Isolation and Identification of α-Tocopherol as an Inducer of the Parasitic Phase of *Ustilago violacea*

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ABSTRACT

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Plant extracts induce the smut fungi *Ustilago violacea, U. scabiosae,* and *U. utriculosa* to change from saprophytic, yeastlike growth to the parasitic, mycelial form. Previously, aqueous extracts were reported to be active when obtained from plant species that host a species of *Ustilago* but generally to be inactive when obtained from nonhost species. However, we report here that all tested plant species, hosts or nonhosts, had active extracts when extracted with methanol. These results indicate that the active agent is more soluble in methanol than in water, is universally distributed in angiosperms, and is possibly more available or accessible in hosts than in nonhosts. Four active peaks were found following reverse phase high performance liquid chromatography of leaf extracts of *Silene alba*, which is a host for *U. violacea*. One peak, found only in aqueous extracts, was weakly active after a 24-hr delay and was identified as ascorbic acid. Some activity was also associated with peaks containing chlorophylls *a* and *b* obtained from methanolic extracts. The major activity was associated with a peak, purified from extracts of *Pastinaca sativa* and also found in *S. alba*, which was identified by UV absorption spectroscopy and mass spectrometry as α-tocopherol (vitamin E). Synthetic α-tocopherol and other tocopherols (β, γ, and δ) as well as phytol were all highly active and induced change to the mycelial growth form even at very low concentrations (10^{-7}–10^{-8} M). The potential advantages of this system for further study of the mechanism of action of vitamin E, for bioassay of vitamin E, and for studies of the host/parasite relationship and smut control are discussed.

The anther smut fungus, *Ustilago violacea*, systemically infects many species of Caryophyllaceae. In the absence of contact with a host (ie, on artificial media) *U. violacea* forms yeastlike sporidia and no mycelium. However, as soon as contact with the plant is made, growth changes from this saprophytic yeastlike form to the mycelial form that is characteristic of the parasitic stage (5).

Previous work (5) established that water or acetone extracts of the host *Silene alba* induce development of the mycelial phase on artificial media. This effect was not restricted to particular physiologic races, as several races of *U. violacea* were induced to form mycelia by extracts from any of a wide range of caryophyllaceous plants that were tested. These extracts induced myceliation only in fungal cells that were potentially pathogenic (ie, heterozygous for the mating-type locus), and had no effect on growth rate or development in haploid cells. The active agents in these extracts were termed "silenins" in the absence of information on their chemical structure (5). Further tests with crudely crushed leaves extracted for 5 min with water showed that all of 38 diverse plant species that are hosts for *Ustilago* species had active extracts, while most (35 of 41) plant species that are not hosts for any smut pathogen had inactive extracts (4).

Two other smut fungus species that attack dicotyledonous hosts, *U. utriculosa* and *U. scabiosae*, responded the same as *U. violacea* to extracts from a variety of plant species, but several species of *Ustilago* that infect monocotyledonous hosts did not respond to any plant extract and indeed produced much mycelial growth in its absence (4).

These results suggested that silenins are universally distributed in plants but are present in more accessible form in angiosperms susceptible to infection by a *Ustilago* species (4). This compound triggers differentiation into the pathogenic mycelial form. We used the term "mycoboethin" to describe plant compounds that induce highly specific morphogenetic changes leading to parasitism by fungal pathogens (4,5).

The purpose of the experiments reported here was to isolate and identify the plant compounds that stimulate transition of *Ustilago* spp. from the saprophytic to the pathogenic state.

MATERIALS AND METHODS

Tissue extraction. *Aqueous extracts.* These were prepared by grinding leaf tissue in distilled H_2O (3 ml/gm fresh weight) in a Waring blender. After centrifugation at 27,000 *g* for 20 min, the supernatant was tested in the bioassay and analyzed chromatographically, and the pellet was discarded.

Acidic extracts. These were obtained by substituting cold *m*-phosphoric acid (0.35 M PO_3, as 2:1 $NaPO_3$:HPO_3, Fisher, Fair Lawn, NJ) and 1.4 M acetic acid for the H_2O in the procedure. Subsequent dilutions were made in cold 50 mM perchloric acid (14) prior to chromatography or in 0.2 M phosphate buffer, pH 6.8, before testing in the bioassay.

Methanolic extracts. These were prepared similarly (3 ml of methanol per gram fresh weight of plant material) except that the supernatant was evaporated to the aqueous residue in a rotary evaporator. Centrifugation (27,000 *g* for 20 min) resulted in the precipitation of most of the active material from the water. The highly active pellet was resuspended in 2–3 ml of HPLC grade methanol (Fisher Chemicals) and recentrifuged to remove any large particulate matter. The entire procedure was repeated on the pellet from the first centrifugation to extract exhaustively the active components. Procedures for extractions with other organic solvents were similar to those used for the crude aqueous extracts, except the organic solvent was substituted for the water.

Chromatography. Separation of the active components in the aqueous and methanolic extracts was achieved by high performance liquid chromatography (HPLC). Most of the chromatography was done with an Altex model 330 isocratic

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system employing either a UV absorbance detector set at 280 nm or a Kratos LC fluorescence detector (model PS970) set at an excitation wavelength of 295 nm. Results were recorded on a Hewlett-Packard 3390A integrator. The remaining separations were carried out with a Hewlett-Packard 1084B chromatograph.

HPLC of aqueous and acidic extracts. One highly polar, weakly active peak in the aqueous extract was resolved on the Hewlett-Packard instrument equipped with a Zorbax-NH_2 column (Dupont) by using a mobile phase of 0.02 M KH_2PO_4, pH 5.5, at 2.0 ml/min. The acidic extracts were analyzed by ion pair chromatography with an Ultrasphere-ODS (Beckman) column. The solvent was 80 mM acetate buffer, pH 4.75, 10 mM tributylamine and 10% methanol in water. A flow rate of 1.0 ml/min was used. This procedure is a modification of the technique developed by Pachla and Kissinger (15).

