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THE UTILIZATION OF EXOGENOUSLY SUPPLIED FERULIC ACID IN LIGNIN BIOSYNTHESIS

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Key Word Index—*Cucumis sativus*; Cucurbitaceae; cucumber; lignification; allelopathy; phenolic acid; ferulic acid.

Abstract—Ferulic acid (FA) acts both as an allelopathic agent and precursor in the endogenous process of lignification. To evaluate the involvement of exogenous FA in lignin biosynthesis, roots of intact, hydroponically grown cucumber (*Cucumis sativus* cv. early Green Cluster) seedlings were exposed to concentrations of FA labelled with [U-ring-^{14}C]FA. Radiotracer was distributed throughout the seedling. At least a portion of this radiolabel was in the form of free acid. A quantitative change in lignification occurred in treated seedlings. In roots and stems the level of lignin increased with the number of exposures and as the concentrations of exogenous FA was increased. Radiotracer was found in the residues of lignin isolated from seedling tissue treated with [U-ring-^{14}C]FA. This suggested the utilization of the exogenously applied FA by whole plants in their endogenous process of lignification. The activities of wall (ionically and covalently) bound peroxidases believed to be involved in the dehydrogenation and condensation of phenylpropanoids into the lignin polymer, were increased in those tissues (i.e. roots and stems) showing greatest concentrations of radiotracer, highest increases in lignification, and incorporation.

INTRODUCTION

Ferulic acid (FA) has often been implicated as an allelopathic agent [1, 2], although endogenously it serves as a direct precursor in the biosynthesis of lignin [3]. Very little is known about the mechanism(s) of allelopathic action or the fate of allelochemical compounds within recipient plants.

Cucumber has been shown to take up exogenously supplied phenolic acids, including FA [4]. Uptake continues over extended periods of time. Glycosylation and esterification [5, 6] of allelopathic phenolic compounds have been reported, but other destinations are both possible and as yet, unidentified.

The objective of this study was to determine whether an exogenous source of FA could be used by the endogenous lignification process of intact, hydroponic-grown cucumber seedlings. To elucidate the extent to which the supplied FA was directly involved, certain questions were experimentally addressed. First, was root-absorbed FA available to the entire seedling (transported)? Secondly, was there a quantitative change in seedling lignification which could be associated with the FA treatments? If so, was the exogenously supplied FA actually utilized as a lignin precursor? Lastly, are the enzymes which regulate the supply of precursor to the endogenous pool (PAL-EC 4.3.1.5) or which are involved in the dehydration and condensation of phenylpropanoids in the formation of the lignin polymer (wall-bound forms of peroxidase-EC 1.11.1.7) affected by the FA treatment of intact seedlings?

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RESULTS

Distribution of radioactivity

In tables and throughout the text, radioactivity is expressed in FA equivalents (based on treatment solution specific activities) and referred to as absorbed FA or radiotracer. The assumption that the radioactivity detected in seedling materials is indicative of the presence of [U-ring-^{14}C]FA (that, in fact, radioactivity = FA), is justified by several lines of evidence. In extracts of the root, stem, and leaf of seedlings exposed to labelled FA solutions 20, 13, and 3% (respectively) of the radioactivity present in the tissues was determined to be free FA. Although not quantified, decapitation of similarly treated seedlings indicated the presence of the radioactive FA in the exudates collected from the cut stem surface. Transport of the root-absorbed FA as free acid was, therefore, evident since a majority of the collected exudate would be from the xylem sap.

Whole plant distribution and percentage distribution of [U-ring-^{14}C]FA (Table 1) was modified by the age of the seedling and the concentration of the FA treatment. FA levels were greater in older seedlings and as treatment concentration of FA increased. Within a treatment concentration, increased solution pH decreased tissue levels of FA. A greater percentage of absorbed FA was transported out of the root as the concentration increased. In older seedlings, the mean percentage of radiotracer contained in the shoots (calculated across all treatments) was 11%, while 30% of the absorbed [U-ring-^{14}C]FA in younger seedlings was localized in the shoot.

