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DIFERULIC ACID AS A COMPONENT OF CELL WALLS OF *LOLIUM MULTIFLORUM**

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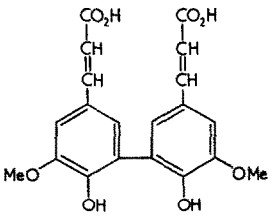
Key Word Index—*Lolium multiflorum*; Gramineae; cell walls; lignin-carbohydrate linkages; *trans,trans*-diferulic acid; *cis,trans*-diferulic acid; *cis,cis*-diferulic acid; *cis* and *trans* ferulic acids; *cis* and *trans* *p*-coumaric acids.

Abstract—*Trans,trans*-, *cis,trans*- and *cis,cis*-diferulic acids were released from cell walls of *Lolium multiflorum* by treatment with sodium hydroxide. The isomers were apparently bound via ester links to the structural carbohydrates of the cell walls. Sodium hydroxide treatment gave, per g of wall, 0.18 mg *trans,trans*-diferulic, 0.02 mg *cis,trans*-diferulic and a trace of *cis,cis*-diferulic acids compared with 5.3 mg *trans*-ferulic, 1.2 mg *cis*-ferulic, 0.78 mg *trans*-*p*-coumaric and 0.12 mg *cis*-*p*-coumaric acids. The significance of these acids in lignin biosynthesis is discussed. The effect of UV light on the *trans,trans* isomer and its fully silylated trimethylsilyl ether derivative was also investigated.

INTRODUCTION

Several workers [1–3] have reported that ferulic and *p*-coumaric acids are released from cell walls of the Gramineae by treatment with NaOH. It had been suggested [4] that these acids are linked to highly polymerised lignin ("core lignin") but it has since been shown [5–7] that treatment of cell walls of leaf laminae of *Lolium multiflorum* with cellulase releases a series of carbohydrate esters of mainly *trans*-ferulic acid (FA) together with some *cis*-FA and *trans* and *cis*-*p*-coumaric acid (PCA). In the work reported here we have examined cell walls of *L. multiflorum* for the presence of bound diferulic acid (DFA, **1**). Although DFA can exist in the *trans,trans*, *cis,trans* and *cis,cis* forms, the separation and properties of these isomers have not been reported. It has therefore been necessary to study these compounds including investigations of changes in isomer composition caused by UV or daylight.



(**1**)

RESULTS AND DISCUSSION

Diferulic acid synthesised by published methods gave one spot when subjected to TLC (Solvent 1, 2 or 3). The acid gave a purple coloration when the plates were exposed to UV light (365 nm) either before or after treatment with ammonia vapour. When the plates were sprayed with diazotised *p*-nitraniline reagent, the acid gave a purple coloration which faded on spraying with sodium carbonate solution. The acid showed low sensitivity to diazotised sulphanilic acid reagent. Mass spectrometry of the acid (probe) 70 eV gave *m/e* (rel. int.): 386 (M^+, 4), 342 (M^+-44; 100), 324 (M^+-62; 90), 298 (M^+-88; 62), 309 (M^+-77; 26), 307 (M^+-79; 24), 343 (M^+-43; 21), 325 (M^+-61; 21), 165 (M^+-221; 18), 281 (M^+-105; 17) and 283 (M^+-103; 14).

TLC of a methanolic solution of the acid which had been exposed to UV light or daylight gave three spots, including a spot corresponding to the original acid, in Solvent 1 or 3, but only one spot in Solvent 2 corresponding to the original acid. All spots showed the same behaviour as the original acid when visualised by UV light or sprayed with diazotised *p*-nitraniline.

This behaviour on TLC might be expected if the original acid was partially converted to its two other geometrical isomers. The *trans* isomers of the substituted cinnamic acids behaved similarly due to their partial conversion to the corresponding *cis* forms ([8] and Table 1), the *trans* isomers having lower R_f values. Hence consideration of R_f values suggests that synthesised DFA is the *trans,trans* isomer (lowest value) and the products of its exposure to UV light are the *cis,trans* and *cis,cis* (highest value) isomers. Solvent 3 is a new basic solvent which gives good resolution of these isomers.

