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Preformed and induced chemical resistance of tea leaf against *Exobasidium vexans* infection

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Introduction

Blister blight caused by *Exobasidium vexans* Massee is a leaf disease in tea, *Camellia sinensis* (L.) O. Kuntze, that is important in tea-growing areas of Asia (Arulpragasam, 1992). *E. vexans* is an obligatory biotrophic fungus that completes its life cycle in the tea leaf (Gadd and Loos, 1948; Agnihothrudu and Moulli, 1991). The initial symptoms of the disease are lime green translucent spots, which appear 7-9 days after the infection. These spots develop into blisters that are ruptured releasing spores at the completion of the life cycle. Tea cultivars have been categorized as resistant and susceptible to blister blight (Balasooriya *et al.*, 1996), and some morphological and anatomical characters have been correlated with resistance (Martosupono, 1991). Little attempt has been made to correlate resistance with the chemical composition of tea leaf, although changes in saccharide metabolism (Pius *et al.*, 1998) and increased activity of polyphenol oxidase and peroxidase, and decrease in chlorophylls and carotenoids (Rajalakshmi and Ramarethinam, 2000) induced by blister blight infection have been shown. A preliminary report (Nagahaulla *et al.*, 1996) discusses the formation of phytoalexins, probably polyphenolic in nature, on blister blight infection. The variation in chemical composition and the quality of tea with increasing severity of blister blight has also been studied (Gulati *et al.*, 1999). Polyphenols are the major chemical constituents of tea, with the catechins being predominant. The role of phenolic substances in disease resistance is well documented (Vidhyasekaran, 1988; Nicholson and Hammerschmidt, 1992). Polyphenols are fungitoxic and antibacterial, with varying levels of toxicity to spore germination, mycelial growth, and fungal enzyme production (Vidhyasekaran, 1988). Caffeine has been shown to play a role in the resistance of tea to attack by the shot-hole borer, *Xyleborus fornicatus* (Kumar *et al.*, 1995). The present study was initiated to investigate the role, of flavan-3-ols (catechins) and methylxanthines on preformed or induced chemical resistance of tea leaf against *E. vexans*.

Proanthocyanidins are widely distributed plant defense compounds and have a general toxicity towards fungi, yeast and bacteria. Therefore another 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.

Although the tea plant is rich in secondary metabolites their role in disease resistance has not yet been elucidated. In this study an attempt was made to identify any chemical / biochemical factors inherent in the tea plant which prevent the blister blight infection. The flavonoid biosynthesis of the tea plant was studied in order to understand how enzymes are regulated during fungal infection and to observe any changes in the flavonoid biosynthetic pathway provoked by *E.vexans* infection.

Methodology

Plant material: Tea plants used in this study were grown in experimental plots at the Tea Research Institute of Sri Lanka, Talawakelle, Sri Lanka. The plants were raised by vegetative propagation using standard procedures. Tender tea leaves (two apical leaves and the bud) from tea cultivars belong to groups resistant (clones DT1, TRI777, TRI2043, N2, TRI4067, TRI4052, NAY3, TRI) and susceptible (clones TRI2025, TRI2024, TRI2023, TRI62/5. TRI3015, TRI3014, TRI62/1) to *E. vexans*.

1. Plant materials were analysed using High Performance Liquid Chromatography (HPLC) for (a) catechin [(+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate and (-)-epigallocatechin gallate] content, (b) caffeine content and c. flavonol content as aglycones (quercetin, kaempferol, myricetin).
2. Blister blight infected leaf materials belonging to 3 stages of infection (Translucent stage, mature blister-1 and mature blister-2) were also similarly analysed for catechin, caffeine and flavonol content. In addition theobromine and proanthocyanidin content of these leaf materials were also determined by HPLC.
3. Presence of proanthocyanidins was confirmed using a. histochemical staining (whole leaf and transverse sections), b. thin layer chromatography, c. radial diffusion assay and acid-catalysed depolymerisation by Butanol/HCl assay.
4. Proanthocyanidins from healthy and blister blight infected tea leaves were separated using LH-20 column chromatography and their structures confirmed using phloroglucinol hydrolysis and through CSIRO, Australia, using matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS).
5. The occurence of leucoanthocyanidins (flavan-3,4-diols) was proved by supplementation experiments with flower petals of mutant *Mattihola incana*
6. The anthocyanidins of red tea cultivars TRI 2043 were identified using paper and thin chromatography

UV-Visible spectroscopy and HPLC.

7. Fungitoxicity of anthocyanin mixture of TRI 2043 was evaluated through the spore fall technique.
8. Enzymology of the flavonoid biosynthetic pathway enzymes (chalcone synthase, chalcone isomerase, flavanone-3-hydroxylase, dihydroflavanone-4-reductase, leucoanthocyanidin-4-reductase, flavanone-4-reductase) was studied using a. radiolabelled substrates (^{14}C) in collaboration with University of Munich, Germany, b. Thin layer chromatography, and c. Bioimage analyser to quantify radioactivity. The enzyme anthocyanidin reductase was assayed using a non-radiolabelled method and the enzyme dihydroflavanone-4-reductase by heterologously expressing it in yeast.

