexans* is of major economic importance in all tea growing areas of Asia (Agnihothrudu and Moulli, 1991). The disease is evident as pale yellow translucent spots which appear on young leaves about nine days after infection. These spots become circular blisters after a further seven to nine days and the upper leaf surface becomes indented corresponding to protru-sion of a blister from the lower leaf surface. Tea is cul-tivated for its leaf and any leaf disease seriously affects production. *E. vexans* attacks only young succulent leaves and stems and reduces the quality and quantity of the leaf harvested. When young stems are infected the damage to the plant is more serious, as the infected stem breaks off and dies back, retarding growth and reducing crop production (Arulpragasam, 1992).

Flavonoids are the most abundant chemical group in tea leaves and of these, flavan-3-ol monomers dominate, contributing about 20-25% by dry weight of the leaf (Yao *et al.*, 2004). In addition to these major compounds, various proanthocyanidins have been reported in tea (Kiehne *et al.*, 1997; Lakenbrink *et al.*, 1999). In tea leaves, proanthocyanidins accumulate in vacuoles of particular cells which form 30-40% of mesophyll cells (Suzuki *et al.*, 2003).

Proanthocyanidins are widely distributed plant defense compounds (Treutter and Feucht, 1999) and have a general toxicity towards fungi, yeast and bacteria (Scalbert, 1991). Proanthocyanidins isolated from *Pinus sylvestris, Anona squamosa* and *Cassia javanice* are fun-gitoxic to *Rhizoctina solani* (Rao and Rao, 1986). Grape proanthocyanidins have also been shown to inhibit the macerating enzymes of *Botrytis cinerea* (Pezet *et al.*, 2003). Proanthocyanidin oligomers specifically and sensitively inhibit protein kinase and stilbene oxidase with IC50s in the micromolar range (Perret *et al.*, 2003; Polya and Foo, 1994). Galloylated flavonoids, such as epigal-locatechin gallate also specifically inhibit fatty acid synthase and lipoxygenase (Wang *et al.*, 2003; Skrzypczak-jankun *et al.*, 2003).

Two pathways lead to the biosynthesis of proanthocyanidins, either through catechin via leucoanthocyanidin reductase (Tanner *et al.*, 2003) or epicatechin via anthocyanidin reductase (Xie *et al.*, 2003). The detection of anthocyanidin reductase activity in tea leaves suggests that anthocyanidins are converted to corresponding epiflavan-3 -1s in this tissue (Punyasiri *et al.*, 2004).

Flavan 3 ols may be involved in the defense of tea leaves against *E. vexans* (Punyasiri *et al.*, 2001; and unpublished data). Higher levels of epicatechin were found in tea cultivars resistant to *E. vexans,* whereas levels of epigallocatechin gallate were higher in susceptible cultivars. The levels of proanthocyanidins increased roughly 10 fold during infection, while free epicatechin and epigallocatechin decreased after infection. The gallate esters, epicatechin gallate and epigallocatechin gal-late were more abundant in the initial stages of infection, but decreased as the blister attained maturity.

The present study was undertaken to isolate and characterise proanthocyanidins in healthy and *E. vexans* infected tea leaves to ascertain if fungal infection had any impact on the stereochemistry of proanthocyanidins in this species.

MATERIALS AND METHODS

Plant material

One cm leaf disks were harvested from field grown tea [*C. sinensis* (Kuntze) L, cv TRI 2025] (average daily temperature 24 ^{0}C, average night temperature 14 ^{0}C). This cultivar was susceptible to infection by *E. vexans* Massee. Leaf disks were harvested either from healthy leaves or blisters infected with *E. vexans* and immediately frozen in liquid nitrogen and freeze dried.

Purification of proanthocyanidin

A single proanthocyanidin extract was prepared from 20 g of freeze dried *C. sinensis* leaf disks from either healthy or *E. vexans* infected leaves. The proanthocyanidins were extracted in 70% (v/v) aqueous acetone and purified by solvent partition and chromatography on Sephadex LH20 as described by Tanner *et al.*, (1994). Reference proanthocyanidins were also similarly isolated and purified from *Hordeum vulgare* (barley) cv Himalaya, *O. viciifolia* (cv Othello) or *M. sativa* (cv Aquarius) seeds.

