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Exobasidium vexans infection of *Camellia sinensis* increased 2,3-*cis* isomerisation and gallate esterification of proanthocyanidins

P.A. Nimal Punyasiri [a,*,1], Gregory J. Tanner [b,1], I. Sarath B. Abeysinghe [a],
Vijaya Kumar [c], Peter M. Campbell [d], N.H.L. Pradeepa [e]

[a] *Biochemistry Division, Tea Research Institute of Sri Lanka, Talawakelle, Sri Lanka*
[b] *CSIRO Plant Industry, GPO Box 1600, Canberra, ACT, 2601, Australia*
[c] *Department of Chemistry, University of Peradeniya, Peradeniya, Sri Lanka*
[d] *CSIRO Entomology, GPO Box 1700, Canberra ACT, 2601, Australia*
[e] *Plant Pathology Division, Tea Research Institute of Sri Lanka, Talawakelle, Sri Lanka*

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 proanthocyanidin, 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 B-rings of proanthocyanidins. The products of acid hydrolysis under oxidative conditions had a slight excess of di-hydroxylated B-rings with cyanidin accounting for $58.3 \pm 0.05\%$ and $60.4 \pm 0.2\%$ of the total anthocyanidin recovered following hydrolysis of proanthocyanidin isolated from infected and healthy leaves, respectively. Similar results were obtained by phloroglucinolysis.

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1. Introduction

Blister blight is a leaf disease of tea (*Camellia sinensis* (Kuntze) IK) caused by the fungus *Exobasidium vexans* Massee, an obligatory parasite spread by wind-borne 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 protrusion of a blister from the lower leaf surface. Tea is cultivated 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).

* Corresponding author.
E-mail address: pnimal@lycos.com (P.A. Nimal Punyasiri).
[1] Joint first authors.

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 fungitoxic 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 epigallocatechin 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-ols 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 gallate 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.

2. 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 *Hordeum vulgare* (barley) proanthocyanidin, suggesting that the proanthocyanidins consisted of a range polymers from degree of polymerisation (Dp) 3 to at least 7 (online supporting data, Fig. 1).

2.1. 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
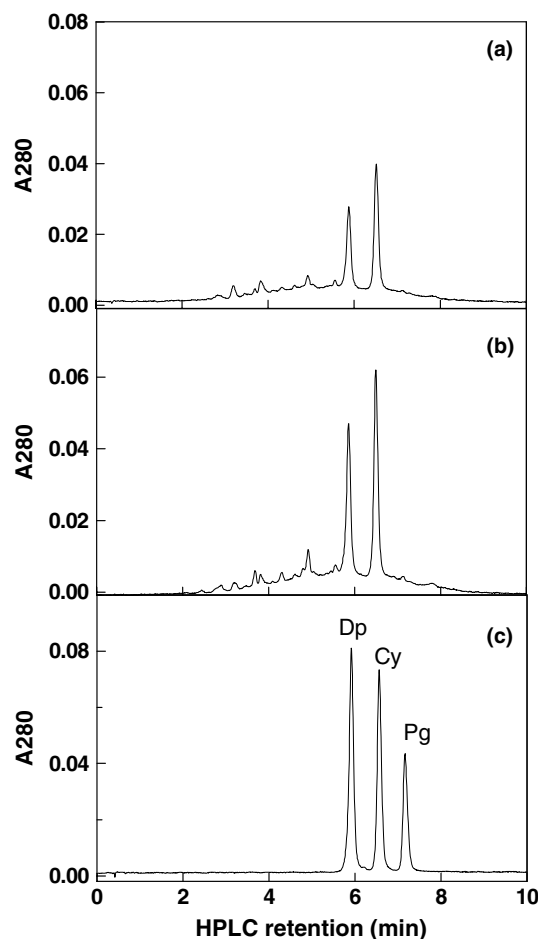

Fig. 1. Acid hydrolysates of *C. sinensis* proanthocyanidins. Proanthocyanidin purified from *C. sinensis* leaves either infected with *E. vexans* (a) or uninfected (b), were hydrolysed under oxidative conditions and the products resolved by HPLC and identified by comparison with authentic standards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

infected and healthy leaves, respectively. This indicates that both proanthocyanidins contained more di- than tri-hydroxylated flavonoids. No pelargonidin was observed in acid hydrolysates of proanthocyanidins isolated from healthy or infected leaves.

