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Specificity of the *cis*-Isomers of Inhibitors of Uredospore Germination in the Rust Fungi

(methyl *cis*-ferulate/methyl 3,4-dimethoxycinnamate/*Puccinia graminis* var. *tritici*/ultraviolet)

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ABSTRACT **The *cis*- but not the *trans*-isomers of the natural inhibitors, methyl ferulate and methyl 3,4-dimethoxycinnamate, inhibit germination of the uredospores of wheat-stem rust. Minimum concentrations for 50% inhibition are about 7 and 140 nM, respectively. Ultraviolet radiation activates the *trans*-isomers by partial conversion to the *cis*-forms, and partially inactivates the *cis*-form by the reverse transformation. About 100 molecules of the germination stimulant, *n*-nonyl alcohol, are required per molecule of methyl *cis*-ferulate to overcome inhibition and restore 50% germination. Seasonal and age differences in sensitivity of spores to inhibitor may be related to variations in content of stimulant.**

The discovery by Macko *et al.* that the natural inhibitor of germination occurring in bean rust uredospores is methyl 3,4-dimethoxycinnamate (1) and the subsequent finding (2) that the self-inhibitor of germination in stem rust uredospores is the closely related compound methyl ferulate (methyl 3-methoxy-4-hydroxycinnamate) have laid the foundations for precise studies of the natural germination inhibitors in fungi. Macko *et al.* (1, 2) reported that preparations of the *cis*- and *trans*-isomers were both inhibitory. There are, however, both theoretical and factual reasons for believing that the two isomers should not have the same activity. Biologically active compounds frequently show a high degree of isomeric specificity and relatives of these germination inhibitors exhibit *cis–trans* specificity in other biological systems. Growth and epinastic responses in flowering plants are produced by *cis*-cinnamic acid, whereas the *trans*-isomer is inactive (3, 4). Although all previous reports on the properties of germination inhibitors from rust uredospores have indicated the occurrence of two inhibitory components (1, 2, 5, 6), the activity of preparations is subject to rapid changes during experimental treatment, and the two chromatographically separated components are interconvertible (6). Furthermore, it is known that *cis*- and *trans*-isomers are readily interconverted by exposure to ultraviolet radiation, and that the *cis*- and *trans*-isomers of both the stem rust and bean rust inhibitors are sensitive to ultraviolet radiation (1, 2). Since most of the work so far reported on the activity of germination inhibitors was performed under conditions that did not completely exclude extraneous radiation, it was possible that radiation-induced interconversion of components of the natural inhibitor occurred, and that the same effect was responsible for an apparent nonspecificity of isomers of the synthetic inhibitor. This paper reports the results of a repeated investigation of isomer specificity, conducted with rigorous exclusion of ultraviolet radiation during all of the operations from the beginning of the process of separating the *cis*- and *trans*-isomers from one another.

METHODS AND MATERIALS

Dihydroxycinnamic acid and its derivatives were obtained from Sigma Chemical Co. and were methylated at 65° in methanol with 10% BF_3 for 5–15 min; 0.2 g of the dry compound was used per 3 ml of solvent. The reaction was stopped by addition of 7 times this volume of cold water; the mixture was then partitioned into about an equal volume of ether, which was washed 3 times with water, separated off, and evaporated to dryness. The resulting product was essentially pure *trans*-isomer. The *cis*-isomer of each compound was obtained by a 30-min irradiation with about 1×10^4 ergs/cm^2 per sec at 254 nm of the *trans*-isomer either in solution (1), or after streaking thin-layer cellulose plates with a methanol solution. The isomers were separated by developing plates with water in darkness. The position of each isomer was located with guide spots detected by ultraviolet irradiation, while protecting the rest of the plate with a cover of aluminum foil. Cellulose in the zone containing a compound was scraped off and eluted with methanol; the solution was clarified by centrifugation, and the compound was stored in methanol or dried as required. Samples for determination of extinction coefficients were further purified by sublimation at about 90° (1). All operations were performed either in complete darkness or with a red safety lamp, which had been shown not to promote the interconversion of isomers. Exposure to very low intensities of white light, for a few minutes, was likewise found to have no detectable effect in converting the isomers. Preparations stored for extended periods of time were checked chromatographically for purity and, if necessary, chromatographed again before using them in any critical experiments. When stored at 3°, slight conversion of the *cis*-isomer of methyl ferulate to the *trans*-isomer occurred. The *cis*-isomer of 3,4-dimethoxycinnamate was quite stable, however, as were the *trans*-isomers of both compounds.