Results

Levels of (-)-epicatechin in tea cultivars (*Camellia sinensis*) resistant to blister blight leaf disease (*Exobasidium vexans*) were significantly higher than those in susceptible cultivars, while the reverse was true for (-)-epigallocatechin gallate suggesting that epicatechin was involved in the resistance mechanism. The content of the methylxanthines, caffeine and theobromine in the leaf increased significantly in the initial translucent stage of the disease, probably as a defense response to fungal attack. Epicatechin and epigallocatechin levels were significantly less than in healthy tissues at this stage, but increases in the corresponding gallate esters suggested that they were being converted into esters. Although epicatechin and epigallocatechin levels decreased from translucent to mature blister stages, the decrease was not significant. The decrease in levels of epicatechin, epigallocatechin and their esters on infection and the formation of cyanidin and delphinidin on oxidative depolymerization of the blisters suggest that proanthocyanidins may play a role in the defense mechanism. The very high resistance of a purple-green leafed cultivar is attributed to the additional catechin source provided by the high levels of anthocyanins present. Cyanidin and delphinidin were identified as two anthocyanidins in the red tea cultivar TRI 2043 (Punyasiri *et al.*, 2005)

Infection of leaves of tea which were susceptible to blister blight 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 ± 0.05% and 60.4 ± 0.2% of the total anthocyanidin recovered following hydrolysis of proanthocyanidin isolated from infected and healthy leaves, respectively. Similar results were obtained by phloroglucinolysis (Punyasiri *et al.*, 2004a).

Leaves of tea contain extraordinarily large amounts of (-)-epigallocatechin, (-)-epicatechin, (+)-gallocatechin, and (+)-catechin and derivatives of these compounds that show positive effects on human health. The health-promoting effects of flavan 3-ols, especially those of green tea, are of scientific and public interest. Furthermore, they play a crucial role in defense against pathogens of tea. Therefore, biosynthesis of these flavonoid compounds was investigated. The anthocyanidin reductase enzyme recently described from *Arabidopsis* and *Medicago* was shown to be present in tea with very high activity and produces epicatechin as well as epigallocatechin from the respective anthocyanidins, thus explaining the very high contents of these compounds. A strong combined dihydroflavonol-4-reductase/leucoanthocyanidin-4-reductase activity was demonstrated and catalyzes the key steps in catechin and gallocatechin formation. Therefore in this study we have elucidated the activities of chalcone synthase (CHS), chalcone isomerase (CHI), flavanone-3-ß-hydroxylase (FHT), dihydroflavonol-4-reductase (DFR), leucoanthocyanidin-4-reductase (LAR), flavanone-4-reductase (FNR), and anthocyanidins reductase (ANR) in the tea plant for the first time (Punyasiri *et al.*, 2004c)

Conclusion

This study provides us with an elegant tool for use in plant breeding programmes to screen blister blight resistance of tea cultivars. Breeding for resistance is very important in a crop where transgenic or genetic manipulations are not permitted and use of pesticides discouraged. In addition, the metabolic profiling of catechins present could be employed as a chemotaxonomic yard stick to classify tea cultivars. The formation and enhancement of proanthocyanidins as a defense response after blister blight attack is a relatively new finding, although the occurrence of these compounds in tea leaf has been known for a long time. The finding of the shift in stereochemistry upon fungal infection from 2,3-*trans* to 2,3-*cis* is reported for the first time. The biological activity of the stereoisomers differ markedly. The fungitoxicity of various types of proanthocyanidins on *E.vexans* has not yet been studied and is worthy of investigation. The partial characterisation of the pigments of the red cultivar TRI2043 was also carried out for the first time and the anthocyanins present in this cultivar were shown to be

fungitoxic.

The flavonoid biosynthetic pathway in tea leaf has not been reported although the pathway for many other polyphenol accumulating plants is well documented. This is the first detailed study on the enzymology of flavonoid biosynthesis in the tea plant, where the activities of CHS, CHI, FHT, DFR, LAR, FNR and ANR have been demonstrated. The combined DFR/ LAR reaction was separately studied by heterologous expression of tea DFR, and the requirement of LAR for catechin formation was confirmed. The DFR sequence has been deposited to the GenBank (AY49420).

Anthocyanidin reductase which was first reported in 2003 from *A. thaliana* has been shown in this study to occur in tea. This provided an explanation for the presence of high levels of catechins with 2,3-*cis* stereochemistry like epicatechin and its derivatives in tea. With the information gathered on the enzymology of the flavonoid pathway of the tea plant it would be possible to engineer a tea plant with either catechin or epicatechin type flavan-3-ol by manipulating the expression of corresponding enzymes LAR or ANR respectively.

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Ceremonial Inauguration of the Graduateship Programme in Chemistry, 2006

Ceremonial inauguration of the Graduateship Programme in Chemistry 2007/2011 will be held at the PPGL Siriwardene Auditorium, Adamantane House on the 14th October 2006 at 10.30 a.m..

Prof. E R Jansz, Senior Professor of Biochemistry, Department of Biochemistry, Faculty of Medicine, University of Sri Jayewardenepura will be the Chief Guest at the Inauguration.

'Entrance Scholarship & Merit Bursary Test' will be held for all registered students at 1.00 p.m.