Accumulation of Caffeoyl-D-quinic Acids and Catechins in Plums Affected by the Fungus *Taphrina pruni*

Claus T. Fuchs and Gerhard Spiteller

Institut für Organische Chemie I, Universität Bayreuth, Universitätsstr. 30, D-95440 Bayreuth, Germany

Z. Naturforsch. **53c,** 799–805 (1998); received February 20/April 16, 1998

Taphrina pruni, Plums, Catechins, Caffeoyl-D-quinic Acids, Photometric Determination

Plums (*Prunus domestica*) affected by the fungus *Taphrina pruni* and healthy ones were harvested in intervals of about four days. Photometric comparison of their methanolic extracts proved that infected fruits contained ten times more compounds with phenolic hydroxyl groups. Further structure elucidation and quantification of these phenolic differences by gas chromatography and gas chromatography / mass spectrometry revealed that the content of caffeoyl-D-quinic acid isomers and (+)-catechin had changed: The amount of chlorogenic acid and its isomers was increased in infected fruits about 15 times compared to non-affected ones. Contrary, (+)-catechin content was decreased. Additional photometric assays demonstrated that (+)-catechin reduction is accompanied by a corresponding increase of proanthocyanidins in infected fruits. All the compounds identified in infected plums in increased concentrations had a common structural feature: they were *o*-diphenols. After oxidation to corresponding *o*-quinones they are able to add to substances with active hydrogen atoms, e.g. fungal enzymes. Consequently, the accumulation of a high concentration of *o*-diphenols may be a defence response directed towards fungal enzymes.

Introduction

Plum trees (*Prunus domestica*) were attacked by the fungus *Taphrina pruni*: Just after fruit formation the diseased plums grew abnormally fast. They showed a curved shape and their surface became yellowish green. Within three weeks the affected plums were three times bigger than unaffected ones. Finally they desiccated and fell off the tree.

These striking external symptoms of affected plums stimulated us to investigate if the abnormal growth was linked to corresponding differences in chemical constituents.

Changes in plum constituents caused by *Taphrina pruni* have not been investigated so far. Nevertheless, some fungal metabolites were previously reported: Straight chain fatty acids (Sancholle *et al.,* 1977), indolyl acetic acid (IAA) (Hirata, 1971) and 4-pentadecylpyridine (Fuchs *et al.,* 1995) were detected.

Results

Three weeks after plum blossom the symptoms of fungal infection were clear enough to enable an unambiguous separation of affected and non-affected plums. From that time on both kinds of fruits were harvested from the same tree every fourth day and processed exactly in the same way by extraction with aqueous methanol. Apart from a doubling in growth and fresh weight, infected fruits showed an increase in the amount of extractable substances (Fuchs, 1996) indicating enhanced metabolic activity.

Since previous reports demonstrated the formation of various phenolic defence compounds in *Rosaceae* (Kokubun *et al.,* 1994; Treutter *et al.,* 1990a; Treutter *et al.,* 1990b), we investigated the total phenolic concentration using a photometric determination of phenolic hydroxyl groups in analogy to Price and Butler (Price and Butler, 1977). This assay is based on the generation of a darkgreen prussiate complex which is detected at 720 nm.

Abbreviations: GC: Gas chromatography; GC/MS: Gas chromatography/Mass spectrometry; DMACA: Dimethylamino cinnamic aldehyde; PPO: Polyphenoloxidase.

Reprint requests to Prof. Spiteller.
Fax: 0921/552671.
E-mail: gerhard.spiteller@uni-bayreuth.de.

0939–5075/98/0900–0799 $ 06.00

After derivatisation with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) the obtained fractions were separated by GC and GC/MS. Caffeoyl-D-quinic acid isomers **(1)**, **(2)**, **(3)** and (+)-catechin **(4)** (Scheme 1) were identified due to their characteristic mass spectra (Fuchs *et al.*, 1996; Fuchs, 1996):

The amount of these phenols was determined by addition of standard compounds (dihydrochlorogenic acid for caffeoyl-D-quinic acids **(1)**, **(2)**, **(3)** respectively (-)-epicatechin for (+)-catechin **(4)**) as internal standards.