The amount of ascorbic acid in these extracts was determined by HPLC analysis of 100-μl samples. The integrator area units obtained were compared to a standard curve relating amount of synthetic ascorbic acid (Fisher Chemicals) to integrator area units.

HPLC of organic solvent extracts. Methanolic extracts were analyzed on the Ultrasphere-ODS column by using a mobile phase of 100% methanol at a flow rate of 2.0 ml/min. Normal phase chromatography was used to confirm the identity of one of the peaks obtained from the methanolic extracts. Two silica columns requiring slightly different solvent systems were used in the course of this work. For a Brownlee Spheri-5 column, the solvent was 0.5% methanol in *n*-hexane, and for a Hewlett-Packard SI-100 column, 0.2% methanol in *n*-hexane (19). With both columns, a flow rate of 2.0 ml/min was used. In all chromatographic procedures, the k' values for the peaks of interest were calculated according to the formula $k' = (RT_a - RT_0)/RT_0$ in which RT_a = retention time of peak and RT_0 = retention time of an unretained compound, or the void volume.

TLC analysis of a phytol preparation. A commercial preparation of phytol (Sigma Chemical Co., St. Louis, MO) was chromatographed on thin layer silica gel plates (K5F, 250 μm, Whatman). A mobile phase of 17:3 *n*-hexane:ethyl acetate (v/v) was used; this has been reported to produce an R_f of 0.35 for phytol and 0.50 for isophytol (7).

Several bands were observed after the plate was sprayed with concentrated sulphuric acid and heated at 60 C for 20 min. Control plates run at the same time but not acid treated were used for bioassay tests. Material scraped from the plate at the position of each band was placed in a test tube and washed with the 17:3 hexane:ethyl acetate solvent. The insoluble silica was pelleted by centrifugation and the supernatant was diluted into water and tested in the bioassay.

TABLE 1. Effect of aqueous and alcoholic extracts of host and nonhost species on induction of myceliation in *Ustilago violacea*

Species	Activity of H_2O extract[a]	Activity of methanol or ethanol extract[a]
Hosts for *Ustilago* species		
Silene alba	+++	+++
Hordeum vulgare	+++	+++
Avena sativa	+++	+++
Zea mays	++	+++
Scabiosa perfecta	+++	+++
Nonhosts		
Hesperis matronalis	−	+++
Taraxacum officinale	−	+++
Lactuca sativa	−	+++
Malva neglecta	−	+++
Plantago major	−	+++
Lycopersicon esculentum	−	+++
Lobelia siphilitica	−	+++
Glechoma hederacea	−	+++
Cichorium intybus	−	+++
Thalictrum dioicum	−	+++
Trifolium pratense	−	+++

[a] Extracts were not concentrated; 20 ml of solvent per gram fresh weight of tissue. Scoring system: +++, 60–100% of conjugated sporidia produced hyphae; ++, 10–60%; +, 1–10%; and −, <1%.

Bioassay. Small amounts of mated haploid cells (6) were spread on water agar in petri plates. Aqueous extracts were applied directly to the cells; acidic or basic extracts were first neutralized. Samples in organic solvents were either diluted 10-fold in H_2O (most solvents were not toxic to the cells at this concentration) or dried by blowing air followed by resuspension into H_2O. In all tests the cells were flooded with 0.2 ml of test solution. The treated cells were examined microscopically for the presence of hyphae after an incubation period of 18–24 hr at 22 C. The scoring system used was: +++, 60–100% of the conjugated pairs produced hyphae; ++, 10–60%; +, 1–10%; and 0, <1% (no greater than control cells treated with water (5).

UV spectroscopy. The UV spectrum of the active peak in the aqueous extract was obtained by using the scanning function (210–400 nm) of the Hewlett-Packard chromatograph. This isolated peak was either too dilute or was degraded too quickly to obtain a better quality spectrum with a conventional spectrophotometer. All other UV and visible spectra were measured on a Pye-Unicam 1800 or a Shimadzu UV 250 spectrophotometer. Samples were routinely scanned from 210 to 700 nm.

Mass spectrometry. A 2-mg sample of the active peak from methanolic root extracts was analyzed on a Varian MAT 311A mass spectrometer in the Department of Chemistry, University of Western Ontario. The instrument was set at a potential of 70 eV and a probe temperature of 60 C. Two milligrams of synthetic α-tocopherol (Sigma Chemical Co.) was used for comparison. Later HPLC analysis of this synthetic sample indicated that it was not pure.

RESULTS

Distribution of the active agent in plant species. When crushed leaves were extracted with water for 5 min, extracts from some species were active, while those from others were inactive (4,5). However, when inactive aqueous extracts of a variety of species were highly concentrated by lyophilization, all were active. The efficiency of extraction of the active agent in leaves of *S. alba* in