GLC separation was only possible on the TMSi ethers using a column in which the concentration of stationary phase (OV-25) was reduced to 0.2%. The R_t (min) of the *trans,trans* isomer was 10.2, *cis,trans* 6.3 and *cis,cis* 4.4. The order of separation was as expected from comparison with the relative R_t's of the TMSi ethers of the monomeric cinnamic acid isomers using OV-25 stationary phase where the *trans* isomers have higher R_t's [8]. GLC–MS of the TMSi ethers of the DFA isomers was carried out with a spectrometer having a two-stage jet separator (LKB 9000) which allowed the effluent of each component in the mixture from GLC to enter directly: an AEI 902 spectrometer with membrane separators showed low sensitivity to these compounds. *M/e* (rel. int.) of the TMSi ether derivative of *trans,trans*-DFA was 674

* Part 4 in the series "Lignin-carbohydrate linkages in plant cell walls." For Part 3 see ref. [7].

Table 1. TLC of the geometrical isomers of diferulic acid and of substituted cinnamic acids

Acid	R_f value in Solvent 1	Solvent 2	Solvent 3
Trans,trans-diferulic	0.24	0.08	0.22
Cis,trans-diferulic	0.31	0.08	0.39
Cis,cis-diferulic	0.47	0.08	0.68
Trans-p-coumaric	0.34	0.10	0.64
Cis-p-coumaric	0.67	0.10	0.88
Trans-ferulic	0.24	0.38	0.63
Cis-ferulic	0.59	0.38	0.86
Trans-sinapic	0.17	0.22	0.52
Cis-sinapic	0.53	0.22	0.99
Trans-caffeic	0.20	0	0.55
Cis-caffeic	0.57	0	—*

For solvent key, see Experimental. * Streak of R_f 0.1–0.6 possibly due to decomposition.

(M^+, 41), 73 (M^+-601; 100), 675 (M^++1; 31), 676 (M^++2; 19), 75 (M^+-599; 19), 659 (M^+-15; 8), 407 (M^+-267; 7), 558 (M^+-116; 6), 379 (M^+-295; 6), 147 (M^+-527; 6) and 381 (M^+-293; 4): of *cis,trans*-DFA 674 (M^+, 41), 73 (100), 675 (31), 75 (21), 676 (19), 558 (9), 659 (8), 147 (8), 407 (7), 379 (5) and 381 (4): and of *cis,cis*-DFA 674 (M^+, 5), 75 (77), 558 (40), 559 (20), 381 (15), 560 (13), 543 (7), 147 (6), 675 (3) and 659 (1).

Attempts were made to isolate the geometrical isomers of DFA using preparative TLC. Only small amounts of the acid (0.1 mg/plate of width 20 cm) could be separated by Solvent 1, larger amounts causing streaking. Good separations were obtained with Solvent 3 using 4 mg per plate but partial conversion of the *cis,trans* isomer to *trans,trans* and of the *cis,cis* isomer to *cis,trans* occurred during subsequent manipulation even though daylight was excluded [8].

Confirmation of the structures of the suspected geometrical isomers was obtained by PMR spectroscopy by comparison of the *trans,trans*-DFA with mixtures of all three isomers obtained from it by treatment of solutions with UV or daylight. The PMR signals (270 MHz; Fourier Transform) of synthesised DFA in $(CD_3)_2CO$ were: δ4.10 (6H, *s*, -OMe), 6.53 (2H, *d*, J = 16.2 Hz, *trans* –CH=CH–), 7.32 (2H, *d*, J = 2.2 Hz, aromatic H), 7.46 (2H, *d*, J = 2.2 Hz, aromatic H), 7.76 (2H, *d*, J = 16.2 Hz, *trans* –CH=CH–) confirming it as the *trans,trans* isomer. The deuterated acetone solution was exposed to daylight for 30 min, and TLC then indicated a ratio of *trans,trans*- to *cis,trans*- of 3:1, traces of the *cis,cis* isomer also being present. The PMR spectrum of this mixture showed the same signals as the *trans,trans* isomer together with δ4.01 (*s*, –OMe), 5.97 (*d*, J = 13.2 Hz, *trans*, –CH=CH– of the *cis,trans* isomer), 7.02 (*d*, J = 13.2 Hz, *trans* –CH=CH– of the *cis,trans* isomer), 7.30 (*d*, aromatic H), 7.41 (*d*, aromatic H), 7.45 (shoulder on δ = 7.46, *cis* –CH=CH– of the *cis,trans* isomer), 8.03 (broad *s*, *cis* –CH=CH– of the *cis,trans* isomer). The ratio of amounts of isomers indicated by peak areas agreed with the ratio estimated by TLC: the *cis,cis* isomer was not detected by PMR.