Lignification

The lignification of roots and stems significantly increased with the concentration of FA in treatment

Table 1. The distributed and percentage distribution of total FA equivalents within cucumber seedlings exposed for 5 hr to FA solutions labelled with [U-ring-^{14}C]FA

Seedling Age (days)	Treatment solution FA (mM)	pH	FA equivalents (μg and % respectively)* Root	Stem	Coty	Leaf
8	0.25	5.5	75.5 (75.5)	14.0 (14.1)	3.2 (3.2)	7.1 (7.1)
	0.50	4.0	136.0 (79.3)	15.5 (9)	6.4 (3.7)	13.7 (8.0)
		5.5	119.5 (73.8)	18.8 (11.6)	6.4 (4.0)	17.2 (10.6)
		7.0	44.5 (65.4)	8.0 (11.8)	4.8 (7.1)	10.7 (15.7)
	1.00	5.5	123.5 (55.2)	40.2 (17.9)	16.7 (7.5)	43.2 (19.4)
18	0.10	5.5	215.0 (96.7)	3.5 (1.6)	0.7 (0.3)	3.2 (1.4)
	0.50	4.0	601.2 (85.4)	46.9 (6.7)	9.7 (1.4)	46.3 (6.6)
		5.5	438.0 (91.4)	20.4 (4.3)	3.4 (0.7)	17.4 (3.6)
		7.0	246.0 (86.2)	15.5 (5.4)	3.5 (1.2)	20.4 (7.1)
	1.00	5.5	729.4 (87.3)	55.6 (6.7)	7.3 (0.9)	43.4 (5.2)

*Equivalents were based on the specific activity of treatment solutions. Mean seedling dry weights were: (8 day old) root = 30 mg, stem = 20 mg, coty (cotyledon) = 40 mg, leaf = 110 mg, (18 day old) root = 90 mg, stem = 50 mg, coty = 50 mg, leaf = 300 mg.

solutions and the number of treatments given (Table 2). Leaf lignin content was not significantly changed by concentration after two exposures, but decreased in seedlings that received three treatments. The stems of control or 0.5 mM FA treated seedlings had lignin levels less than or equal to those of the leaf. The pattern was different for seedlings treated with the highest concentration of FA (1.0 mM). In these seedlings, the lignin concentration was significantly higher in the stem than in the leaf.

Analysis of isolated lignin (Klasson) residues indicated an incorporation of the radiotracer into the native polymer (Table 3). In seedlings allowed to remain in labelled treatment solutions for longer periods of time (8–10 hr), and in those transferred to non-labelled solutions after five hr, the greatest incorporation of absorbed FA into lignin occurred in the root and stem. Increasing the FA concentration of treatment solutions resulted in greater levels of root and stem lignin radioactivity. Over a two day period, an overall increase in lignin was observed and a steady ratio held between root and stem lignin content of untreated seedlings (Table 2). If the exogenously supplied FA was being used as an endogenous precursor, it would follow that, over the experimental period used in these incorporation studies (5–10 hr), radiotracer found in the lignin residues should also increase and result in a steady root to stem relationship. Total radiotracer incorporated into lignin did increase with time (while the root:stem radioactivity ratios remained constant) for all treatments in which seedlings were held in radiolabelled solutions until harvested. Those seedlings given a pulse of [U-ring-^{14}C]FA and then transferred to cold solutions of the same concentration showed a relative increase in stem lignin content of radiotracer.

No detectable FA or other phenolic acid was released

Table 2. Lignification of 12 day old seedlings given two (on days 10 and 12) or 15 day old given three (on days 10, 12 and 14) root exposures to FA

No. of exposures	Plant tissue	Treatment solution FA mM 0.00	0.50	1.00
		mg lignin/mg		
2	Root*	0.181 a	0.259 a	0.275 a
	Stem*	0.045 c	0.076 b	0.098 b
	Leaf	0.176 b	0.060 b	0.068 c
3	Root*	0.348 a	0.393 a	0.529 a
	Stem*	0.096 b	0.109 b	0.139 b
	Leaf*	0.113 b	0.106 b	0.076 c

Lignin is on an extractive-free basis. Within columns and exposures, values followed by the same letter are not different at 0.001 level of significance.

*Within the row, all values are different at an 0.001 level of significance.

Table 3. Radiotracer incorporation into lignin (residues), isolated from seedlings root-exposed for various periods of time to FA solutions labelled with [U-ring-^{14}C]FA

FA (mM)	Exposure* (hr)	Tissue			
		Root	Stem	Coty†	Leaf
	FA equivalents μ g/g extractive-free material				
		Experiment 1			
0.1	5	70.8	29.3	1.7	3.1
	5/3	65.6	50.3	1.8	4.5
0.25	5	96.7	47.5	2.0	5.6
	5/3	87.2	82.1	2.0	5.4
		Experiment 2			
0.1	5	59.3	27.9	nd	2.1
	8	74.5	37.8	1.3	2.6
	10	81.9	45.0	1.8	3.2
0.25	5	70.7	30.9	1.1	2.0
	8	88.5	41.9	1.1	2.3
	10	106.2	53.0	1.7	2.4

*In pluse/chase experiments (5/3); seedlings were given labelled solutions for 5 hr and then chased with cold solutions for 3 hr. Seedlings used in Experiment 1 were grown at a later date than those used in Experiment 2. FA equivalents are absolute values for pooled seedling.

nd, below detectable levels.