The changes in contents of caffeoyl-D-quinic acids **(1)**, **(2)**, **(3)** and (+)-catechin **(4)** during the growth of infected fruits are illustrated in figures 1 and 2.

Since gain in chlorogenic acid isomers **(1)**, **(2)**, **(3)** outweighs (+)-catechin **(4)** reduction for infected fruits, total concentration of phenolic hydroxyl groups is increased.

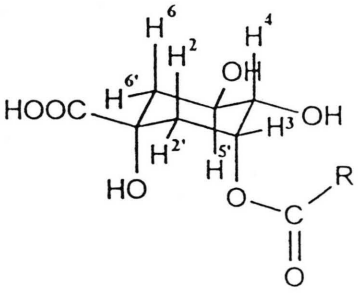
Previous investigations (Kokubun *et al.*, 1994; Treutter *et al.*, 1990a; Treutter *et al.*, 1990b) demonstrated an increase of catechin concentration in fruits after elicitor treatment for *Rosaceae*. Therefore we checked if the observed reduction in plums affected with *Taphrina pruni* is a result of further catechin metabolism to condensed tannins (proanthocyanidins) (Scalbert, 1991) and catechinglycosides (Harborne, 1988), well-known high molecular metabolites of (+)-catechin **(4)**.

After acidic depolymerisation proanthocyanidins were determined as red coloured anthocyanidins (Porter *et al.*, 1986). Fortunately, the monomer ((+)-catechin **(4)**) produces no colour reaction due to its reduced stability under the applied conditions (Watterson *et al.*, 1983).

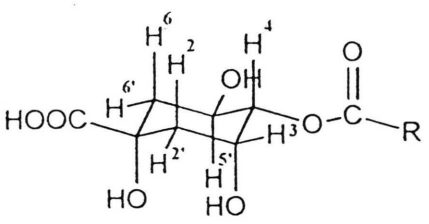
Contrary to the procedure of Porter *et al.* (Porter *et al.*, 1986), the total amount of catechins ((+)-catechin **(4)**, catechinglycosides and proanthocyanidins) is achieved by electrophilic addition of vanillin (Price *et al.*, 1978).

This reaction is restricted to flavonoids with two metasubstituted hydroxyl groups, a saturated C-2 – C-3 bond and no carbonyl group at C-4 (Sarkar *et al.*, 1976).

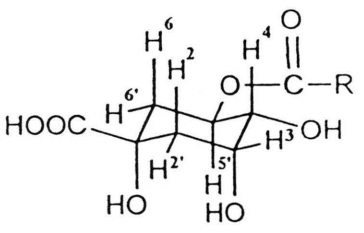
If all catechins are summed up – (+)-catechin, catechinglycosides and proanthocyanidins – the content in infected fruits surmounts that in non-infected ones for a factor of about 8 (Fig. 3A).



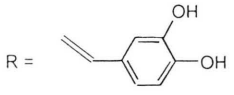
3-*O*-caffeoyl-D-quinic acid (neochlorogenic acid) **(1)**

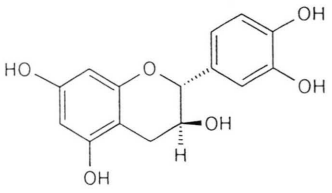


4-*O*-caffeoyl-D-quinic acid (cryptochlorogenic acid) **(2)**



5-*O*-caffeoyl-D-quinic acid (chlorogenic acid) **(3)**





(+)-Catechin **(4)**

Scheme 1. Phenolic compounds identified in different amounts in infected and non-infected plums.