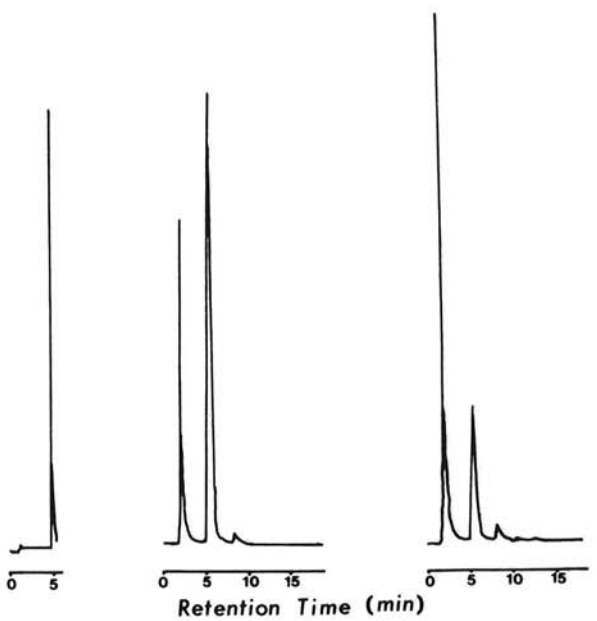

Fig. 1. Cochromatography of peak A and ascorbic acid. Ascorbic acid and plant extract were chromatographed on a Hewlett-Packard high performance liquid chromatograph equipped with a Zorbax-NH_2 column. UV absorbance detector was set at 264 nm. The tracing on the left is ascorbic acid, center is ascorbic acid plus plant extract and on the right is the plant extract alone. The peak of interest (peak A) appears at a retention time of 5 min.

aqueous as compared to organic solvents was investigated. Extracts of fresh material with methanol and acetone or extracts of freeze-dried material with ethyl ether or chloroform were more active than water extracts and remained active when diluted 10^3-fold. In general, most water extracts were weakly active at 10-fold dilution. Methanol gave the most active extracts and thus was used for further work. Several plant species that yielded inactive unconcentrated aqueous extracts yielded active methanolic extracts (Table 1).

Isolation and purification of the active agents. Extracts from leaves of *S. alba* were analyzed primarily by reverse phase HPLC, and four active peaks were found. Peak A was highly polar and was found only in aqueous extracts. It was moderately active in the bioassay and characteristically induced mycelia only after a delay of 24 hr after treatment compared to 6 hr with whole extracts. In an investigation of several common polar plant compounds, it was noted that synthetic L-ascorbic acid induced moderate mycelial growth with the same long delay as peak A. Cochromatography of ascorbic acid and peak A in the Zorbax-NH_2 column (Fig. 1) and the Ultrasphere-ODS column by using ion pair chromatography, and analysis of the UV spectrum of peak A (Fig. 2) supported the conclusion that the unknown active compound was ascorbic acid. Only two other plant compounds (cysteine and sorbic acid) from a wide range of tested commercially available amino acids, sugars, phenolics, and plant acids were active (*unpublished*). Again the activity was weak and there was a long delay before mycelia were induced. Spectral and chromatographic analyses of peak A were not consistent with the presence of cysteine or sorbic acid. Analysis of numerous plant species showed no correlation between extract activity in the bioassay and ascorbate concentration (Table 2), and it was concluded that peak A was not the primary contributor to the activity of extracts.

Methanolic extracts of leaves of *S. alba*, analyzed by reversed phase HPLC, produced three peaks (termed B, C, and D) of varying activity. All three peaks induced hyphal growth after a 6-hr lag period as did the crude extracts, but peak D appeared to be more effective as an inducer than either peaks B or C.

Peaks B and C were green solutions and corresponded in retention time (k' = 1.8 and 3.1) and spectral properties to chlorophylls *a* and *b*. Commercial preparations of chlorophyll *a* were inactive as inducers of mycelia at the concentrations found in leaf extracts, so it seemed likely that peaks B and C represented either mixtures or complexes of an active agent with these chlorophylls. However, no success was achieved in several attempts to separate activity from the chlorophyll peaks. Peak D was clearly the major inducing component, but it was small and difficult to purify from these leaf extracts.

Root extracts were therefore analyzed in an attempt to circumvent these difficulties. When analyzed with the same chromatographic conditions used with leaf extracts, extracts of roots of *S. alba* contained a single large active peak (Fig. 3). This

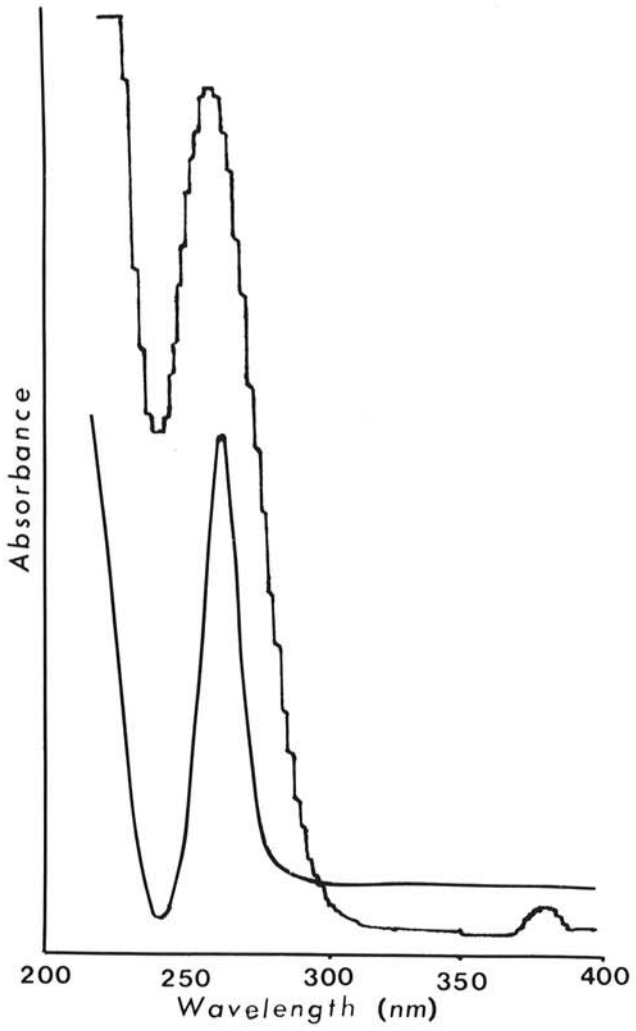

Fig. 2. UV absorption spectra of the material from peak A and ascorbic acid. The stepped UV absorption spectrum of the material from peak A was obtained using the scanning function of the Hewlett-Packard chromatograph. Chromatographic conditions were the same as outlined in Fig. 1 and Materials and Methods. The lower curve is the spectrum of L-ascorbic acid in 50 mM $HClO_4$ run on a Shimadzu UV250 spectrophotometer. Both spectra show a strong absorption peak at 264 nm.

