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Caffeic Acid Identified as an Inhibitory Compound in Asparagus Root Filtrate

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Abstract. **A potential allelochemical was isolated and identified from methanol extracts of asparagus *(Asparagus officinalis* L.) fresh root tissue. Fractions were collected by cellulose column chromatography and tested for inhibition by an asparagus seed germination bioassay. The fraction showing the greatest inhibition contained caffeic acid, as identified by melting point, thin-layer chromatography, and infrared spectrum analysis. Seed germination bioassays and greenhouse pot tests showed depression of seedling emergence when asparagus seeds were exposed to various dosages of crude filtrate, a methanol extract from crude filtrate, and caffeic acid.**

The decline of asparagus beds and subsequent difficulty in reestablishing stands has been attributed to pathogenic organisms, such as *Fusarium oxysporum* (Schlecht) f. sp. *asparagi* (Cohen), ***F. moniliforme*** (Sheld.) emend Snyd & Hans, and *Rhizoctonia violacea* (Tul.) (Yang, 1982). Factors other than fungal pathogens also have been implicated in asparagus decline. Asparagus roots have been shown to contain autotoxic and allelopathically active compounds. Soils amended with asparagus root tissue fragments were inhibitory to tomato, lettuce, and asparagus seed germination (Shafer and Garrison, 1977), whereas soils amended with crown tissue or stem tissue were less inhibitory or not inhibitory, respectively (Laufer and Garrison, 1977).

Hartung and Stephens (1983) suggested that there may be an additive toxic effect of *Fusarium* and asparagus autotoxin. Peirce and Colby (1987) investigated the interaction between asparagus root filtrate and ***F. oxysporum*** f. sp. *asparagi.* Either root filtrate or *Fusarium* alone depressed seedling emergence, but the severity greatly increased when both were present. Hartung et al. (1989) suggested that the autotoxic chemicals might predispose plants to *Fusarium* infection by physiological or biochemical effects.

Hartung and Putnam (1985) indicated that the inhibitory fractions from roots are of low molecular weight, nonproteinaceous, nonlypophilic, and very polar. Young and Chen (1988) confirmed that asparagus is an autotoxic species and that the phenolic content of asparagus root and associated soil extracts, as detected with Folin-Denis reagent, was related to inhibition. Purification of a crude asparagus extract by solvent partitioning, charcoal adsorption, cation exchange, and thin-layer chromatography showed a fraction that fluoresced under ultraviolet light, reacted with phenolic-sensitive localization reagents, and inhibited the growth of lettuce and asparagus radicles (Hazebroek et al., 1989). Aqueous extracts of dried asparagus roots have been isolated and characterized by sequential solvent extraction, column (silica gel, flash) and high-pressure liquid chromatography, and gas chromatography-mass spectroscopy (Hartung et al., 1990). Allelopathically active fractions, tested by a curly cress germination bioassay, included ferulic, isoferulic, malic, citric, fumaric, methylenedioxycinnamic (MDA), and caffeic (CA) acids. No single compound was found responsible for the allelopathic activity of asparagus extracts, although MDA did severely inhibit curly cress growth at concentrations of 25 ppm or above. No further characterization of asparagus root extracts has been reported.

The purpose of this study was to purify and characterize the most inhibitory component of the asparagus autotoxin and confirm autotoxicity of the component using a greenhouse seedling study.

Laboratory extraction and toxin identification. An asparagus seed germination bioassay was employed to identify inhibitory activity in various fractions extracted from root tissues. 'Viking' asparagus seeds (Harris-Moran Seed Co., Salinas, Calif.) stored at 2C were imbibed in distilled water 12 h before surface disinfestation in 10% sodium hypochlorite for 15 min. Seeds were rinsed