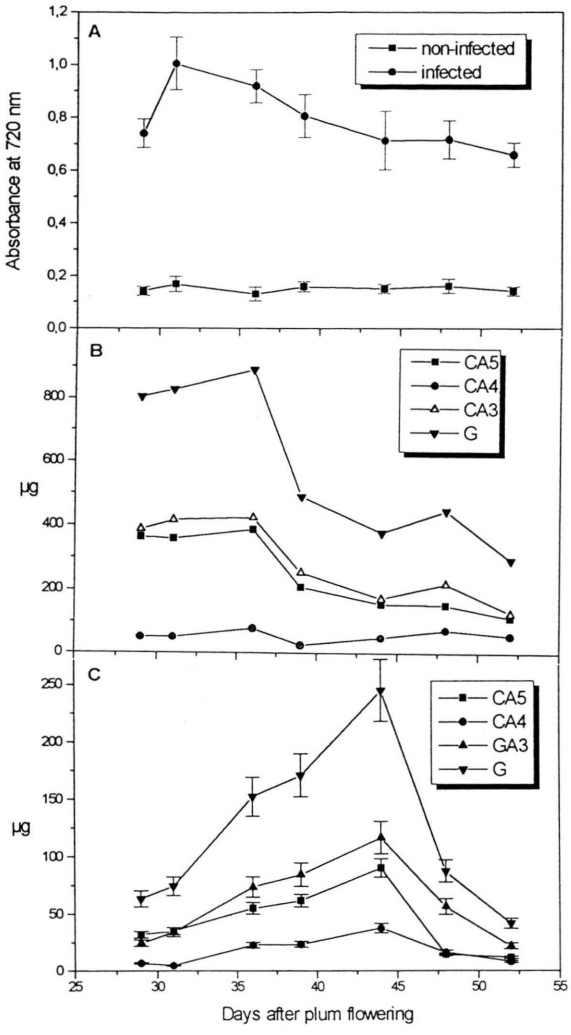


Figure 1A: Total concentration of phenolic hydroxyl groups in infected and non-infected plums. 1B: Change in the content of caffeoyl-D-quinic acids (**1**), (**2**), (**3**) in infected fruits. 1C: Change in the content of caffeoyl-D-quinic acids (**1**), (**2**), (**3**) in non-infected fruits.

Dimethylamino cinnamic aldehyde (DMACA) shows analogous reactivity to metasubstituted diphenols. Nevertheless, the more reactive dimethylamino group shifts the absorbance maximum towards higher wavelength (reduced probability of disturbing absorbance) and makes the reaction less sensitive to traces of water and acid (Sierotzky and Gessler, 1993; Treutter, 1989). The measurement with DMACA confirms the results of the vanillin assay: Infected plums have eight times more total catechins than non-infected ones (Fig. 3B).

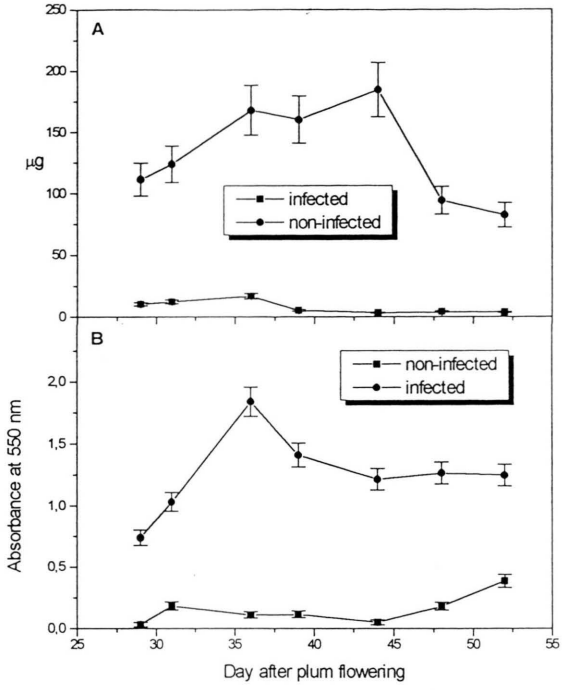
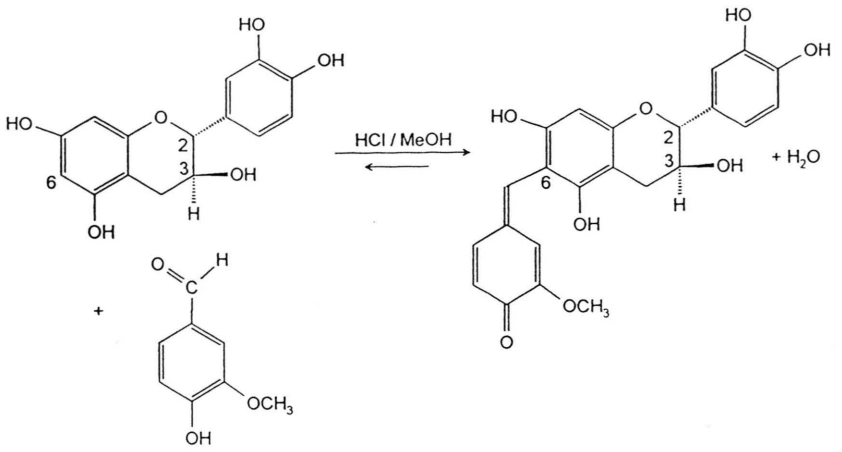