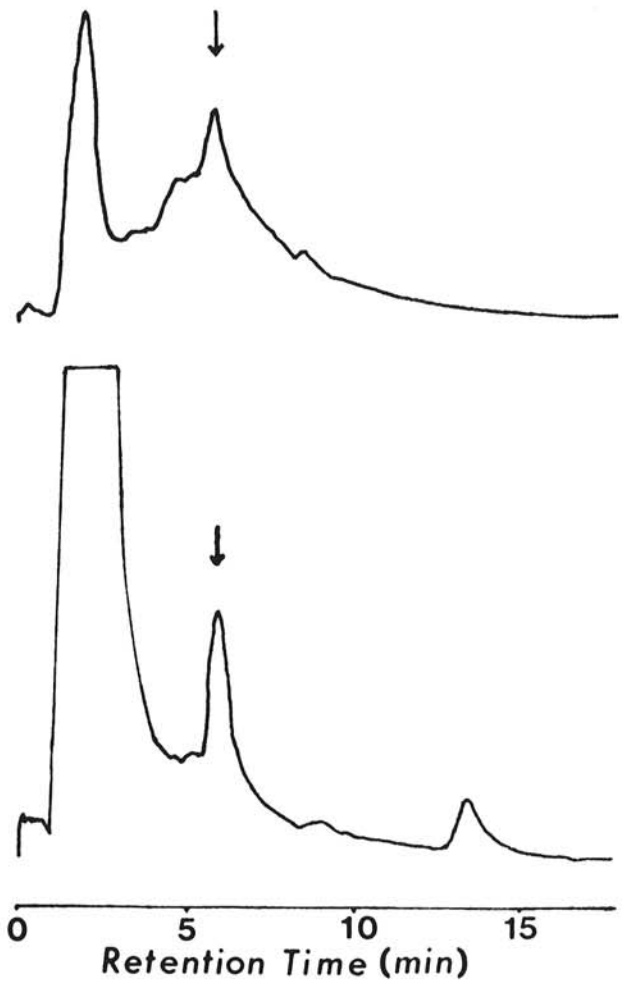

Fig. 3. Reverse phase chromatography of root extracts of *Silene alba* and *Pastinaca sativa*. Methanolic extracts of roots of *S. alba* (top) and *P. sativa* (bottom) were chromatographed on the Ultrasphere-ODS column with a mobile phase of 100% methanol at a flow rate of 2.0 ml/min. The UV absorbance detector was set at 280 nm. The active peak D appears at a retention time of 6 min and is indicated by the arrows.

peak corresponded very closely to peak D in terms of retention time ($k' = 5.9$) and had high activity in the bioassay. Extracts of *S. alba* and other plant species that had lost activity because they had been stored for some time no longer exhibited this peak when chromatographed. Peak D appeared to be the main, if not sole, determinant of activity in these extracts. Attempts were made to purify sufficient quantities of this compound for identification.

During the winter months, shortage of plants of *S. alba* forced the use of an alternative species. Commercially obtained roots of parsnip (*Pastinaca sativa*) were found to produce highly active methanolic extracts. Reverse phase HPLC analysis showed that parsnip root extracts contained a peak with the same retention time (Fig. 3) and UV absorption spectrum (Fig. 4) as the peak in roots and leaves of *S. alba* (Peak D). The absorption maxima were at 212 and 294 nm. The k' values of the active peaks from these two species remained very similar in two other HPLC chromatographic systems, and it was clear that the same compound was responsible for activity in both species.

The fractions of the HPLC eluates from *P. sativa* containing the active peaks were pooled before being run twice more through the same HPLC system. A single, large, apparently homogeneous peak containing ~2 mg of active material was obtained.

Identification of the active agent. A sample of this purified material was analyzed by mass spectroscopy (Fig. 5) and the spectrum obtained was characterized by a strong peak at m/e 430 along with signals at m/e 446, 204, and 165 (Fig. 5). Precise molecular weight determinations gave the values 430.3811 and 430.3807 for the first peak, corresponding to the molecular formula $C_{29}\ H_{50}\ O_2$ (calculated precise molecular weight, 430.3811). This molecular formula, along with the presence of the other ions and UV absorption maxima at 212 and 294 nm (Fig. 4), identified the active agent as α-tocopherol. The m/e 446 ion arises from the presence of a small amount of the oxidation product, α-tocopherolquinone ($C_{29}\ H_{50}\ O_3$), commonly found in samples of α-tocopherol exposed to air. The m/e 204 and 165 peaks represent the known major ion fragments of α-tocopherol (20). Further confirmation of this identification was obtained in two other ways. Firstly, aqueous emulsions of synthetic α-tocopherol were highly active and induced mycelia 6 hr after treatment (Fig. 6). Secondly, α-tocopherol and purified active peak D material from leaves or roots of *S. alba* or roots of *P. sativa* had the same or very similar k' values under several reverse phase and normal phase HPLC conditions. Moreover, "spiking" experiments in which α-tocopherol and the active peak were run at the same time gave a single peak equal to the combined integrator areas of the two peaks run separately (Table 3). In later experiments, an active peak was found in aqueous extracts of leaves with the same k' value as α-tocopherol.