Purified *C. sinensis* proanthocyanidins were dissolved in methanol, separated by TLC on cellulose HPTLC plates (Merck) in water-saturated butan-2-ol and visual-ised by spraying with a 20 fold dilution in acetone of 2% (w/v) DMACA dissolved in 6 M HCl in ethanol (Li *et al.*, 1996).

Degradation of proanthocyanidins

Duplicate aliquots of methanol containing 1 mg purified *C. sinensis* proanthocyanidins were dried under nitrogen and hydrolysed under oxidative conditions in 0.1 ml aqueous 2 M HCl at 100 °C for 20 min, and 10 µl diluted with 90 µl of water and analysed by HPLC or TLC.

Purified *C. sinensis* proanthocyanidins were also subjected to phloroglucinolysis (Abrahams *et al.,* 2003). Flavan 3 ols were identified by monitoring the effluent at A280, and quantified by comparison with authentic standards (Sigma). Phloroglucinol adducts were identified by comparison with retention times produced following phloroglucinolysis of standard proanthocyanidins as described online. The average Dp of proanthocyanidins was calculated as the ratio of the sum of total extension and initiating units divided by the sum of total initiating units.

Mass spectroscopy of proanthocyanidins

Purified proanthocyanidins were dissolved in MeOH and mixed with 3β-indoleacrylic acid matrix solution as described by Hedqvist *et al.* (2000). Positive ion MALDI-TOF mass spectra were collected in reflectron mode averaging about 250 scans on a Voyager Elite mass spectrometer (perseptive Biosystems) with delayed extraction and an accelerating voltage of 20,000 V. Masses in the range 800—3000 Da were examined with external calibration against peptides of mass 927.4940 and 2045.0285, from a trypsin digest of bovine serum albumin (Campbell *et al.*, 2001).

RESULTS AND DISCUSSION

TLC and HPLC showed proanthocyanidins purified from infected *C. sinensis* leaves were uncontaminated by other flavonoids, however proanthocyanidins purified, from healthy leaves contained some epigallocatechin gallate. TLC showed both *C. sinensis* proanthocyanidin preparations appeared as a ladder of individual bands similar to *H. vulgare* proanthocyanidin, suggesting that the proanthocyanidins consisted of a range polymers from degree of polymerisation (Dp) 3 to at least 7.

Acid hydrolysis under oxidative conditions

Acid hydrolysis under oxidative conditions produces characteristic anthocyanidins from the extension units of proanthocyanidins, allowing an estimation of total di-and tri-hydroxylated B-rings. The products of acid hydrolysis of *C. sinensis* proanthocyanidin were cyanidin and delphinidin by TLC and HPLC (Fig. 1). On a molar basis there was a slight excess of cyanidin over delphinidin, with cyanidin accounting for 58.3 ± 0.05% and 60.4 ± 0.2% of the total anthocyanidin recovered following hydrolysis of proanthocyanidin isolated from infected and healthy leaves, respectively. This indicates that both proanthocyanidins contained more dithan tri-hydroxylated flavonoids.

No pelargonidin was observed in acid hydrolysates of proanthocyanidins isolated from healthy or infected leaves.

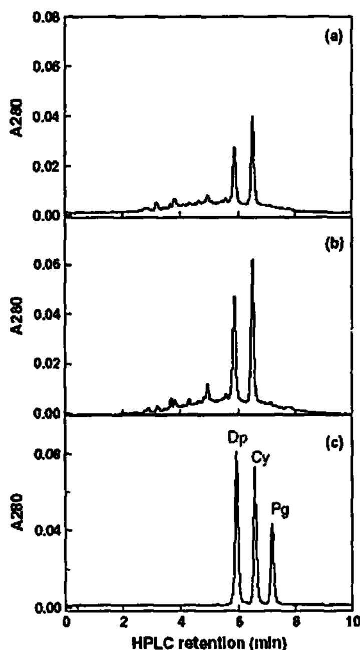

Fig. 1. Acid hydrolysates of *C. sinensis* proanthocyanidins. Proanthocyanidin purified from *C. sinensis* leaves either infected with *E. vexans* (a) or uninfected (b), were hydrolysed under oxidative conditions and the products resolved by HPLC and identified by comparison with authentic standards (c) cyanidin (Cy), delphinidin (Dp), or pelargonidin (Pg).