Figure 2A: Comparison of the content of (+)-catechin (**4**) in infected and non-infected fruits. 2B: Concentration of proanthocyanidins (condensed tannins) in infected and non-infected fruits.

Our results prove that the total amount of catechins ((+)-catechin **(4)**, catechinglycosides and proanthocyanidines) (Figs 3A and 3B) – especially proanthocyanidins (Fig. 2B) – increased in plums after infection with *Taphrina pruni*. Obviously this fact is caused by a shift of plum metabolism from the monomeric (+)-catechin **(4)** to polymeric proanthocyanidins.

The biological relevance of these flavanols is corroborated to their ability for protein complexation (Bae *et al.*, 1993; Balde, 1993). Whereas monomeric (+)-catechin **(4)** has low effects (Mason *et al.*, 1987; Okuda *et al.*, 1985; Ezaki-Furnichi *et al.*, 1987), binding affinity considerably increases with molecular weight of proanthocyanidins (polymeric catechins) (Mason *et al.*, 1987; Ezaki-Furnichi *et al.*, 1987; Porter *et al.*, 1984; Kumar, 1986; Artz, 1987). This increasing binding affinity is paralleled by enhanced fungal toxicity (Brownlee *et al.*, 1990; Haars *et al.*, 1981; Grant *et al.*, 1976). In general, it seems reasonable that polymerisation of monomeric (+)-catechin **(4)** to condensed tannins is a defence strategy towards fungal infection.



Scheme 2. Electrophilic addition of vanillin to catechins.

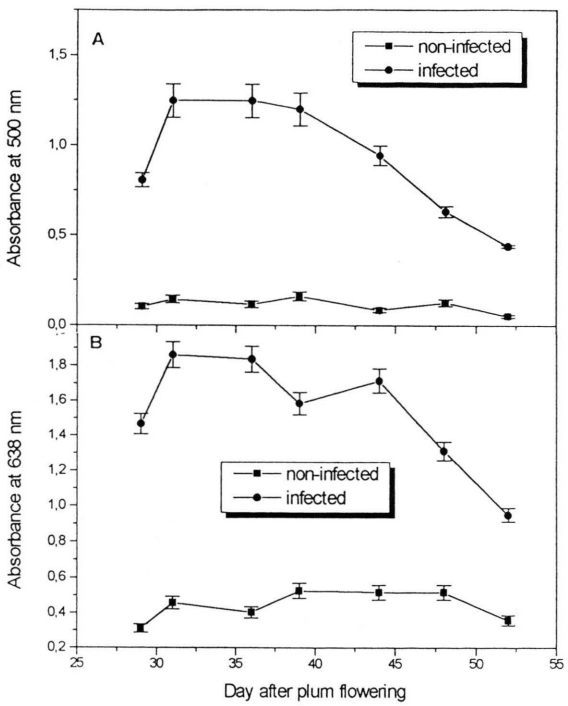


Figure 3A: Total amount of catechins ((+)-catechin (**4**), catechinglycosides and proanthocyanidins) in infected and non-infected fruits by the vanillin assay. 3B: Total amounts of catechins ((+)-catechin (**4**), catechinglycosides and proanthocyanidins) in infected and non-infected fruits by the DMACA-assay.