Effective dose of tocopherols and other phytyl compounds. α-Tocopherol was active from undiluted (hyphae grew through the oily layer) down to 5×10^{-8} M (Fig. 7). Other forms of tocopherol (β, γ, and δ) were isolated from a mixed isomer oil by normal phase HPLC (Fig. 8), and all were highly active at an estimated concentration of $\sim 3 \times 10^{-6}$ M. Other phytyl-containing compounds showed activity in the bioassay. The most potent of these was a commercial preparation of phytol, active down to 10^{-7} M (Fig. 7). As this phytol preparation was crude (estimated 60% phytol, Sigma Chemical Co.), thin layer chromatography was used to isolate the components. Phytol, identified as the major band with an R_f of 0.32 compared to the reported value of 0.35 under these chromatographic conditions (7) was highly active. Lesser activity was also associated with minor bands of higher R_f values, but the identities of these bands were not investigated. Chlorophyll *a* and vitamin K_1 (3-phytylmenadione) were only weakly active: no myceliation was induced by concentrations $< 10^{-2}$ M. Vitamin K_3 (menadione) was completely inactive.

During the course of this investigation, over 200 compounds known to occur in plants were screened for activity. Nearly all proved negative. The active compounds were classified into three categories: highly active (tocopherols and phytol), weakly active (vitamin K_1 and chlorophyll *a*), and weakly active with a delay in the induction of hyphal growth (ascorbic acid, cysteine, sorbic acid, Fe^{2+}, Fe^{3+}, selenium dioxide, and sodium selenide).

DISCUSSION

The difference in ability to induce myceliation in *U. violacea* between extracts derived from host plants and extracts derived

TABLE 2. Determination of ascorbate content of various plant species

Species	Ascorbic acid (mg/100 g)[a]	Content (cited in [13]) (mg/100 g)[a]	Activity after 24 hr[c]
Hosts for an *Ustilago* species			
Lychnis flos-cuculi	43.5		+++
Lychnis coronaria	21.0		+++
S. alba	56.5		+++
Silene maritima	25.5		+++
Tragopogon pratensis	65.0		++
Zea mays	18.5		++
Nonhosts			
Petroselinum pastinaca	185.0	170	NT[b]
Brassica oleracea (broccoli)	107.5	120	NT
Brassica oleracea (brussels sprouts)	115.0	100	NT
Brassica oleracea (kohlrabi)	49.5	70	−
Gentiana crinita	157.0		+++
Erysimum cheiranthoides	125.0		−
Lobelia siphilitica	152.0		++
Parnassia glauca	86.0		−
Chenopodium album	212.0		++
Rheum rhaponticum	54.0		++
Lemna sp.	51.0		++
Taraxacum officinale	52.0		−
Glechoma hederacea	55.0		+
Apium graveolens (celery leaves)	35.5		−
Apium graveolens (celery stalk)	3.0	8	−
Echium sp.	4.0		NT

[a] Acidic extracts chromatographed in ion-pair system. Units: mg per 100 g fresh weight.
[b] NT = not tested in this determination. Extract from *Petroselinum pastinaca* was active in separate test.
[c] Scoring system: +++, 60–100% of the conjugated pairs of sporidia produced hyphae; ++, 10–60%; +, 1–10%; and − < 1%.

from nonhost species reported previously (4) was found to be quantitative rather than qualitative. Highly concentrated aqueous extracts or extracts employing organic solvents from any plant species were all active. Thus, it is clear that inducer compounds (silenins) are not unique to host plants but are probably universally distributed in angiosperms. The results with aqueous extracts suggest that the active compounds have low water solubility and that host plants either have a greater concentration of inducer or have it in a form more accessible to extraction by water.

The UV and MS data on an active compound isolated by HPLC chromatography identify it as α-tocopherol. It was detected as the major active peak in both root and leaf extracts of two species that were active in the bioassay. The only other active peaks corresponded to those for ascorbic acid (leaves, aqueous extracts) and chlorophylls *a* and *b* (peaks B and C leaves, methanolic extracts). Ascorbic acid was weakly active and acted only after a long delay, while commercially available chlorophyll *a* was not active at the concentration involved in plant extracts. It is likely therefore that the activity of peaks B and C in leaf extracts is due to tocopherols or phytols complexed with the phytyl moiety of the chlorophyll molecule. While phytol and some other tocopherols (β, γ, and δ) were shown to be highly active inducers, they were not detected in plant extracts. Clearly, α-tocopherol was the active agent in plant extracts and thus is likely to be the active plant agent that induces *U. violacea* to change to its parasitic mycelial form. However, this cannot be proven conclusively and it remains possible that some other compound (probably with a phytyl moiety) is the plant compound that comes into contact with the pathogen during host infection. Inducers would have to be located on the leaf surface as myceliation is initiated prior to penetration (*unpublished*). Continued growth of mycelia depends on a