2.2. 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. 2(a)), which were absent from chromatograms of untreated controls. The major peak represents the extension units (Fig. 2(a), peak 6), and co-chromatographed with epicatechin-(4β-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-(4α-2)-phloroglucinol (Fig. 2(a), peak 5), produced by phloroglucinolysis of procyanidins B3 and C2. 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 co-chromatographed with authentic epigallocatechin gallate (Fig. 2(a), peak 8), and represents the units which initiate proanthocyanidin polymerisation. A smaller peak corresponded to gallocatechin-(4α-2)-phloroglucinol (Fig. 2(a), peak 3) produced by phloroglucinolysis of proanthocyanidins from *H. vulgare* and *Onobrychis viciifolia* (sainfoin) and represents gallocatechin extension units. There were also smaller peaks corresponding to gallocatechin, and epicatechin (Fig. 2(a), 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. 2(b)). 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-(4β-2)-phloroglucinol and epigallocatechin gallate and the decreased levels of catechin-(4α-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 di-hydroxylated B-rings (51% and 55% di-hydroxylated 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 flavonoids. 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-*O*-gallate-(4β-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
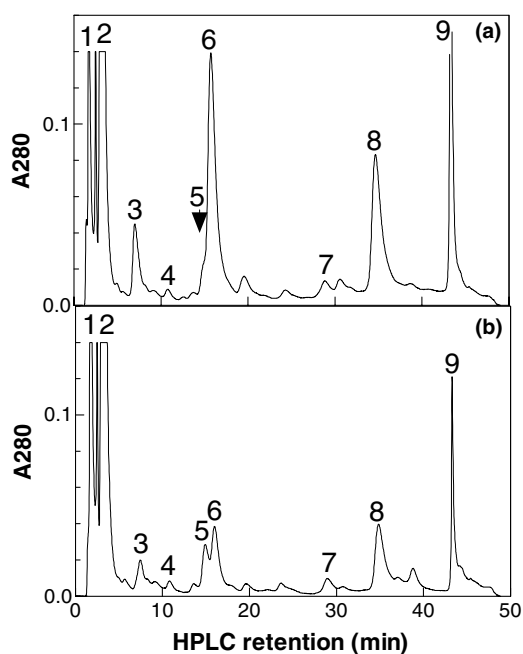

Fig. 2. Phloroglucinolysis of *C. sinensis* proanthocyanidins. Fifty micrograms of proanthocyanidin purified from *C. sinensis* leaves either (a) infected with *E. vexans* or uninfected (b), were subject to phloroglucinolysis and the products resolved by HPLC. Peaks were identified by comparison with 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).

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 ± 4.3	(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.

was seen in this area of our chromatograms. It is therefore likely that there were no significant amounts of this compound in the hydrolysates.

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 phloroglucinolysis ± SE) degradation of proanthocyanidins 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).

2.3. 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 (Fig. 3(a)–(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 distinguish 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 (online supporting Table 1). Proanthocyanidins extracted from in-

fected 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 tri-hydroxylated B-rings (Figs. 3(a)–(c); online supporting Tables 2, 3 and 4). Observed masses for oligomers were within 0.5 mass units of the calculated mono-isotopic mass, e.g. masses of 1177.6, 1177.3 and 1177.5 were observed for (epi)catechin homo-tetramers from proanthocyanidin isolated from infected or healthy *C. sinensis* leaves, or *H. vulgare*, respectively (online 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. 3(a) and (b)).

In addition to the series of hetero-oliogomeric proanthocyanidins 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. 3(a); online supporting Table 6), corresponding to the calculated masses of 1041.21, 1057.20, 1073.20 and 1089.19 for the corresponding sodiated ions (online supporting Table 5). Similarly, gallate esters of tetramers, pentamers, hexamers and heptamers were also observed in the spectra of proanthocyanidins isolated from infected *C. sinensis* leaves (Fig. 3(a); online supporting Table 6). The intensity of the corresponding gallate es-