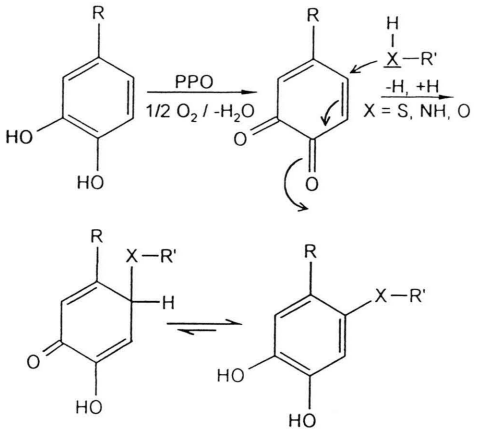
The second main difference was a dramatic increase in caffeoyl-D-quinic acid concentration in infected fruits.

Chlorogenic acid **(3)** is a widespread phenol (Dickinson *et al.*, 1954). It accumulates within several plants as a response to infection (Smith *et al.*, 1981; Lyons *et al.*, 1990).

Both, caffeoyl-D-quinic acids as well as catechins, have a common structural feature: They are *o*-diphenols and may be oxidised to the corresponding quinones by polyphenoloxidase (PPO) (Lerch, 1987).

Several reports demonstrate such an increase in polyphenoloxidase (PPO)-activity after infections or wounding (Matta *et al.*, 1970; Pitt, 1975), oxidising *o*-diphenol derivatives like caffeoyl-D-quinic acids and catechins to toxic *o*-quinones. Consequently, host resistance respectively pathogen infectibility is directly correlated to enhanced PPO-activity (Bashan *et al.*, 1987; Leon, 1971).

Though chlorogenic acid has relatively low toxicity itself (Rhodes, 1978), the corresponding quinone is an efficient defence compound towards pathogen infection (Felton *et al.*, 1990; Felton *et al.*, 1987; Felton *et al.*, 1989). The assumption that these oxidation reactions participate in defence reactions is corroborated by the observation that susceptibility is enhanced by quinone reduction (Moustafa *et al.*, 1970; Mukherjee *et al.*, 1975). Trajkowski found that host resistance in *Ribes* to the pathogen *Spaeotheca* depends on the levels and interaction of polyphenoloxidase, chlorogenic acid **(3)** and a reducing agent such as ascorbic acid (Trajkowski, 1976). This host resistance is directly correlated to quinone toxicity and their ability to add to substances with active hydrogen atoms, e.g. lysine-, cysteine- or tyrosine residues (Pierpoint *et al.*, 1977; Pierpoint, 1966; Auf' Mkolk, 1985) (Scheme 3):



Scheme 3. Toxicity of *o*-diphenols: Addition of *o*-diquinones to S-, N- and O-containing nucleophils. PPO: Polyphenoloxidase (EC 1.14.18.1).

Considering that fungal enzymes are S- and N-containing compounds, they are inactivated by the corresponding quinones (Mason and Wassermann, 1987; Goldstein and Swain, 1963; Hayes, 1989). Thus the accumulation of a high concentration of *o*-diphenols in infected plums is a defence response directed to the enzymes of the pathogen. Nevertheless, the effectiveness of this defence response is highly dependent on quinone formation respectively PPO-activity.

In summary, one can assume that the increase in the concentration of caffeoyl-D-quinic acids and catechins in infected fruits and subsequent oxidation to toxic ortho-quinones is the response of plums to pathogen infection.

Experimental

(-)-Epicatechin, vanillin, dimethylamino cinnamic aldehyde and chlorogenic acid were obtained from Fluka Chemie AG, Neu-Ulm, Germany.

MSTFA was purchased from Macherey and Nagel, Düren, Germany.

Analytical GC was carried out using a WCOT glass capillary OV 101 column (30 m x 0.3 mm), temperature program from 80 °C to 280 °C at 3 °C min^{-1}. The temperature of injector and detector (FID) were kept at 270 °C and 290 °C, respectively. Carrier gas was H_2 and the split ratio 1:30. Retention indices were calculated in a linear approximation according to Kováts (Kováts 1958) with n-alkanes (C_{10}-C_{36}) as reference compounds.