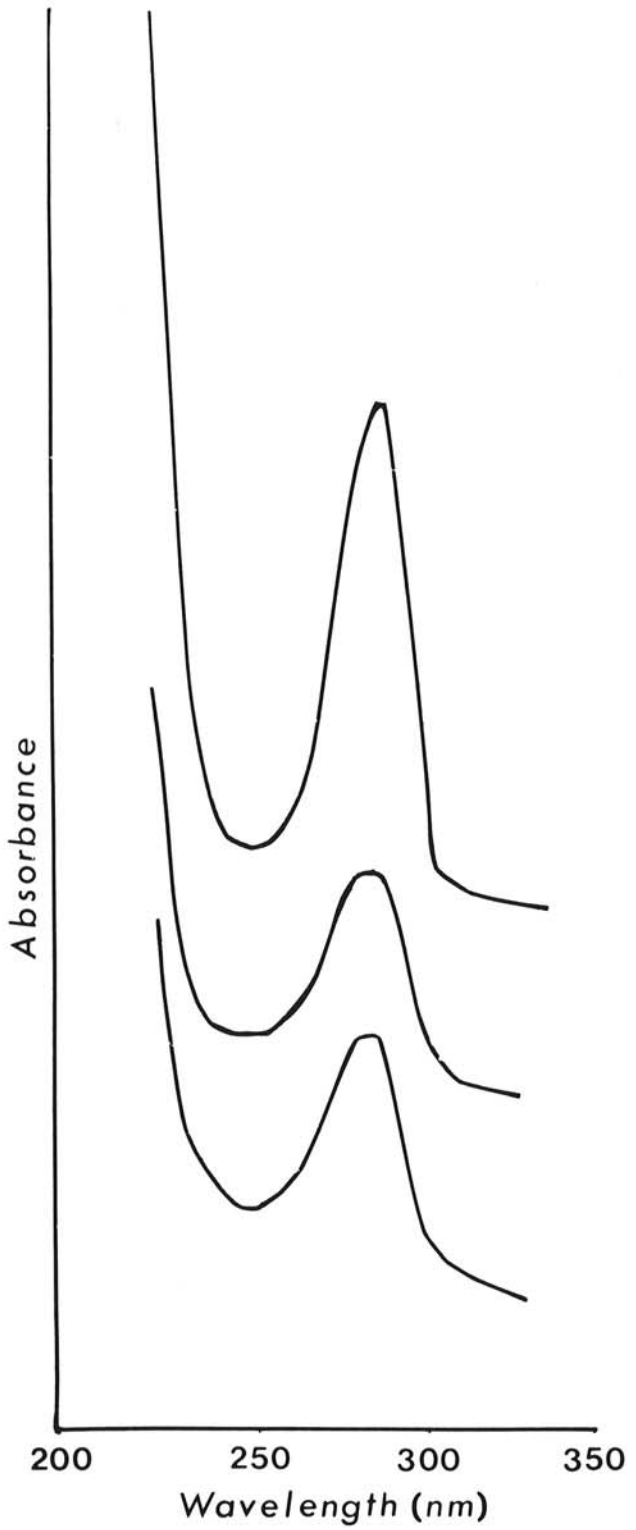

Fig. 4. UV absorption spectra of α-tocopherol and the compound at peak D from *Silene alba* and *Pastinaca sativa* root extracts. Material from peak D from *S. alba* (bottom) and *P. sativa* (middle) and a sample of α-tocopherol (top) were purified with normal phase chromatography. The isolated peaks, dissolved in 0.5% methanol in n-hexane, were scanned with a Pye-Unicam 1800 spectrophotometer. All spectra showed a characteristic maximum at 294 nm.

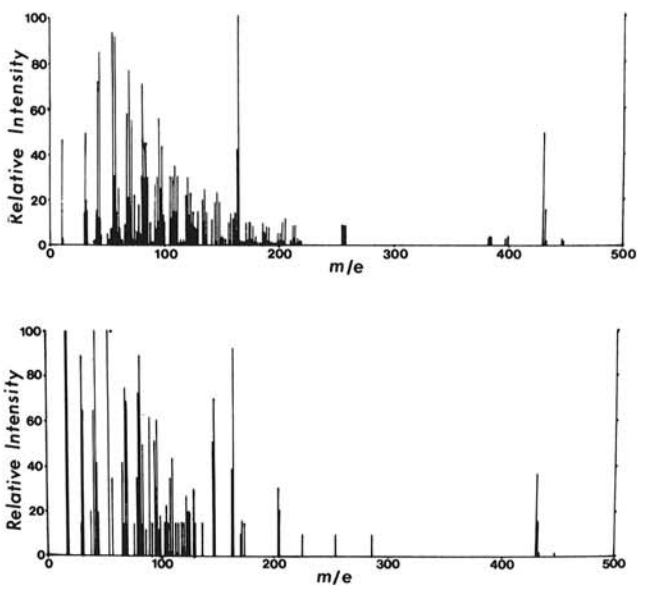

Fig. 5. Mass spectra of α-tocopherol (top) and the material from peak D from *Pastinaca sativa* (bottom). The peaks marked with the dot were set at a value of 100% and all other peaks reported as a percentage relative to that peak.

TABLE 3. Cochromatography of α-tocopherol and the active compound (peak D) isolated from parsnip extracts

	k'	Integrator area units
Reverse phase[a]		
10 μl α-tocopherol	5.14	55,840
25 μl parsnip extract	5.13	101,560
25 μl parsnip extract + 10 μl α-tocopherol	5.14	189,710
Normal phase[b]		
10 μl α-tocopherol	3.18	142,350
100 μl parsnip extract	3.30	176,080
10 μl α-tocopherol + 100 μl parsnip extract	3.30	335,200

[a] Ultrasphere ODS column, 2.0 ml/min, 100% methanol.
[b] Hewlett-Packard SI-100 column, 2.0 ml/min, 0.2% methanol in *n*-hexane.

continued supply of inducer (5) so that later mycelial colonization of the penetrated plant would involve inducer molecules within the plant tissue. This second inducer need not be the same compound as the leaf surface inducer.