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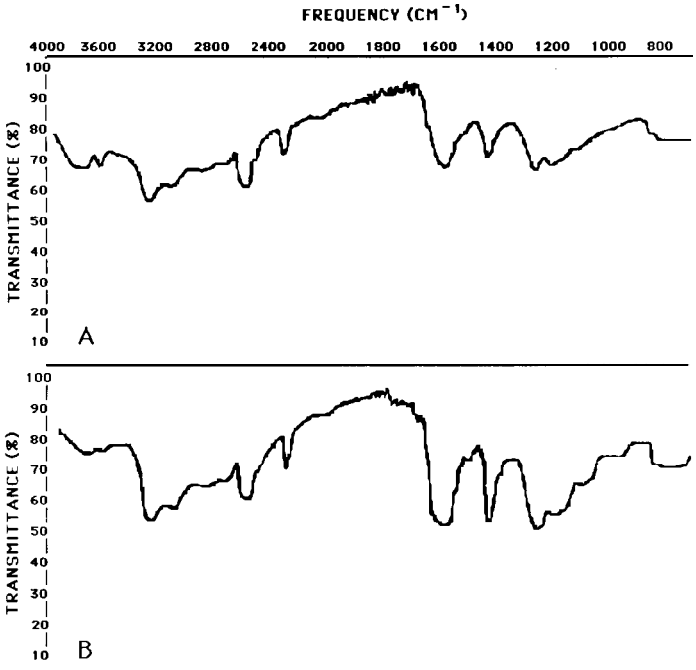

Fig. 1. Infrared spectra (KBr pellet) of (A) recrystallized caffeic acid from a commercial source (Sigma) and (B) caffeic acid isolated from MeOH extract of asparagus root tissue.

Table 1. Mean germination and radicle length of asparagus seeds exposed to fractions of methanol extract obtained from cellulose column chromatography.

Fraction no.	Elution agent (MeOH : HOAc : H_2O)	Fraction description	Germination (mean %)	Mean radicle length (mm)
Control	---	---	66.7 ab[z]	15.75 a
1	98:1:1	Yellow	68.3 ab	10.80 abc
2	97:1:2	Yellow, turbid	43.3 b	3.37 d
3	94:1:5	Yellow, clear	70.0 ab	6.48 bcd
4	89:1:10	Yellow, clear	68.3 ab	5.15 cd
5	79:1:20	Yellow, clear	60.0 ab	12.18 ab
6	49:1:50	Colorless	88.3 a	14.32 a
7	0:1:99	Colorless	65.0 ab	10.22 abc

[z]Mean separation by Duncan's multiple range test, P = 0.05.

Table 2. Mean germination of asparagus seeds exposed to eluates from preparative paper chromatography of methanol extract.

Zone	Zone description	Germination (mean %)
Control		60.0 a[z]
1	Contains origin	20.0 ab
2	Narrow band of yellow fluorescence followed by two blue fluorescent bands	33.0 ab
3	Yellow fluorescence, followed by an intense blue fluorescence, visible brown	0.0 b

[z]Mean separation by Duncan's multiple range test, P = 0.05.

in sterile water and placed in sterile petri plates (9 cm) on Whatman no. 1 filter paper (7 cm). Before treatment, each fraction was filter-sterilized through a 0.2-µm syringe filter unit to remove any residual *Fusarium* spores. Twenty-five seeds and 3 ml of each test fraction were used per plate in a completely randomized design with three replications. Distilled water was used as a control treatment. All plates were sealed with parafilm to retain moisture and incubated for 12 days in a growth chamber at 25C with 16 h of light (cool-white fluorescent, 75 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Radicle lengths and number of germinating seeds were recorded.

In the summer, roots in initial stages of decay were removed from plants harvested from diseased field plots. During the winter, roots were harvested from diseased greenhouse plants. Washed and macerated root tissue was extracted for 1 week at 12C in methanol (0.37 kg fresh weight in 1 liter of solvent). Methanol extracts were filtered and concentrated to dryness in vacuo, resulting in a brown syrup (yield, 3.3% of the original root mass). For bioassays, the methanol extract syrup was reconstituted in 1 liter of cold water and the pH adjusted to 5.6 (the pH of original root tissue) if necessary.