GC-MS measurements were performed on a fused silica gel column DB1 (30 m x 0,3 mm) with the same temperature program used for analytical GC. The column was connected to a double-focusing mass spectrometer. EIMS were recorded at 70 eV.

Extraction

50 g of infected and non-infected plums were harvested near Bayreuth at intervals of about 4 days from one tree. The fruits were frozen in liquid nitrogen, homogenised and extracted four times with 500 ml MeOH/H_2O (9:1 v/v) at 20 °C.

Quantification

For quantification 200 µg of (-)-epicatechin and 200 µg dihydrochlorogenic acid per g fresh weight were added. After evaporation of the solvent in vacuum the residue was redissolved in H_2O and extracted three times with 300 ml of each of the following solvents: cyclohexane (Cy), ethylacetate (EA) and butanol (BuOH).

The EA fractions were subjected to GC and GC/MS measurement. The FID signals were calculated with a Merck Hitachi Chromatointegrator D-2500.

Hydrogenation of chlorogenic acid was performed as previously described (Fuchs, 1996).

Colour reactions

Proanthocyanidin method

Proanthocyanidin determination was performed in analogy to Porter *et al.* (Porter *et al.*, 1986):

Butanol reagent: 0.7 g ferrous sulphate heptahydrate was added to 50 ml of conc. HCl. Then the flask was filled up with *n*-butanol to 1 l.

2 ml butanol reagent and 200 µl sample (solution: 10 ml MeOH/g of the methanolic extract) were added to a reaction vessel. After mixing, the closed vessel was heated for an hour in a boiling water bath. Finally, the solution was cooled to room temperature and the absorbance was measured at 550 nm.

Blank: 50 ml of HCl was substituted by 50 ml of water.

Vanillin method (Price *et al.*, 1978; Sarkar *et al.*, 1976)

Vanillin reagent: Two solutions were prepared: One containing 8% HCl in methanol and another containing 1% vanillin (0.15 g vanillin in 15 g methanol) in abs. methanol. Equal volumes of both solutions were combined.

The sample (dissolved in 10 ml abs. methanol / g methanolic extract) and 1.5 ml reagent solution were tempered separately in a thermomixer (600 rpm; 20 minutes at 30 °C). After the addition of 200 µl sample to 1.5 ml reagent solution the mixture was kept for another 20 minutes at 30 °C. Finally the absorbance was read at 500 nm.

Blank: Mixture of 1.5 ml reagent solution and 200 µl abs. methanol.

Price and Butler method (Price and Butler, 1977)

10 µl sample (10 ml MeOH/H_2O 1:1 per g of the methanolic extract) were dissolved in 25 ml of distilled water in a Erlenmeyer flask. Then 3 ml of a 0.1 M $FeCl_3$ * 6 H_2O solution were added and mixed. 3 minutes later 3 ml of a 0.008 M aqueous $K_3Fe(CN)_6$ solution were added and mixed. After 15 minutes the absorbance was read at 720 nm.

DMACA assay (Sierotzky and Gessler, 1993; Treutter, 1989)

25 µl sample (10 ml absolute methanol per g methanolic extract) was incubated with 1 ml DMACA-reagent (0.1% DMACA in HCl/MeOH 8%). After three minutes the absorbance was read at 638 nm.

Trimethylsilylation

The samples were dissolved in dry ethylacetate and MSTFA was added. The mixture was allowed to stand at room temperature for at least 8 h.

Mass spectra

(+)-catechin **(4)**: 650(19), 383(4), 355(24), 282(8), 267(11), 179(10), 147(13), 73(68). RI = 2900.

5-*O*-Caffeoyl-D-quinic acid (chlorogenic acid) **(3)**; RI 3150

786(19), 771(3), 462(2), 397(9), 396(7), 345(100), 307(42), 255(41), 219(12), 191(6), 147(8), 73(34).

4-*O*-Caffeoyl-D-quinic acid (cryptochlorogenic acid) **(2)**; RI 3210

786(9), 771(3), 579(4), 489(14), 447(12), 419(3), 396(11), 373(12), 324(24), 307(100), 255(51), 219(9), 191(4), 147(5), 73(17).