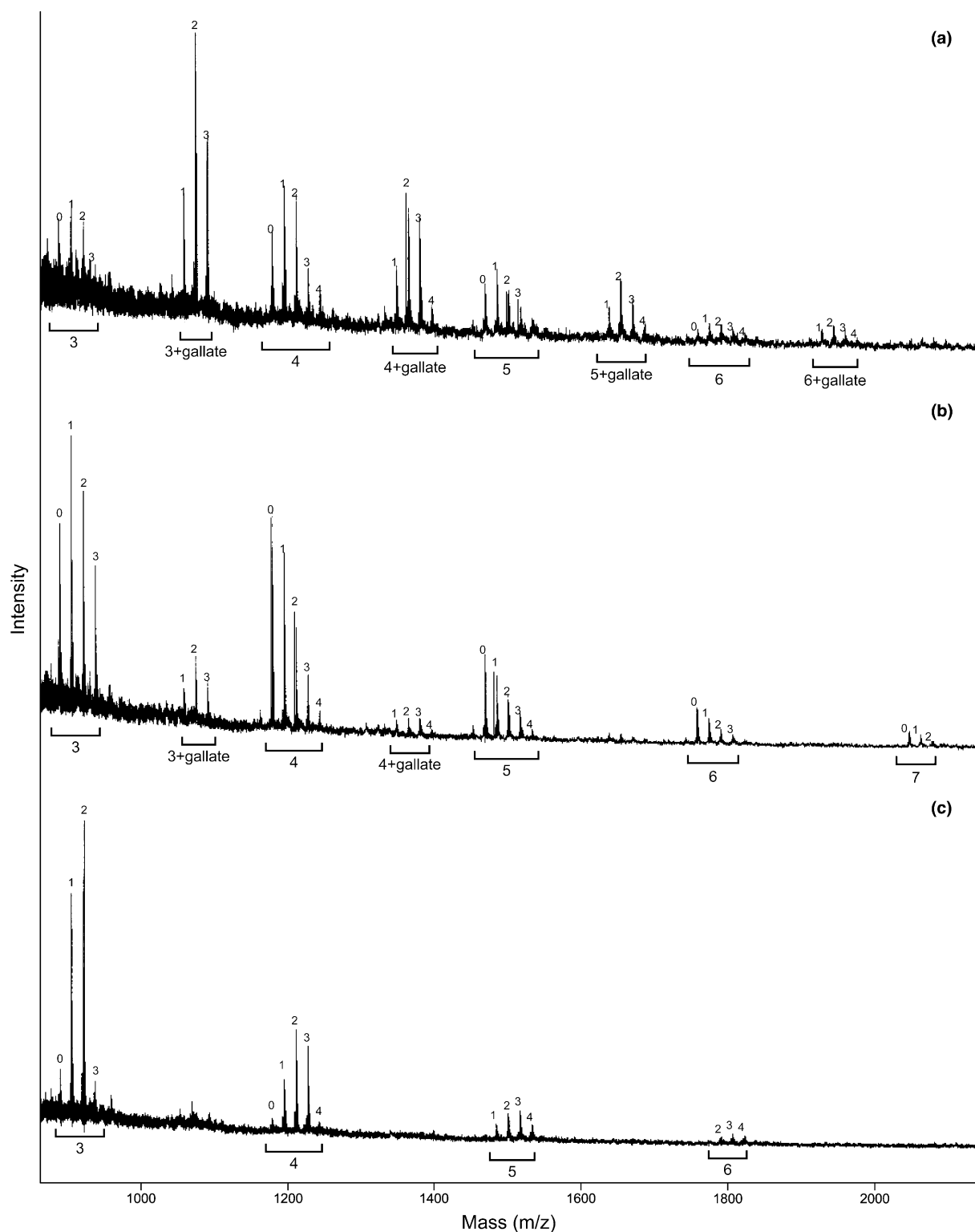Fig. 3. Mass spectroscopy of *C. sinensis* proanthocyanidins. Proanthocyanidins were purified from *C. sinensis* leaves either infected with *E. vexans* (a) or uninfected (b) and examined by mass spectroscopy. Control proanthocyanidins were also isolated from *H. vulgare* (c). The degree of polymerisation 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.

ters was significantly less in the mass spectrum of pro-anthocyanidins isolated from healthy *C. sinensis* leaves, and galloylated proanthocyanidin oligomers were not detected with Dp above 4 in proanthocyanidins from healthy leaves (Fig. 3(b); online supporting Table 7).

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 homo-pentamer and hexamer, respectively. These peaks could represent a polymer with one mono-hydroxylated B-ring unit (pelargonidin like) with the rest of the units consisting 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.

3. Conclusion

Infection resulted in a relative shift of the proanthocyanidin stereochemistry away from a 2,3-*trans* stereochemistry (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 spectra 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* stereochemistry 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 similar 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 mono-hydroxylated B-ring (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 flavonoid-3'-hydroxylase enzyme is blocked by mutation (e.g. in Arabidopsis, Abrahams et al., 2002).

Acid hydrolysis detected subunits with only di- or tri-hydroxylated 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 chromatograms 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 membrane fluidity more than 2,3-*trans* isomer, catechin (Tsuchiya, 2001). Different isomers of catechin have vastly different phytotoxicity; with (−)catechin being phytotoxic at micromolar 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 proanthocyanidin composition (Punyasiri et al., 2001; and unpublished data).

4. Material and methods

4.1. Plant material

One cm leaf disks were harvested from field grown tea (*C. sinensis* (Kuntze) L, cv TRI 2025) (average daily temperature 24 °C, average night temperature 14 °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.

4.2. 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 *H. vulgare* (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 visualised 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).

4.3. 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 as described in online supporting material.

Purified *C. sinensis* proanthocyanidins were also subjected to phloroglucinolysis (Abrahams et al., 2003) as described in online supporting material. 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.

4.4. 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 (Perspective 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).

Acknowledgements

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version at doi:10.1016/j.phytochem.2004.09.004.

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