†Cotyledons.

by the saponification of materials from seedlings either root exposed or not exposed to FA. This would suggest that FA was not esterified in the cell wall areas of the seedlings. In the isolation of lignin residues, the pre-extraction and sulphuric acid condensation should remove all components of the plant with the exception of the lignin. The Klasson procedure, although a quantitative assay, is thought to be harsh enough to potentially extract even a small portion of the actual lignin. If any non-lignin associated phenolic acid were present in the wall it would probably be ester-linked. Linked in this manner it would be the only wall compound likely to condense with the lignin rather than be removed by the extractants. The lack of release of FA with saponification reduces the probability that the detection of radiotracer in the lignin residue was a result of associations formed during the extraction and condensation procedures rather than incorporation of absorbed FA through the endogenous biosynthetic pathway. In dicotyledons, ferulic and sinapic acid are the precursors for the alcohols which then randomly form the lignin polymer. Since the lignin residues isolated in the incorporation studies are most likely composed of native lignin components, it follows that the source of the radioactivity found in the residues is the absorbed [U-ring-^{14}C]FA. This lends additional support to the assumption that the radioactivity present is in the form of FA.

Enzyme assays

Regardless of how activity was expressed, soluble forms of peroxidase were generally higher than the bound forms. On a unit sample basis, the activities of the ionic and covalent forms of peroxidase increased in the roots of treated seedlings (Table 4). With the exception of one mean ionic value, (at 0.5 mm FA) these forms also increased with treatment in stems. Leaf activity of covalent peroxidase increased with FA concentration, but the ionic form showed no clear pattern. Differences in the activities of the soluble root versus leaf peroxidases were not significant while soluble stem activity increased only at the highest treatment concentration. In general, the protein level increased in the bound enzyme preparations while it decreased slightly in the soluble fraction with FA treatment. Peroxidase activities on a protein basis (Table 4) reflect this difference.

PAL is involved in the supply of precursors for lignin biosynthesis and, as such, might have been regulated by the presence of one of these precursors (FA). It was anticipated that the activity would decrease by a feedback (product) inhibition. Since lignification was continuing and actually increasing in some treated tissues, a decrease in the enzyme which normally provided the precursors would support the idea that the exogenous FA was instead being utilized. However, PAL activity in the root, stem and leaf tissue of FA exposed seedlings was not significantly decreased or increased over controls (Data not shown).

DISCUSSION

This study is unique in that it demonstrated an exogenously supplied allelopathic agent utilized by an intact plant as a precursor in an endogenous process. FA was well suited to this study as it inhibits growth of cucumber seedlings [7, 8], since it is taken up at relatively high rates by seedlings over extended periods [4] and because it functions as a lignin precursor in most dicotyledonous plants.

Table 4. Peroxidase activity of tissues from 14 day old seedlings roots exposed to FA for 24 hr

Plant Tissue	FA (mM)	Peroxidase activity*					
		Soluble		Ionic		Covalent	
		% of Control activity per					
		sample	mg Protein	Sample	mg Protein	Sample	mg Protein
Root	0.1	87	99	122	100	102	98
	0.5	85	100	164	100	159	129
	1.0	77	100	156	99	290	114
Stem	0.1	126	107	132	100	98	105
	0.5	123	105	96	68	186	114
	1.0	189	180	266	106	222	110
Leaf	0.1	112	109	94	80	135	99
	0.5	107	135	80	80	155	101
	1.0	127	135	132	82	231	107

*Activity is expressed as a percentage of the control (0.0 FA) seedling on both a sample or mg protein basis. Three seedlings were used per treatment, and each was sampled twice per tissue. Values represent means from one experiment. Standard errors were below 10% of the mean.

For the whole plant to utilize exogenous FA in the biosynthesis of lignin, the compound must be taken up and distributed to lignifying tissues. The successful isolation of free radiolabelled FA from shoot tissues and from the xylem sap in this study indicated that exogenous FA was translocated within seedling. This is in agreement with Leather and Einhelling [9] who saw uniform distribution of [^{14}C]salicylic acid (16% as free acid) in root exposed sorghum seedlings.