Phloroglucinolysis

Phloroglucinolysis (Jorgensen *et al.*, 2004) was used to quantitate the initiating and extension units of the PA polymer from both infected and healthy leaves. Individual purified and characterised phloroglucinol adducts could not be obtained to calibrate the phloroglucinolysis, which was instead calibrated using the retention times produced by phloroglucinolysis of well characterised proanthocyanidins.

Phloroglucinolysis of proanthocyanidin isolated from infected *C. sinensis* leaves yielded three dominant HPLC peaks (Fig. 2a), which were absent from chromatograms of untreated controls. The major peak represents the extension units (Fig. 2a, peak 6), and co-chromatographed with epicatechin (4ß-2) phloroglucinol, produced by phloroglucinolysis of procyanidin B2 and *Medicago sativa* (alfalfa) proanthocyanidin. This peak was resolved, but not completely separated from a minor peak which co-chromatographed with catechin-(4á-2)-phloroglucinol (Fig. 2a, peak 5), produced by phloroglucinolysis of procyanidins B3 and C2.

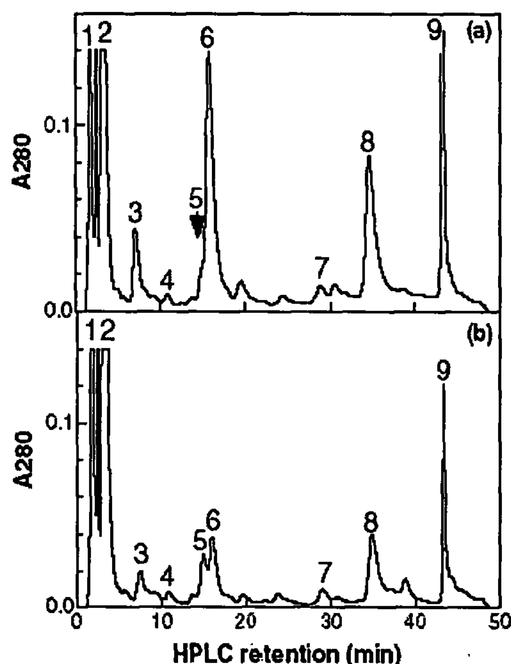

Fig. 2. Phloroglucinolysis of *C. sinensis* proanthocyanidins. Fifty micrograms of proanthocyanidin purified from *C. sinensis* leaves either (a) infected with *E. vexans* or uninfected (b), were subject to phloroglucinolysis and the products resolved by HPLC. Peaks were identified by comparison with authentic commercial standards or phloroglucinolysis products of known proanthocyanidins; ascorbate (1), phloroglucinol (2), gallocatechin-(4α-2)-phloroglucinol (3), gallocatechin (4), catechin-(4α-2)-phloroglucinol (5), epicatechin-(4β-2)-phloroglucinol (6), epicatechin (7), epigallocatechin gallate (8), and residual proanthocyanidin (9).

Unsuccessful attempts were made to improve the separation between peaks 5 and 6 (Fig. 2) by repeating or adapting published methods. The other major peak cochromatographed with authentic epigallocatechin gallate (Fig. 2a, peak 8), and represents the units which initiate proanthocyanidin polymerisation. A smaller peak corresponded to gallocatechin-(4á-2)-phloroglucinol (Fig. 2a, peak 3) produced by phloroglucinolysis of proanthocyanidins from *H. vulgare* and *Onobrychis viciifo-lia* (sainfoin) and represents gallocatechin extension units. There were also smaller peaks corresponding to gallocatechin, and epicatechin (Fig. 2a, peaks 4 and 7, respectively), representing flavan-3-ol initiating units. The initial peaks corresponded to ascorbate and phloroglucinol and the last peak contained residual proanthocyanidin.