UV spectra (Table 2) showed, in general, that the maxima of *trans,trans*-DFA were higher than those of the mixture of isomers. This could be due to the presence, in the mixture, of isomers containing the *cis* –CH=CH– group [9]. Major bands in the IR spectrum of *trans,trans*-DFA were as follows (KBr, ν_{max} cm^{-1}): 3483 (-OH), 1692 (-COOH), 1627 (>C=C< conjugated to aromatic ring), 1594 (>C=C< conjugated to aromatic ring), 1494 (aromatic >C=C<), 1454, 1412, 1377, 1284, 1262, 1142, 1077, 1048, 957, 859 and 812. In addition, there was a band at 980 cm^{-1} which could be due to the *trans* –CH=CH– group [10].

The relative amounts of the three isomers of DFA obtained by treatment of the *trans,trans* isomer with UV light for 2 hr were determined by conversion to the fully silylated derivatives followed by GLC: the ratio of *trans,trans*-DFA to *cis,trans*-DFA to *cis,cis*-DFA was 4.4:4.7:1. The TMSi ether derivative of *trans,trans*-DFA was much less affected by UV light than the free acid: after exposure of the derivative for 9 hr, the ratio of *trans,trans* to *cis,trans* to *cis,cis* TMSi derivative was 26:15:1.

Treatment of cell walls of *L. multiflorum* with NaOH followed by two-dimensional TLC (first experiment, Solvent 2 followed by Solvent 1, second experiment Solvent 2 followed by Solvent 3) of the liberated phenols, indicated the presence of *trans,trans* and *cis,trans*-DFA together with the *cis* and *trans* isomers of FA and PCA. Small amounts of other phenols were also detected. Confirmation of the presence of DFA was obtained by subjecting a mixture of the liberated phenols to preparative

Table 2. Comparison of UV spectra of *trans,trans*-diferulic acid with those of a mixture of geometrical isomers of the acid

Isomer	UV absorption* in H_2O–MeOH (11.5:1) λ_{max}(nm)	Log ϵ	MeOH λ_{max}(nm)	Log ϵ	0.1 N NaOH λ_{max}(nm)	Log ϵ	N NaOH λ_{max}(nm)	Log ϵ
Trans,trans-diferulic acid	218	4.37	216 sh	4.33	270	4.34	244 sh	4.26
	241	4.38	243	4.35	320	4.46	322 sh	4.32
	306 sh	4.43	311 sh	4.44	329 sh	4.45	358	4.58
	318	4.44	324	4.47				
Mixture of the 3 geometrical isomers of diferulic acid	218	4.47	216 sh	4.39	270	4.50	244 sh	4.39
	241	4.47	243 sh	4.43	318	4.50	310 sh	4.38
	310	4.45	305 sh	4.42			355	4.56
			324	4.46				

sh—shoulder. * 16 mg diferulic acid/l soln, 1 cm cells.

TLC (Solvent 2) and silylating the material in the band having the R_f value of DFA. GLC–MS showed the presence of the TMSi ethers of both *trans,trans* and *cis,trans*-DFA, the mass spectra being similar to the reference spectra. Traces of the corresponding *cis,cis* isomer were also present.

The total amount of DFA liberated was 0.20 mg/g cell wall as estimated by TLC (Solvent 2) by comparison with reference *trans,trans*-DFA. Total DFA was not estimated by GLC as the TMSi derivatives of the acid had similar R_t values to TMSi derivatives of some other components released from the cell walls. The ratio of *trans,trans* to *cis,trans* isomers was 9:1 estimated by preparative TLC (Solvent 2) followed by GLC of the TMSi derivatives. It should be pointed out that, even though exposure to daylight was avoided, some conversion could have occurred of the *cis,trans* isomer to *trans,trans* and of the *cis,cis* isomer to the *cis,trans*. The amounts of other liberated phenolic acids estimated by GLC of their fully silylated TMSi derivatives in comparison with the corresponding derivatives of the reference *trans* acid were, per g of cell wall, *trans*-FA 5.3 mg, *cis*-FA 1.2 mg, *trans*-PCA 0.78 mg and *cis*-PCA 0.12 mg.

The bonding of DFA units to cell walls was also investigated. After cell walls of *L. multiflorum* were treated with a commercial cellulase preparation (*Oxyporus* sp., a Basidiomycete), no phenolic material could be extracted from the filtrate with ether. NaOH treatment of the filtrate yielded *trans,trans* and *cis,trans*-DFA together with *cis* and *trans* isomers of both FA and PCA. Analytical TLC indicated that the ratio of these components was similar to that obtained by direct NaOH treatment of the cell walls. The total yield of phenolic acids, determined by UV absorption as the sodium salt of *trans*-FA at λ_{max}345 nm, was 6.3 mg (calculated as *trans*-FA)/g cell wall compared with 7.5 and 9.0 mg/g cell wall obtained by NaOH treatment of cell walls for 45 min and 20 hr respectively.