3-*O*-Caffeoyl-D-quinic acid (neochlorogenic acid) **(1)**; RI = 3240

786(4), 771(6), 447(38), 419(8), 396(14), 345(93), 307(100), 255(28), 219(16), 147(9), 73(21).

Mass spectra of caffeoyl-D-quinic acids were assigned as previously reported (Fuchs *et al.*, 1996).

Acknowledgements

We are grateful to Deutsche Forschungsgemeinschaft for financial support of this work. Furthermore, we are obliged to Mr. M. Gläßner for measurement of the mass spectra and Mr. W. Kern for destillation of the solvents.

Artz W. E., Bishop P. D., Dunker A. K., Schanus E. G. and Swanson B. G. (1987), Interaction of synthetic proanthocyanidin dimer and trimer with bovine serum albumine and purified bean globulin fraction G-1. J. Agric. Food Chem. **35**, 417–421.

Bae H. D., McAllister T. A., Muir A. D., Yanke L. J., Bassendowski K. A. and Cheng K.-J. (1993), Selection of a method of condensed tannin analysis for studies with rumen bacteria. J. Agric. Food Chem. **41**, 1256–1260.

Balde A. M. (1993), Proanthocyanidins from stem bark of *Paretta owariensis*. Part 2. Dimeric and trimeric proanthocyanidins possessing a doubly linked structure from *Paretta owariensis*. J. Nat. Prod. **56**, 1078–1087.

Brownlee H. E., McEuen A. R., Hedger J. and Scott I. M. (1990), Anti-fungal effects of cocoa tannin on the witches' broom pathogen *crinipellis perniciosa*. Physiol. Mol. Plant Pathol. **36**, 39–48.

Dickinson D. and Gawler J. H. (1954), Chemical constituents of Victoria plums: preliminary qualitative analysis. J. Sci. Food Agric. **5**, 525–529.

Ezaki-Furnichi E., Nonaka G., Nishioka I. and Hayashi K. (1987), Affinity of proanthocyanidins (condensed tannins) from the bark of *Rhaphiolepis umbellata* for proteins. Agric. Biol. Chem. **51**, 115.-120

Felton G. W., Duffey S. S., Vail P. V., Kaya H. K. and Manning I. (1987), Interaction of nuclear polyhedrosis virus with catechols. J. Chem. Ecol. 13, 947–957.

Felton G. W., Donato K., Del Vecchio R. J. and Duffey S. S. (1989), Activation of plant foliar oxidases by insect feeding reduces nutritive quality of foliage for noctuid herbivores. J. Chem. Ecol. **15**, 2667–2694.

Felton G. W. and Duffey S. S. (1990), Inactivation of *Baculovirus* by quinones formed in insect-damaged plant tissue. J. Chem. Ecol. **16**, 1221–1236.

Fuchs C. and Spiteller G. (1995), 4-Pentadecylpyridine: A metabolite from *Taphrina pruni*. Z. Naturforsch. **50c**, 766–768.

Fuchs C. and Spiteller G. (1996), Rapid and easy identification of isomers of coumaroyl- and caffeoyl-D-quinic acid by Gas Chromatography / Mass Spectrometry. J. Mass Spectrom. **31**, 602–608.

Fuchs C. (1996), Ph. D. Thesis, University of Bayreuth.

Grant W. D. (1976), Microbial degradation of condensed tannins. *Science* **193**, 1137–1139.

Haars A. Huettermann A. and Chet I. (1981), Effect of phenolic compounds and tannin on growth and laccase activity of *Fomes annosus*. Eur. J. Forest Pathol. **11**, 67–76.

Hirata S. (1971), Comparison of the abilities of phytopathogenic fungi to produce indoleacetic acid. CA, **77**:85387.

Kokubun T. and Harborne J. B. (1994), A survey of phytoalexin induction in leaves of the *Rosaceae* by copper ions. Z. Naturforsch. **49c**, 628–634.

Kováts E. (1958), Gaschromatographische Charakterisierung organischer Verbindungen. Helv. Chim. Acta **41**, 1915–1932.