If tocopherols are the major inducers in vivo, the following questions arise: do tocopherols occur on the leaf surface in host plants?, and how does tocopherol availability/accessibility differ in plants and thus account for the observed differences between the host plant group (crude water extracts active) and nonhost plant group (crude water extracts inactive)?. While little is known of the distribution and role of α-tocopherol within plant cells and tissue, the following points are relevant. Firstly, α-tocopherol appears to be present universally in angiosperms in levels far above the 5×10^{-8} M threshold required in the bioassay system for *U. violacea* (8). Secondly, it is located mainly in the chloroplasts; how much is

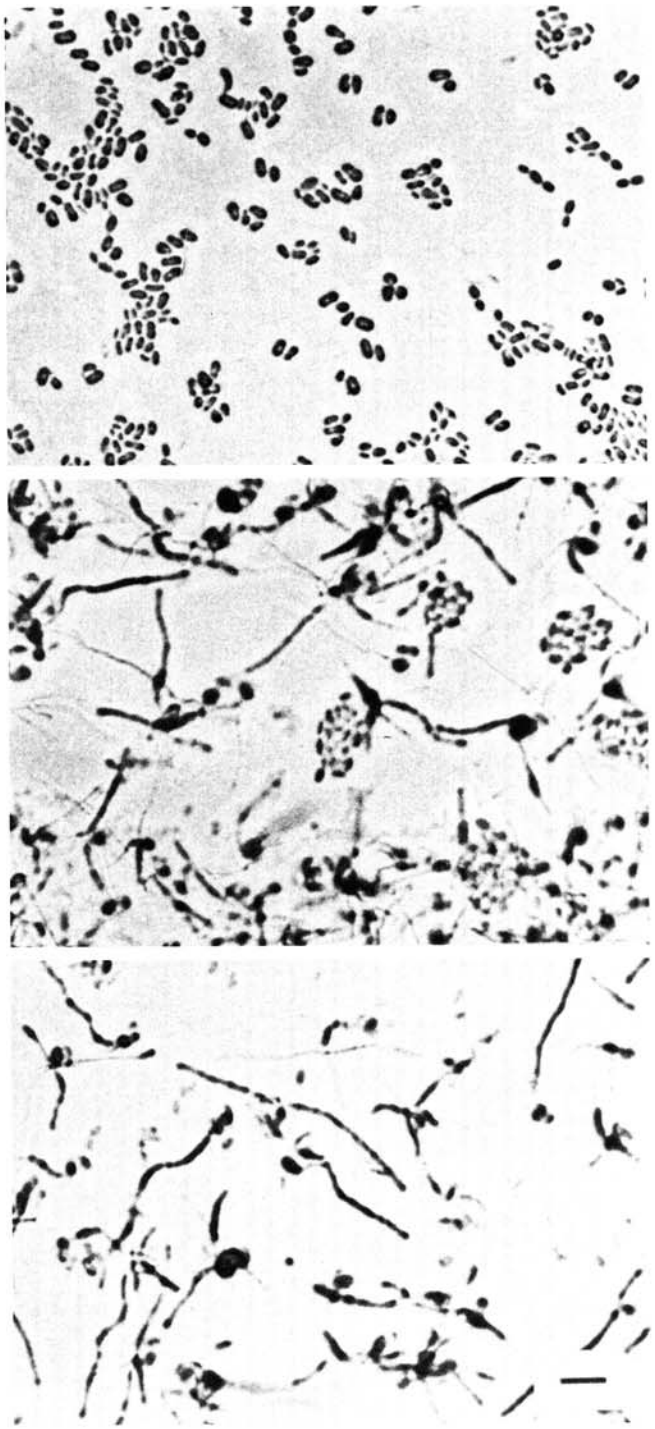

Fig. 6. Response of *Ustilago violacea* to α-tocopherol, extract from *Silene alba*, and water. Mated haploid sporidia were spread on water agar and flooded with 0.2 ml of distilled water as a control (top), a distilled water emulsion of 10^{-6} M α-tocopherol (bottom), or a methanolic extract from roots of *S. alba*; dried; and resuspended in distilled water (center). These pictures were taken after 24 hr of growth at 22 C. The sporidia were photographed directly on the surface of the medium. Bar = 20 μm.

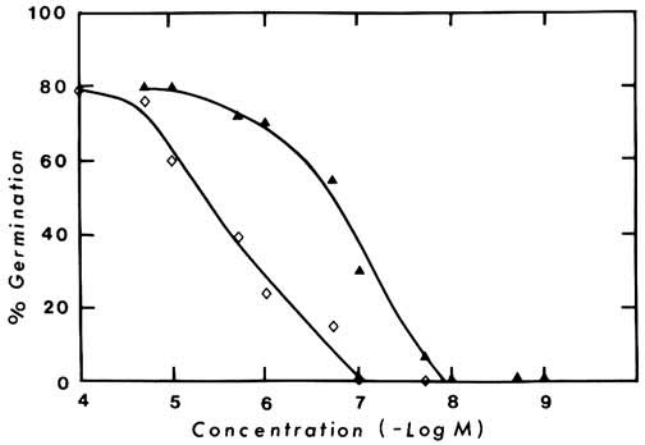

Fig. 7. Initiation of hyphal growth in response to various concentrations of α-tocopherol and phytol. For each test, $\sim 10^6$ conjugated haploid pairs were spread over a small surface of water agar. Stock solutions of α-tocopherol or phytol were serially diluted in 80% ethanol. Each stock dilution was diluted a further 100× in H_2O, and 0.1 ml of these emulsions were used to flood the cells. After 24 hr, 200 mated pairs were scored for the percentage of conjugated cells producing hyphae (= percent germination). Each spot represents the mean of three determinations. ▲ = tocopherol; ◊ = phytol.