A cellulose column (38 x 2.5 cm) was prepared with a slurry of cellulose powder (Schleicher and Schuell, grade 286) and methanol and equilibrated in 98 methanol : 1 acetic acid : 1 water (by volume). Methanol extract syrup (3.0 g) was applied to the top of the column and eluted initially with 98 MeOH : 1 HOAc : 1 H_2O (by volume). Seven fractions were collected using a stepwise gradient elution (Table 1). All fractions were concentrated in vacuo to dryness and aliquots reconstituted to a solution (4 x) four times more concentrated than the original aqueous root extract. After centrifugation, the supernatant was adjusted to pH 5.6 and used for the bioassay,

Methanol extract residue (250 $mg \cdot ml^{-1}$ in water) was streaked along the origin (28 cm direction) of Whatman 3MM sheets (18.5 x 28 cm, 0.4 ml/sheet) and developed ascendingly in 2 nBuOH : 1 HOAc : 1 H_2O (by volume) solvent. Eluates of each of three zones were concentrated in vacua, aliquots reconstituted to a 4 x concentrated solution, pH adjusted, and bioassayed.

The syrup (3.4 g) from the methanol extract was diluted in 20 ml of water and acidified to pH 2.0 with HCl before being extracted with ethyl acetate. The extracts were concentrated to dryness in vacuo (yield of residue from ethyl acetate extraction = 0.53% of original root). This residue (0.51 g) was dissolved in ethanol and streaked along the origin of preparative silica gel 60 thin-layer chromatography plates (20 cm x 20 cm x 2 mm thickness)(Merck, Rahway, N.J.). The plates were developed in 40 benzene : 25 chloroform : 35 acetone (by volume) solvent and visualized under ultraviolet light. The wide yellow, blue-fluorescing band (Rf $\approx$ 0.25) was scraped and eluted with 85% ethanol and dried in vacuo. The residue was extracted with warm water and concentrated in vacuo to yield the crude active autotoxin fraction (yield = 0.06% of the original root). The crude fraction (82 mg) was extracted with ethyl ether and the insoluble residue washed with water to yield a "clean" residue (yield = 0.02% of the original root). The cleaned residue was recrystallized from hot water to yield a yellow solid (yield = 0.006% of the original root).

Of the fractions collected from the cellulose column, inhibitory activity, as indicated by reduced asparagus radicle growth, was confined largely to 2, 3, and 4, with fraction 2 the most active (Table 1). Thin-layer chromatography of these fractions revealed CA in fraction 2. No other phenolic compounds were identified.

Bioassays of the fractions from preparative paper chromatography of the methanol extract showed the greatest inhibitory activity in zone 3, which contains a blue fluorescent band visible under long-wave ultraviolet light (Table 2). Thin-layer chromatography of zone 3 eluate showed the presence of CA. No CA was found in zone 2 eluate.

The cleaned residue melted at 194 to 196C (corrected) with decomposition, which corresponds to the cited value for CA (Wind-

Table 3. Emergence in greenhouse pot tests of asparagus seeds treated with caffeic acid (CA), a methanol extract from this filtrate (MeOH), or crude root filtrate (RF).

	Fusarium inoculated			Sterile medium		
Dosage	CA	MeOH	RF	CA	MeOH	RF
Control	44.7	37.3	48.0	75.3	76.7	77.3
20%	0.7	15.3	18.0	45.3	76.0	78.0
40%	0.7	3.3	3.3	72.0	71.3	50.7
60%	0.0	2.0	0.7	0.0	44.0	47.3
80%	0.0	0.0	2.7	8.0	40.7	25.3
100%	0.0	0.0	8.7	0.0	12.0	35.3
R^2 values[z]						
Linear	0.34	0.56	0.38	0.59	0.62	0.40
Quadratic	0.61	0.76	0.72	0.58	0.66	0.41

[z]All R^2 values significant at $P = 0.01$.

Table 4. Mean germination of asparagus seed when exposed to root filtrate, methanol extract, or two sources of caffeic acid (CA).

Treatment	Germination [mean %)
Control	78.0 a[z]
Crude filtrate	59.0 b
MeOH extract	52.0 bc
CA (asparagus)	47.0 c
CA (Sigma)	45.0 c

[z]Mean separation by Duncan's multiple range test, $P = 0.05$.

holz, 1983), and showed no melting point depression when mixed with a commercial source of CA (Sigma, St. Louis). On thin-layer chromatography silica gel 60 (Merck) plates developed in 40 benzene : 25 chloroform : 35 acetone (by volume), the material moved identically with a CA standard (Sigma), showing the same blue fluorescence and reaction to diazotized sulfanilic acid spray (Pauly reagent). Further evidence that the material is CA was the similarity of its infrared spectrum (Fig. lB) with that of CA (Fig. 1A).