The process of lignification in the root and stem was quantitatively altered by FA treatment (Table 2) in a manner that generally corresponded to the distribution (Table 1) of this precursor. The decrease in levels of lignin in the leaf of seedlings exposed to multiple doses of FA may represent an overall slowing of metabolic processes and/or reduction of leaf turgor pressure potential rather than an inability to utilize the supplied FA. Phenolic compounds are known [7, 8] to reduce the leaf area, induce stomatal closure, and produce short-term wilting of *Cucumis*.

The demonstration of FA distribution and the observed increase in lignification do not themselves prove that root-absorbed FA acted as a precursor. Many environmental stresses and pathogens are known to affect lignin levels. However, the incorporation of radiolabel into the condensed residue of the native lignin strongly supports a conclusion that root-absorbed FA served as a precursor for lignin synthesis. As was observed for the distribution and quantitative lignin analysis, incorporation of radiotracer into lignin also varied with external levels of FA supplied.

Since lignification is primarily a wall process, and it is a bound peroxidase which mediates its synthesis [10, 11], the wall-located forms of this enzyme might be expected to vary with an increase in precursor supply. In fact, when put on a unit sample basis, the covalently and ionically bound peroxidase activities of the root and stem (and to some extent leaf) did increase with exogenous application. The significant change in the wall forms of this enzyme, particularly in the root and stem, paralleled the responses in lignification previously discussed. Protein levels increased for the wall bound extractions with treatment. When activity was reported on a protein basis, it remained fairly steady across treatments. The increase in activity per unit of material may then be due to the presence (synthesis or decreased degradation) of more enzyme rather than any inherent increase in enzyme efficiency. The soluble form of the peroxidase is involved in numerous porcesses and, as such, was not necessarily expected to vary with lignin biosynthesis.

The lack of a significant change in PAL activity in this whole plant study was not wholly unexpected. The activity of PAL has been shown to be inhibited by its hydroxylated cinnamic products [12–14], but there are problems in demonstrating this in intact seedlings [15]. In plant systems the inhibitory effects of FA on the enzyme are often very low or nonexistent [16]. It appears likely that the role of PAL in the shunting of carbon into the phenylpropanoid pathway is independent of the size of the endogenous FA pool.

In summary, in intact seedlings of *Cucumis*, lignification is increased with FA treatment, absorbed FA is available to all plant tissues and is incorporated into lignin. This FA utilization may well represent a destination within the plant for exogenous FA, a compound which may constantly occur in the environment at a low level and periodically rise to concentrations inhibitory to growth.

EXPERIMENTAL

Distribution and form of radioactivity. Cucumber seedlings were grown hydroponically as previously described [4] and exposed for 5 hr to 0.1, 0.25, 0.50, or 1.0 mM FA solutions labelled with [U-ring-^{14}C]FA and adjusted to pH 4.0, 5.5 or 7.0. The [U-ring-^{14}C]FA stock solution was 253 μCi/mM and all treatment solutions were made to contain 0.001 μCi/ml. Seedlings were used either 8 or 18 days after germination. Tissue radioactivity was determined [4] and the FA equivalents calculated from the specific activity of the treatment solutions.

To determine the form of the radioactivity detected in the tissues, methanol and water extracts of the root, stem and leaf of seedlings exposed for 5 hr to 0.5 m, [U-ring-^{4}C]FA labelled solutions were run on HPLC MeOH gradient, C-18 semi-

preparatory column (Waters, Milford, MA), and the fractions collected. The radioactivity of the FA peaks was determined.

In a separate experiment, seedlings were placed in radiolabelled treatment solutions and then decapitated just below the cotyledons. A capillary tube was used to collect the exudate coming from the cut surface over a 2 hr period. The collected material was separated by HPLC and the FA fractions assayed for radioactivity.

Lignin analysis (spectrophotometric). Seedlings were given two (on days 10 and 12) or three (on days 10, 12 and 14) treatments of 0.10, 0.50, or 1.00 mM FA and harvested on day 13 or 15. After harvest, seedlings were separated into root, stem and leaves, freeze-dried and ground to pass through a 60 mesh sieve.