The same compounds were present in chromatograms following phloroglucinolysis of proanthocyanidin from healthy *C. sinensis* leaves (Fig. 2b). However in this case, untreated control chromatograms of proanthocyanidin from healthy leaves contained 60% of the epigallocatechin gallate in the phloroglucinolysis chromatograms, indicating that the proanthocyanidin preparation from healthy leaves was contaminated with free epigallocatechin gallate. This contamination was subtracted from the total amount observed in the phloroglucinolysis extract. Quantitatively, the composition of the two proanthocyanidins differed, most notably in the relative increase of epicatechin-(4ß-2)-phloroglucinol and epigallocatechin gallate and the decreased levels of catechin-(4á-2)-phloroglucinol in proanthocyanidin isolated from infected leaves (Table 1).

The hydroxylation state of the B-ring of proanthocyanidin extension units may also be estimated by summing the total di- and tri-hydroxylated compounds derived from extension units (Table 1). This confirms that on a molar basis the proanthocyanidins contained mainly dihydroxylated B-rings (51% and 55% dihydroxylated B-ring in healthy and infected leaves, respectively) and compared well with the estimates produced by acid hydrolysis.

The majority of the peak area in chromatograms following phloroglucinolysis was assigned to known flavo-noids. In chromatograms from both infected and healthy *C. sinensis* leaves, the areas assigned to known standards accounted for over 70% of the total peak area, excluding ascorbate, phloroglucinol and undegraded proanthocyanidin. No suitable standard was available for unequivocal identification of epicatechin-3-*0*-gal late-(4ß-2)phloroglucinol resulting from epicatechin gallate extension units. This adduct has been reported to elute on HPLC between catechin and epicatechin (Kennedy and Jones, 2001) and no significant peak was seen in this area of our chromatograms. It is therefore likely that there were no significant amounts of this compound in the hydrolysates.

Table 1
Composition of *C. sinensis* proanthocyanidins

Compound	Uninfected nmol/50 μg proanthocyanidin (% of total recovered)		Infected nmol/50 μg proanthocyanidin (% of total recovered)	
Initiating units				
Gallocatechin	11.5 ± 3.8	(46.5%)	5.2 ± 1.1	(21.8%)
Epigallocatechin gallate	5.8 ± 1.6	(23.5%)	12.6 ± 1.0	(52.7%)
Epicatechin	5.8 ± 1.0	(23.5%)	5.1 ± 0.8	(21.3%)
Catechin	1.6 ± 0.3	(6.5%)	1.0 ± 0.5	(4.2%)
Extension units				
Gallocatechin-(4α-2)-phloroglucinol	21.1 ± 4.5	(38.8%)	31.3 ± 2.7	(36.7%)
Epicatechin-(4β-2)-phloroglucinol	21.1 ± 4.1	(38.8%)	51.4 ± 3.0	(60.3%)
Catechin-(4α-2)-phloroglucinol	12.2 ± 2.5	(22.4%)	2.6 ± 0.3	(3.1%)
Dp	3.2		4.6	

The composition of initiating or extension units of proanthocyanidins purified from *C. sinensis* leaves either uninfected or infected with *E. vexans*, was determined by phloroglucinolysis. Compounds were identified by comparison with authentic commercial standards or phloroglucinolysis products from known proanthocyanidins, and quantitated as described in methods. The means of triplicate hydrolyses ± SE are shown as nmol/50 μg proanthocyanidin, or in brackets as a percentage of the respective total recovered initiating or extension units. The Dp is calculated as described in the online supporting material.

For purified proanthocyanidins, an estimate of the degree of degradation is obtained by comparing the peak area due to residual proanthocyanidins in treated extracts with that in untreated controls. This suggested that there was 46.2 ± 4.8% and 38.9 ± 8.4% (mean of three phoroglucinolysis ± SE) degradation of proanthcyanidins from infected or healthy leaves, respectively. These values are typical of the yields observed with complex proanthocyanidins. It is therefore likely that the products of phloroglucinolysis are representative of the overall composition of the proanthocyanidins.

The average Dp calculated following phloroglucinolysis, was slightly greater for proanthocyanidins from infected tea leaves (Dp 4.6) compared to those isolated from healthy leaves (Dp 3.2) (Table 1).