The volatility of germination inhibitors has been indicated in several studies with crude extracts (5, 7–9). Significant amounts are lost from chromatographic plates dried at elevated temperatures or from weighing vessels left in a desiccator for prolonged periods. Plates were therefore dried at room temperature, and all drying operations were completed as quickly as possible. When these precautions were observed, close to 100% recovery was achieved.

Absorption curves for each isomer in absolute methanol

TABLE 1. *Extinction coefficients, absorption maxima, and inhibitor activity toward germination of stem rust uredospores for the isomers of several methyl cinnamate derivatives*

Compound	$E^{320}_{1\%}$*		Absorption maximum*		ED_{50}†
	cis	*trans*	*cis*	*trans*	*cis*
Methyl 3,4-dimethoxy-cinnamate	520	790	316	320	140 nM
Methyl 3-methoxy-4-hydroxycinnamate (methyl ferulate)	540	840	326	325	7 nM
Methyl 3-hydroxy-4-methoxycinnamate (methyl iso-ferulate)	150	750	318	325	>500 μM‡§
Methyl 3,4-dihydroxy-cinnamate (methyl caffeiate)	190	730	320	327	>500 μM‡

Data for inhibition are taken from tests with summer-grown spores.

* In absolute methanol.

† Concentration for 50% inhibition.

‡ No inhibition at this concentration.

§ Compound not all dissolved.

were obtained with a Gilford Recording Spectrophotometer over the range from 400 to 200 nm. Change in absorption during the scan was not significant, as indicated by the identity of curves obtained with a single sample scanned first from long to short wavelengths and then in the reverse direction. The extinction coefficient of each isomer was calculated from samples dried in a desiccator and weighed with a Sartorius balance sensitive to 0.01 mg. The extinction coefficient at 320 nm for a 1% solution and a light path of 1 cm ($E^{320}_{1\%}$) is given in Table 1 for each of the compounds tested. Also shown for these compounds are the absorption maxima in the long-wavelength ultraviolet region. None of the *cis*-isomers show any fluorescence when exposed to ultraviolet radiation, but because of the rapid transformation to the fluorescent *trans*-isomer, a light blue fluorescence emerges quickly at the locus of any spot containing a *cis*-isomer.

Bioassays were conducted on distilled water solutions as described (6), with uredospores of *Puccinia graminis* Persoon var. *tritici* Eriksson & E. Henning, race 56, grown in a greenhouse.

TABLE 2. *Effect of irradiation of solutions of methyl trans-ferulate on inhibitor activity*

Conc., nM	% Germination at 90 min	
	Dark throughout	5 min at 367 nm
0	95	95
250	95	70
500	95	30
1000	95	25
2000	95	10
5000	95	5

Ultraviolet radiation was from a mineral lamp at a distance of 2.5 cm above the vessels. Tests were with summer-grown spores.

RESULTS

Although assays of the *trans*-isomer of methyl ferulate performed with material kept in ordinary laboratory light showed activity at concentrations as low as 10 nM, similar preparations prepared and tested with rigorous exclusion of radiation were inactive, even at concentrations as high as 1 mM. The purified *cis*-isomer, prepared and tested with similar precautions, still showed activity as high as that reported for any preparation of methyl ferulate (Table 1).