Greenhouse pot tests of CA. Serial dosages of CA (10 mg·liter^{-1}, Sigma), methanol extract (0.36 kg fresh weight/liter), and crude filtrate (0.36 kg fresh weight/liter) were tested in a split-plot design in six replicates. Pots were filled with premoistened peat-lite mix (Redi-Earth; W.R. Grace, Cambridge, Mass.) to the lower rim of the pot and firmed. Each pot was seeded with 25 asparagus seeds, covered with peat-lite mix to within 1 cm of the top of the pot, and firmed again. Each pot of the main plots received either 10 ml of water or *F. oxysporum* inoculum (600 spores/ml). The CA, methanol extract, or crude root filtrate solutions were each filter-sterilized through a 0.2-µm filter, then added in 50-ml doses to each pot. Each pot was placed in a marked plastic bag after treatment, sealed, and randomized.

To confirm that isolated CA is as active as the commercial form (Sigma), seeds were also bioassayed in multiwell plates. Treatments included crude root extract (50 g fresh weight/140 ml), methanol root extract (50 g fresh weight/140 ml), CA isolated from the methanol extract (3.6 mg/140 ml), CA purchased from Sigma (3.6 mg/140 ml), and a distilled water control. All liquids were applied in amounts that reflected active ingredients about equal to the 10 mg of CA obtained from the processing of 1 kg of fresh root tissue and were proportional to dosages used in greenhouse pot tests. All solutions were filter-sterilized as before. Four milliliters of each treatment was added to wells containing peat-lite mix and 10 asparagus seeds. The multiwell plates were covered, and seeds were allowed to germinate at room temperature under normal light conditions. After 14 days, germination was assessed.

In all experiments, percentage data were arcsin-transformed for analysis of variance. Standard errors were computed for each dose, and all other mean comparisons were tested with Duncan's multiple range test.

Seedling emergence was depressed when asparagus seeds were exposed to a range of dosages of CA, methanol extract, and crude filtrate (Table 3). CA suppressed emergence more than the others. However, it is difficult to duplicate effects among treatments by dosage, since pure CA concentrations most likely were considerably higher than those present in either the methanol extractor root filtrate.

Regression analysis of data from the pot test showed that dosage results from *F. oxysporum* - inoculated medium were consistent with previous data (Peirce and Colby, 1987) showing a quadratic relationship. Dosage data from sterile medium also were consistent with previous results. Best-fit regression is complicated by two factors: 1) inclusion of the control treatment, which is quite different from values for the treatments, and 2) a threshold effect above which the treatment completely suppressed emergence of seedlings.

The multiwell bioassay confirmed that CA isolated from methanol extracts was as active as commercially obtained CA (Sigma) (Table 4). All treatments significantly inhibited asparagus seed germination compared to the water control. Serial dosages (100%, 50%, 25%, and 12.5% of stock solutions) did not show any clear trend within a treatment (data not shown); however, in previous studies, the effect of dose was most evident in medium inoculated with *F. oxysporum.*

Since highly purified forms of the root extract and CA alone both inhibit asparagus seed emergence, CA appears to be a significant factor in asparagus autotoxicity. However, its biochemical role has not yet been elucidated. Synergistic or additive interactions with other unidentified compounds from less inhibitory fractions of fresh asparagus root extract cannot be ruled out. Research on asparagus allelopathy by Hartung et al. (1989) indicated that CA alone was not significantly phytotoxic to curly cress *(Lepidiurn sativum* L.). Hartung's asparagus root extracts were prepared from aqueous extracts of dried roots as opposed to direct solvent extraction of fresh root material. The difference in extraction procedures and in species used for bioassays may account for the difference in results. Also, the original plants from which the root material was obtained may have been subject to different environmental stresses or may have exhibited variable CA production based on seasonal changes. The plant roots for our experiments were obtained from field-grown material in which *F. oxysporum* previously had been isolated. It is not known if *Fusarium* induces increased production of CA in response to infection or if the concentration passively accumulates while root tissue is destroyed by the pathogen. Our data do confirm, however, that CA is a synergist, increasing the activity of *Fusarium oxysporum* f. sp. *asparagi*

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