Mass spectroscopy of proanthocyanidins

The mass spectra of proanthocyanidins from both infected leaves and healthy leaves were compared with the spectra of proanthocyanidins isolated from mature *H. vulgare* grains (Figs. 3a-c). The position of dimeric proanthocyanidins was obscured by matrix molecules and is not shown. Only very low abundance mass peaks were seen above 2200 and are not shown. *H. vulgare* testa/pericarp accumulates heteropolymers of catechin or gallocatechin units with Dp up to about 6 (Jende -Strid and Moller, 1981). Mass spectrometry does not distin-guish between catechin and its 3-hydroxyl epimer, epicatechin. However increasing B-ring hydroxylation of proanthocyanidin oligomers gives rise to a mass series which increase by 16, the weight of oxygen. Proanthocyanidins extracted from infected or healthy *C. sinensis* leaves, gave similar spectra to that obtained from *H. vulgare* proanthocyanidins, with (epi)catechin heteropolymers of Dp up to seven, containing one or more trihydroxylated B-rings (Figs. 3(a-c). Observed masses for oligomers were within 0.5 mass units of the calculated monoisotopic mass, e.g. masses of 1177.6, 1177.3 and 1177.5 were observed for (epi)catechin homotetramers from proanthocyanidin isolated from infected or healthy *C. sinensis* leaves, or *H. vulgare*, respectively (on-line supporting Tables 2, 3, and 4, respectively). These compare well to a mass of 1177.26 calculated for

the sodiated catechin homotetramer (online supporting Table 1). There was a similar correspondence between calculated and observed mass for larger polymers. The higher order oligomers were more, intense in the spectra of proanthocyanidins from healthy *C. sinensis* leaves compared with the spectra from infected leaves (Fig. 3a & b).

In addition to the series of heterooliogomeric pro-anthocyanidins seen in *H. vulgare,* another series of oligomers was observed in *C. sinensis,* formed by the addition of a single gallate ester to (epi)catechin oligomers. For example, masses corresponding to (epi) catechin trimers carrying 0, 1, 2, or 3 B-ring trihydroxyls and a single gallate moiety were observed in the spectra of proanthocyanidins isolated from infected *C. sinensis* leaves at masses of 1041.5, 1057.5, 1073.5, and 1089.5 (Fig. 3a), corresponding to the calculated masses of 1041.21, 1057.20, 1073.20 and 1089.19 for the corresponding sodiated ions. Similarly, gallate esters of tetramers, pentamers, hexamers and heptamers were also observed in the spectra of proanthocyanidins isolated from infected *C. sinensis* leaves (Fig. 3a). The intensity of the corresponding gallate esters was significantly less in the mass spectrum of proanthocyanidins isolated from healthy *C. sinensis* leaves, and galloylated proanthocyanidin oligomers were not detected with Dp above 4 in proanthocyanidins from healthy leaves (Fig. 3b).

In the spectra of proanthocyanidins from infected *C. sinensis* leaves, relatively small additional peaks were seen at masses of 1449.3 and 1737.4 corresponding to loss of one oxygen atom from an (epi) catechin homopentamer and hexamer, respectively. These peaks could represent a polymer with one mono-hydroxylated B-ring unit (pelargonidin like) with the rest of the units consist-ing of di-hydroxylated B-rings.

In spectra of all proanthocyanidin samples, low intensity peaks were observed two mass units below the more intense polymer peaks. For example, these peaks were seen in the spectra of the proanthocyanidins purified from infected leaves at masses of 1175.65, 1191.61, and 1207.62 each two mass units below the main peaks observed for tetramers with 0, 1, or 2 additional tri-hydroxylated B-rings, respectively. These might correspond to the loss of two hydrogens from the adjacent proanthocyanidin in the spectrum or two hydrogens and an oxygen (a dehydration) from the next molecule in the hydroxylation series. Such products have not previously been reported and it is not clear if this represents an artifact of preparation or an unreported native proanthocyanidin structure.