We have found that several cellulase preparations from *Aspergillus niger* sources and one from a *Trichoderma viride* source contain esterase activity. Treatment of cell walls of *L. multiflorum* with these preparations gave rise in each case to a mixture of phenolic acids which contained similar proportions of the acids to those obtained by NaOH treatment of the walls. The acids could be extracted directly with ether. Such preparations could be useful for removing phenols from cell walls without the use of alkali which can react with hemicellulose components. In contrast to the cellulase from *Oxyporus*, treatment of cell walls of *L. multiflorum* with any of these preparations did not give $\beta(1\rightarrow4)$-xylobiose, the main sugar components detected by TLC being xylose, arabinose and glucose [7].

Hence it appears that the DFA isomers are bound to the carbohydrates of cell walls via ester links, involving one or both of their carboxyl groups, in a similar manner to that shown earlier for *trans*-FA [5]. The DFA units in cell walls might be formed *in situ* in the growing plant from FA units by oxidative coupling reactions involving peroxidases. It is of interest that Geissmann and Neukom [11] have converted water-soluble carbohydrates containing ester-linked FA units to the corresponding DFA compound by treatment with a mixture of peroxidase and hydrogen peroxide. Such reactions are believed to be of importance in the gelling of wheat flours to form doughs. The same workers have very recently identified DFA in the insoluble pentosan fraction of wheat flour [12]. Treatment of our *L. multiflorum* cell walls with a similar mixture of peroxidase and peroxide did not lead to a detectable increase in the number of DFA units, possibly because the FA units of the walls were not suitably orientated to allow coupling to occur.

The biosynthesis of lignin in plants is thought to proceed via water-soluble sugar esters of phenolic acids, including FA and PCA, to the corresponding free phenolic alcohols (e.g. coniferyl alcohol) followed by oxidative coupling reactions [13]. Nevertheless, in the Gramineae it is possible, from our results, that lignin biosynthesis involves oxidative coupling reactions of phenolic acids (e.g. *trans*-FA) linked to the carbohydrates of the wall, forming dimers and higher polymers: further phenolic acid units might be transported to the wall as their soluble sugar esters and participate in the coupling process.

Fluorescence microscopy [14,15] has indicated that bound phenolic acids occur in most of the cell walls of the Gramineae and not only in those that are lignified, i.e. that react with phloroglucinol–hydrochloric acid reagent. It is likely that only monomers and low polymers, for example DFA, are present in the non-lignified cell types. It is probable that the oxidative coupling of phenolic acids is at least partially responsible for causing the carbohydrates of the walls to be less easily attacked by carbohydrases. It is well known that as the lignin content of cell walls of the Gramineae increases, the carbohydrates of the walls are less easily degradable by ruminants [e.g. 16]. Much of the carbohydrate of cell walls which is unavailable to ruminants can be released by pre-treatment with alkali [e.g. 17–19], suggesting the possibility that the rupturing of ester bonds between phenolic acids and wall carbohydrates allows enhanced degradation of the carbohydrates by carbohydrases.

EXPERIMENTAL

All manipulations of solns of geometrical isomers of phenolic acids and their TMSi derivatives were carried out in "white" fluorescent light as UV and daylight cause isomerisation.

Plant material. Primary growth of shoots of Italian ryegrass (*Lolium multiflorum L.*), cv. RVP, were harvested on June 21, 1972 before ear emergence, freeze-dried, ground, and cell walls separated as previously reported [16]. The yield of cell walls was 34.4% of dry matter.

Source of cellulase (Oxyporus *sp., a Basidiomycete*), trans-*FA and* trans-*PCA.* These were described previously [8,16].

Source of cellulases having esterase activity. These were from *Aspergillus niger* (Sigma Types I and II, Koch-Light and Calbiochem) and from *Trichoderma viride* (Mayvil, Cheshire).

Preparation of authentic DFA. The *trans,trans* isomer was synthesised from vanillin using established methods [20,21].