Lerch K. (1987), Monophenol monooxygenase from *Neurospora crassa*. Methods Enzymol. **142**, 165–169.

Lyons P. C., Wood K. V. and Nicholson R. L. (1990), Caffeoyl ester accumulation in corn leaves inoculated with fungal pathogens. Phytochemistry **29**, 97–101.

Lyr H. (1965), Inhibition by oxidized polyphenols. Phytopathol. Z. **52**, 229–235.

Mason T. L. and Wasserman B. P. (1987), Inactivation of red beet ß-glucan synthase by native and oxidized phenolic compounds. Phytochemistry **26**, 2197–2202.

Matern U. and Kneusel R. E. (1988), Phenolic compounds in plant disease resistance. Phytoparasitica **16(2)**, 153–170.

Matta A. and Abbattista G. I. (1970), Differential inhibition and activation of polyphenoloxidase activity in healthy and *Fusarium*-infected tomato plants. Phytopathol. Mediterr. **9**, 168–173.

Moustafa F. A. and Whittenbury R. (1970), Properties which appear to allow phytopathogenic *pseudomonads* to counteract plant defence. Phytopathol. Z. **67**, 214–217.

Mukherjee P. K. and Gosh J. J. (1975), Quantitative changes in pectic substances in rice leaves infected with *Helminthosporum oryzae*. Sci. Cult. **41**, 433–439.

Okuda T., Nonaka G. and Hayashi K. (1985), Effects of the interaction of tannins with coexisting substances. Part III. Relationship of the structures of tannins to the binding activities with hemoglobin and methylene blue. Chem. Pharm. Bull. **33**, 1424–1433.

Pierpoint S., Ireland R. J. and Carpenter J. M. (1977), Modification of proteins during the oxidation of leaf phenols: Reaction of potato virus x with chlorogenoquinone. Phytochemistry **16**, 29–34.

Pierpoint W. S. (1966), The enzymic oxidation of chlorogenic acid and some reactions of the quinone produced. Biochem. J. **98**, 567–580.

Pitt D. (1975), Changes in the subcellular location of catalase and o-diphenol oxidase during infection of potato tubers by *Phytophthora erythroseptica*. Trans. Br. Mycol. Soc. **65**, 91–95.

Porter A. J. and Woodruffe J. (1984), Haemanalysis: The relative astringency of proanthocyanidin polymers. Phytochemistry **23**, 1255–1260.

Porter L. J., Hristich L. N. and Chan B. C. (1986), The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry **25**, 223–230.

Price M. L., van Scoyoc S. and Butler L. G. (1978), A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. J. Agric. Food Chem. **26**, 1214–1218.

Price M. L. and Butler L. G. (1977), Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. J. Agric. Food Chem. **25**, 1268–1273.

Rhodes J. M. (1978), Biochemistry of Wounded Plant Tissues. Berlin: Walter de Gruyter & Co.

Sancholle M. and Schneider A. (1977), Multiplication cellulaire et production lipidiqui en culture in vitro chez *Taphrina institutiae* et *Taphrina pruni*, parasites de *Prunus domestica*. Physiol. Plant. **40**, 250–253.

Sarkar S. K. and Haworth R. E. (1976), Specifity of the vanillin test for flavanols. J. Agric. Food Chem. **24**, 317–320.

Scalbert A. (1991), Antimicrobial properties of tannins. Phytochemistry **30**, 3875–3883.

Smith B. G. and Rubery P. H. (1981), The effects of infection by *Phytophthora infestans* on the control of phenylpropanoid metabolism in wounded potato tissue. Planta **151**, 535–540.

Trajkovski V. (1976), The mechanism of resistance of *Ribes nigrum* to *sphaerotheca mors-uvae* (Schw.) Berk. Swed. J. Agric. Res. **6**, 215–223.

Treutter D. and Feucht W. (1990a), Accumulation of flavan-3-ols in fungus-infected leaves of *Rosaceae*. Z. Pflanzenkr. Pflanzenschutz **97**, 634–639.

Treutter D. and Feucht W. (1990b), The pattern of flavan-3-ols in relation to scab resistance of apple cultivars. J. Hortic. Sci. **65**, 511–517.