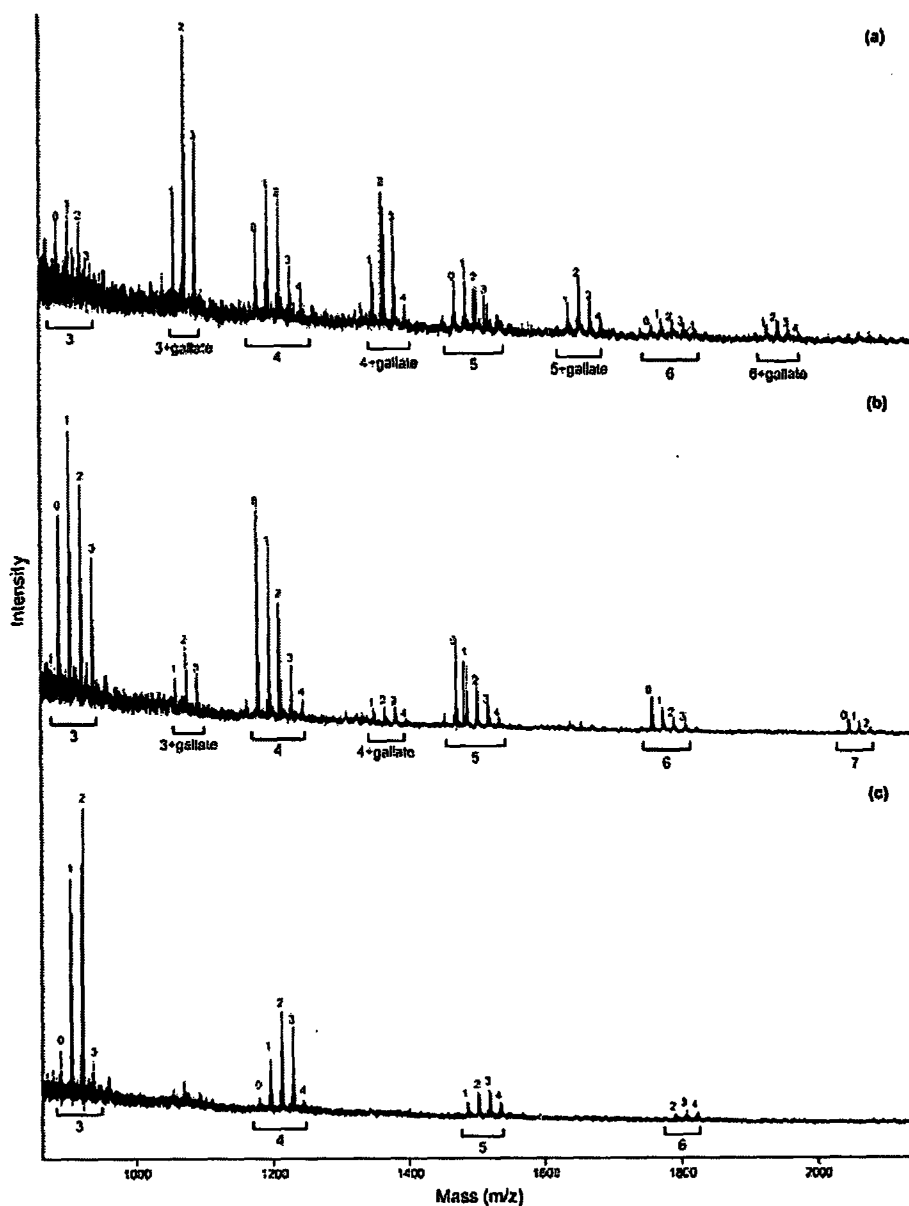

Fig. 3. Mass spectroscopy of *C. sinensis* proanthocyanidins. Proanthocyanidins were purified from *C. sinensis* leaves either infected with *E. vexans* (a) or uninfected (b) and examined by mass spectroscopy. Control proanthocyanidins were also isolated from *H. vulgare* (c). The degree of polymerisation is indicated below each group of peaks while the number of additional B-ring hydroxyls is indicated above each peak. See also online supporting tables of predicted and observed masses. Some very small peaks can be observed above the noise and are noted in the tables but not indicated here.

CONCLUSIONS

Infection resulted in a relative shift of the proanthocyanidin stereochemistry away from a *2,3-trans* stereo-chemistry (e.g. catechin and gallocatechin) and towards *2,3-cis* stereochemistry (e.g. epicatechin and epigallocatechin). Infection also resulted in increased gallic acid esterification of the initiating subunits of proanthocyanidins. These changes were inferred by changes observed in the relative composition of mass spectra of whole proanthocyanidins and changed subunit composition following phloroglucinolysis.