Plant materials were pre-extracted (after Fry, [17]) sequentially with: water (100°, 30 min), 3% SDS (sodium dodecyl sulphate, 120°, 30 min, repeat), 85% EtOH (75°, 30 min), and Me_2CO (56°, 30 min). After the final extraction, the material was filtered and left to dry overnight in a 30° oven. Following Johnson *et al.* [18] and the modifications recommended by van Zyl [19], samples (5–20 mg) of dry, pre-extracted (i.e. extractivefree) tissue were weighed into 50 ml volumetric flasks and digested with 2.5 ml of 25% MeCOBr (30 min, 70° dry heat). After cooling, 15 ml of a 2 N NaOH and glacial HOAc mixture (1:5) was added to the digest. Before bringing the mixture to volume with HOAc 0.25 ml of 7.5 M $H_2NOH{\cdot}HCl$ was added to each volumetric flask. The A_{280} stabilized after 2 hr. Standard curves were based on cucumber tissues for which gravimetric (Klasson) lignin determinations [20] had previously been made. Lignin is reported on the basis of extractive-free sample weight.

Incorporation of [U-ring^{14}C]FA into lignin. Seedlings (14 days old) were exposed for 5 hr to 0.1 or 0.25 mM solutions of FA labelled with [U-ring-^{14}C]FA. Treatment solutions were 0.01 μCi/ml, with the added amount of non-radioactive FA adjusted according to the contribution (to concn) of [U-ring-^{14}C]FA. Pooled plants were dried and ground as before. The dry materials were either pre-extracted as in lignin analysis or subjected to extraction by ETOH and C_6H_6 (1:2) under reflux for 4 hr. After drying, extractive-free material was placed into 72% H_2SO_4 and left, with occasional stirring, at 21° for 2 hr. After dilution to 3% acid, the mixture was refluxed for 2.5 hr and then filtered through a porcelain-sided Selas crucible. After oven-drying, all of the (Klasson lignin) residue was removed from the crucible and ground using a mortar and pestle. The radioactivity of powered residues was determined by liquid scintillation spectrophotometry. In the same experiment, some seedlings were placed into labelled treatment solutions for the 5 hr exposure and then transferred to cold solutions of the appropriate FA concentration. After 3 hr, the seedlings were harvested and the incorporation of radiotracer into lignin determined as above. In separate experiments, seedlings were left in labelled treatment solutions for longer periods of time (5, 8 and 10 hr), before the incorporation of radioactivity into lignin was determined.

Ester linkages of FA in the primary wall have been reported [17, 21] and, if present, might possibly be associated with the condensed lignin residue. To determine the amount of FA esterified in the wall area, a saponification procedure of ref. [17] was used on treated and untreated seedlings materials pre-extracted as above. Dry materials were rehydrated in 0.1 M NaOH (0.1 ml/mg dry wt) in the presence of trace quantities of 2-ethyl-hexan-1-ol. The mixture was incubated at 20° for 18 hr. The pH was adjusted to 2.5 and the mixture partitioned against EtOAc. The presence of FA in the organic phase was determined by both HPLC and scintillation spectrophotometry.

Peroxidase activity. The activity of soluble and wall-(ionically and covalently) bound peroxidase was determined by a method modified from refs [22, 23]. Samples of fresh tissue were homogenized in 10 ml, 0.067 M Pi-buffer (pH 7.0). Soluble peroxidase activity was determined from the supernatants following a 10 min spin at 2000 g. The pellets were resuspended in 1% Triton X-100, spun at 1000 *g* for 1 min, and then washed with H_2O until no activity was detected in the supernatants. The pellets were then washed × 3 with 1 ml of 1M NACl. The washes were pooled and read as ionically bound peroxidase. The NaCl washed pellets were resuspended in 2 ml of 0.05 M buffer (pH 5.5) containing 10 mg cellulase and incubated at 25° overnight. After a 10 min spin at 1000 *g*, the supernatants were used to determine levels of covalently bound peroxidase activity.

The reaction mixture consisted of 1.3 ml (0.05%) guaiacol, 1.3 ml H_2O_2 (0.008 M), and 0.2 ml of the enzyme preparation. Original sample sizes were kept constant (0.50 g) and the activity expressed per sample and per mg protein. Protein in the enzyme preparations was determined using a BioRad reagent and protein standard.

PAL activity. Fresh tissues were cut into 3 cm pieces and placed in a freezer for 1 hr. Acetone powders were prepared using the method of ref. [24] by homogenizing the frozen tissues in cold Me_2CO, filtering the residue and again rising with Me_2CO. The powder was allowed to air dry before being put in a vacuum desiccator and stored at 6°.

PAL activity was determined from 0.05 g of prepared powder, brought to 3 ml with phenylalanine (0.1%) and borate buffer (0.1 M, pH 8.8). The mixture was incubated at 37° for 2–4 hr. The reaction was stopped at various times with the addition of 0.5 ml $HCLO_4$ (2M) and the particulates removed by centrifugation. The A_{290} of the supernatant remained constant (and linear to the amount of powder originally added) after 3 hr.

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