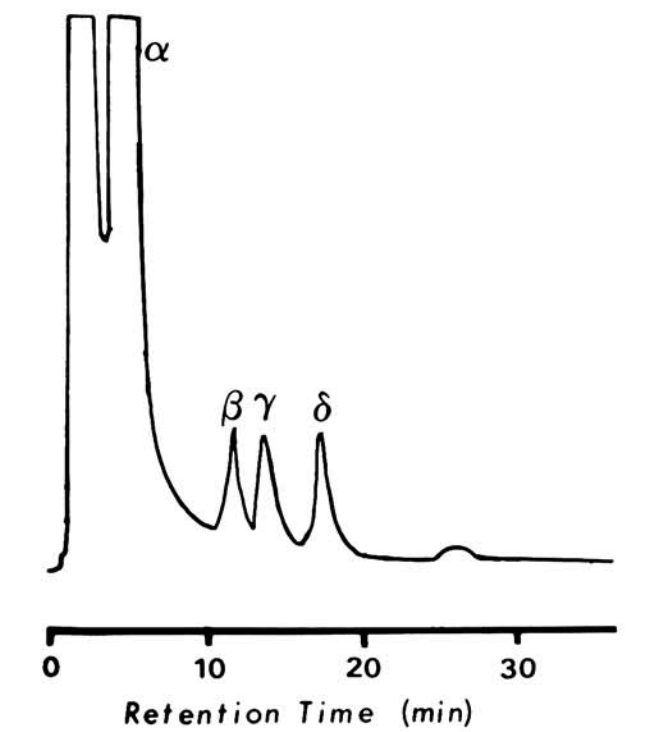

Fig. 8. High performance liquid chromatography separation of the four tocopherols. A mixed-isomer preparation of the four forms of tocopherol (estimated to be 67% α, 1–2% β, γ, and δ by Sigma Chemical Co., St. Louis, MO) was dissolved in 0.5% methanol in *n*-hexane. This solution was injected onto a Brownlee Speri-5 silica column and was eluted with 0.5% methanol in *n*-hexane at a flow rate of 2.0 ml/min. Peaks were detected by UV absorbance at a wavelength of 280 nm. The k' value for each compound was: α, 5.7; β, 12.8; γ, 15.5; and δ, 199. Peaks were identified according to the elution pattern (19) and the UV spectra of the individually collected peaks.

outside these organelles is uncertain (11). Perhaps the host group contains more free tocopherol (outside the chloroplasts, possibly in the leaf surface lipid layers), which could be extracted with water, than nonhost species. Investigations of this point using quantitative HPLC analyses of plant extracts are underway.

The mode of action of vitamin E in plant and animal systems is still controversial despite many years of research (12). While an important antioxidant role of α-tocopherol is established (9,12,17), other more specific mechanisms, including control of gene transcription (2), have been postulated. If α-tocopherol does indeed act as an antioxidant in regulating development in *U. violacea*, then the activity of ascorbic acid, cysteine, and Fe ions, all redox agents, is understandable. However, phytol (which is non-oxidizing) is a potent inducer, while other very common redox agents (reduced glutathione, dithiothreitol, mercaptoethanol) are inactive. It appears more likely that α-tocopherol acts in this system by affecting gene regulation more or less directly, a view that is supported by the very low concentrations required (5×10^{-8} M solution is equivalent to about 10^{-17} g per treated cell), the changes in protein synthesis patterns that occur 3–6 hr after tocopherol treatment (*unpublished*), and the requirement for heterozygosity at the mating-type locus, the developmental master switch gene in this organism (1,3,4). In this respect, the effect of vitamin E on *U. violacea* is very similar to that on the rotifers *Asplanchna sieboldi* and *Brachionus calyciflorus* for which concentrations as low as 5×10^{-7} M induce striking developmental changes (10). These concentrations are much lower than those that would affect mammalian cells (10).

The weak and delayed effects of ascorbic acid and cysteine may be explained by a sparing effect on α-tocopherol, as these compounds can form an oxidation-reduction chain with vitamin E (17). So far, however, we have failed to detect endogenous α-tocopherol (or any other inducer) in *U. violacea* and several other fungi (*unpublished*) so that explanations involving sparing of α-tocopherol may be of dubious validity. Clearly much remains to be done to investigate the specific developmental changes induced in *U. violacea* by α-tocopherol. Indeed, the rapid synchronous response to α-tocopherol and the ease of manipulation of the cells of this simple eucaryote make this an ideal system in which to investigate further the mode of action of this vitamin. Furthermore, the system with *U. violacea* could be developed into a very sensitive bioassay for detecting tocopherols/phytols at concentrations as low as 10^{-8} M, which at present are beyond the range of HPLC detection.

The requirement for phytyl compounds to initiate parasitic development in several species of *Ustilago* is unique in fungi. Vitamin E responses and/or requirements of any kind have not been reported previously in fungi. We have used the term *mycoboethin* to describe host products that act specifically to induce morphogenetic changes favoring pathogenicity (5). Several examples of plant and animal compounds that induce growth of the parasite are listed by Strange et al (16). Many of these compounds involve probable nutritional effects and act to increase the growth rate. In contrast, α-tocopherol acts as a true mycoboethin on *U. violacea*, and a few other *Ustilago* species (4) inducing *morphogenetic* changes at "hormonal" concentrations.

Our results suggest that large groups of pathogens such as the genus *Ustilago* may share certain common needs (nutritional/hormonal) that, in effect, restrict the number of potential host plants. This requirement is entirely compatible with gene-for-gene and protein-for-protein (Vanderplank [18]) mechanisms of host/parasite specificity that apply more particularly at the cultivar/physiologic race level. Investigation of the importance and mode of action of mycoboethins and other compounds that contribute to host *susceptibility* is likely to be important in studies of host/parasite specificity, and in providing possible new methods of disease control.

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