Phloroglucinolysis of *C. sinensis* proanthocyanidins indicated that the dominant terminal unit was gallocatechin in uninfected tissue and epigallocatechin gallate in infected tissue (Table 1). This is consistent with the identification of relatively more intense peaks in mass spec-tra of proanthocyanidins from infected tissue containing gallate esters.

The shift in proanthocyanidin stereochemistry was also reflected in changes in the relative composition of proanthocyanidin initiating and extension units determined by phloroglucinolysis (Table 1). In uninfected tissue these subunits had a predominantly *2,3-trans* sterochemistry which accounted for 53% and 61% of the total initiating and extension units, respectively.

Conversely in infected tissue the situation was reversed and subunits with a *2,3-trans* stereochemistry accounted for only 26% and 40% of the total initiating and extension units, respectively.

Infection had little impact on the hydroxylation state of the B-rings of proanthocyanidins. The products of acid hydrolysis (i.e. the proanthocyanidin extension units) indicated that there was a slight excess of di-hydroxylated compounds. Phloroglucinolysis gave simi-lar results.

Infection also resulted in a slight increase in the average Dp of *C. sinensis* proanthocyanidins. The average Dp in proanthocyanidins isolated from uninfected leaves was 3.2 compared to the average Dp of 4.6 for proanthocyanidins isolated from infected leaves (Table 1). Antioxidant activity has been reported to increase with the Dp for monomers, dimers and trimers of epicatechin (da Silva Porto *et al.*, 2003). It is not known if the differences in Dp observed here are physiologically significant.

MS revealed masses corresponding to heteropolymers of (epi)catechin and (epi)gallocatechin and the gallate esters of these. In addition MS detected relatively small mass peaks corresponding to oligomers containing units with one monohydroxylated Bring (i.e. propelargonidins). This is not unexpected, as mono-hydroxylated intermediates have been isolated from *C. sinensis* (Lakenbrink *et al.*, 1999), however they usually only accumulate in plants where the activity of the flavo-noid 3'-hydroxylase enzyme is blocked by mutation (e.g. in Arabidopsis, Abrahams *et al.*, 2002).

Acid hydrolysis detected subunits with only dior trihydroxylated B-rings, i.e. cyanidin and delphinidin, respectively. No pelargonidin was detected after acid hydrolysis (Fig. 1). Similarly, the corresponding mono-hydroxylated flavan-3-ol, afzelechin, was not detected,

following phloroglucinolysis (Fig. 2). We therefore estimate that the proportion of proanthocyanidin subunits with mono-hydroxylated B-rings was less than 1% of the total intermediate and terminal units. This is consistent with the absence of these compounds in chromato-grams but their presence in mass spectra.

The shift in proanthocyanidin stereochemistry away from *2,3-trans* and towards *2,3 cis* compounds may reflect relative changes in metabolic flux through the respective biosynthetic pathways. This may involve a relative decrease in flux through leucoanthocyanidin reductase (Tanner *et al.*, 2003) and a relative increase in flux through anthocyanidin reductase (Xie *et al.*, 2003). Anthocyanidin reductase activity has been demonstrated in enzyme extracts of *C. sinensis* and it is likely that this enzyme is responsible for production of epi-flavan-3-ols from anthocyanins in this tissue (Punyasiri *et al.*, 2004).

The change in stereochemistry could alter the biological properties of the proanthocyanidins which may have profound effects on invading fungi. The biological properties of flavonoids are sensitive to stereochemical changes. The *2,3-cis* isomer, epicatechin, reduced mem-brane fluidity more than *2,3-trans* isomer, catechin (Tsu-chiya, 2001). Different isomers of catechin have vastly different phytotoxicity; with (-)catechin being phyto-toxic at micro molar levels, whilst (+)catechin is not (Bais *et al.*, 2002, 2003). Gallic esters of catechins show higher antibacterial, antiviral and antioxidant activity than catechins without the galloyl ester (Kajiya *et al.*, 2001, 2002). It is possible that the increased resistance of some *C. sinensis* cultivars to *E. vexans* may be a result of higher levels of epicatechin or changed proanthocy-anidin composition (Punyasiri *et al.*, 2001; and unpublished data).

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