Treatment of cell walls with NaOH. Cell walls (100 mg each time) were treated with N NaOH (5 ml) under N_2 (contg. < 5 ppm O_2) at 20° for 20 hr with occasional shaking. The mixture was filtered (No. 1 porosity glass sinter) and the filtrate acidified with 6N HCl to pH 2.5 and extracted with Et_2O (3 × 10 ml). The combined ethereal extracts were dried, evaporated and dried over Si gel. Methanolic solns of the residue were used for TLC. For GLC the residue was silylated by adding *N,O*-bis-(trimethylsilyl)-trifluoroacetamide ("BSTFA", 100 μl) and heating at 37° for 20 hr with occasional shaking. Similar amounts of *trans*-FA, *trans*-PCA and *trans,trans*-DFA to those released from the cell walls were subjected separately to the same sodium hydroxide procedure as the walls. Acidification followed by ether extraction and TLC (Solvents 1 and 2) or GLC showed that no oxidative coupling

of FA to DFA had occurred and that the geometrical forms of the isomers of the three acids were unchanged.

For UV spectroscopy, cell walls (30 mg) were treated with N NaOH (2 ml) at 20° under N_2, filtered (No. 1 sinter) and diluted to 25 ml with H_2O.

Treatment of cell walls with cellulase (Oxyporus *sp.*). This method has been described previously [16]: buffer was replaced with H_2O of pH 5.2 to enable TLC of sugars to be carried out without interference from Na ions.

Saponification of the H_2O-soluble compounds from treatment of cell walls with cellulase (Oxyporus *sp.*). The soln of H_2O-soluble compounds (2 ml) from 30 mg cell walls was treated with NaOH soln (10N, 0.2 ml) under N_2, shaken, and left at 20° for 45 min. The soln was acidified with 6N HCl to pH 2.5 and extracted with Et_2O (3 × 3 ml). The ethereal extract was dried over dry Na_2SO_4, concentrated to a small vol, then submitted to TLC. For GLC, the ethereal extract from 90 mg of cell walls was evaporated to dryness over Si gel, BSTFA (50 μl) added, and the mixture heated as above. For UV spectroscopy, a similar soln after NaOH treatment was diluted to 25 ml with H_2O.

Treatment of cell walls with cellulases having esterase activity. The above method of incubation was employed using 5 mg of each cellulase preparation instead of the cellulase from *Oxyporus* sp., and H_2O of pH 5.2 instead of buffer. The filtrates were acidified with N HCl to pH 2.5, concentrated to 0.15 ml over Si gel in vac., and subjected to TLC (Solvents 1 and 2).

Separation of plant extracts by TLC. Schleicher and Schüll cellulose plates (F1440) were employed with Solvent 1, HCOOH–H_2O (4:96), Solvent 2, PhMe–HCOOH–H_2O (40:45:15, upper) or Solvent 3, 0.880 NH_4OH–NH_4OAc–H_2O (5:5:95). Spots or preparative TLC layers were located by UV light, diazotised *p*-nitraniline or diazotised sulphanilic acid reagents [22–24]. For preparative TLC (Solvent 2), the residue from NaOH extraction of cell walls (100 mg each time) was applied to a 20 cm-width plate. Phenols were recovered using MeOH–Et_2O (2:1, 3 × 4 ml/cm of layer depth): after filtration (0.2 μ cellulose membrane) the solvent was removed at 30° and the residue dried over Si gel.

Separation of silylated plant extracts by GLC. Before GLC, residues from preparative TLC were silylated using BSTFA (50 μl) as above. TMSi derivatives of DFA were separated using an FID and a 1.50 m × 4 mm glass column packed with 0.2% OV-25 on Diatomite 'CQ'. The flow rate of Ar carrier gas was 50 ml/min. Column oven temp. was 250° and the detector 300°. An inlet heater was not employed, the samples being injected directly into the column packing. Peak areas were measured with a Chromalog 3 integrator. A second GLC method [8] was employed for the separation and estimation of the *cis* and *trans* isomers of FA and PCA.

Exposure of trans,trans-*DFA to UV light and separation of the isomers produced. Trans,trans*-DFA (1.0 mg) was dissolved in MeOH–Et_2O (2:1, 5 ml) and exposed to UV light under N_2 as already described [8]. The *trans,trans*, *cis,cis* and *cis,-trans* isomers were separated by TLC (Solvent 1). TMSi derivatives were prepared and separated by GLC as described above: the ratio of the *trans,trans* to *cis,trans* to *cis,cis* isomer was determined by measurement of peak areas.

Exposure of the TMSi derivative of trans,trans-*DFA to UV light and separation of the isomers produced.* The derivative was exposed to UV light for 9 hr under N_2 and the ratio of *trans,trans* to *cis,trans* to *cis,cis* isomers determined as above.

GLC–MS. These were obtained using the above GLC conditions with the 0.2% OV-25 column programmed from 230° to 260° at 3°/min and maintained at the higher temp. The detector temp. was 300° and He flow rate 50 ml/min. Accelerating voltage for MS was 3.5 kV.

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