Irradiation of the assay vessels after the test solutions were dispensed led to activation of the *trans*-isomer. A comparison of germination on solutions of methyl *trans*-ferulate kept in complete darkness with germination on solutions given a 5-min exposure to ultraviolet irradiation (1.6×10^4 ergs/cm^2 per sec at 367 nm) before addition of spores is shown in Table 2. Similar activation at 254 nm occurred with a shorter exposure. In separate experiments, addition of methyl *trans*-ferulate was shown to have no effect on the activity of the *cis*-isomer.

Production of an active inhibitor also resulted from exposure of methyl 3,4-dimethoxy-*trans*-cinnamate to ultraviolet radiation, as shown by the inhibition of germination of stem rust uredospores. With both compounds, irradiation resulted in the production of the *cis*-isomer, as determined by chromatographic separation on cellulose.

There is a high degree of specificity amongst the derivatives of *cis*-cinnamic acid. Of several closely related compounds differing only in the substitution of groups on the 3- and 4-hydroxy position, only methyl ferulate and the bean rust inhibitor (methyl 3,4-dimethoxycinnamate) were active (Table 1). *Cis*-ferulic acid and 3,4-dimethoxy-*cis*-cinnamic acid were inactive at concentrations up to 25 μM.

As reported previously, the stem rust inhibitor can be counteracted by various stimulatory compounds, of which *n*-nonyl alcohol is one of the most active (10). Higher concentrations of stimulant are required to produce germination in large populations than in small populations. Similarly, higher amounts of nonyl alcohol were required to counteract inhibition by high concentrations of methyl *cis*-ferulate than were required for lower concentrations. In the experiment shown in Fig. 1, an increase in the amount of inhibitor from 11 to 30 nM required an increase in nonyl alcohol from 2.5 to 5.0 μM to give equivalent (50%) germination. On a molar basis, therefore, it took about 200 molecules of nonyl alcohol to counteract one molecule of methyl *cis*-ferulate at threshold

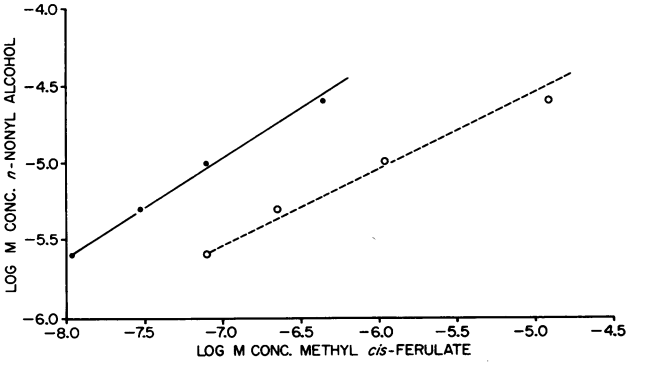

FIG. 1. Effect of increasing concentrations of inhibitor (methyl *cis*-ferulate) on the concentration of *n*-nonyl alcohol required to restore germination to 5% (O——O) and 50% (●——●).

concentrations of the inhibitor (<30 nM). As the amount of exogenous inhibitor was increased, however, the amount of nonyl alcohol required to counteract became relatively less. This is indicated in Fig. 1 by the fact that the slope of the log/log curve is less than 45°. In the higher range of inhibitor concentrations, only about 50 times as much nonyl alcohol as inhibitor was required to achieve 50% germination. Similar relationships were observed for the effects of nonyl alcohol in counteracting inhibition of germination in these spores by the bean rust inhibitor.

We have frequently encountered great variability in the apparent yields of inhibitor at different seasons of the year, a phenomenon that has also been reported by other investigators (9, 11). Spores grown in a greenhouse have consistently been more sensitive to the synthetic methyl *cis*-ferulate in summer than in winter. The differences are large, as shown in Table 3, where a change occurs in ED_{50} (see Table 1) from 125 nM for spores harvested in December to 8 nM for a July harvest. The sensitivity of autumn- and winter-harvested spores appears to increase during storage, as shown for the October harvest in Table 3. We have observed extreme differences of about 100-fold in the sensitivity of different test spores, but even the least sensitive are 50% inhibited by 100–200 nM methyl *cis*-ferulate.

TABLE 3. *Seasonal changes in sensitivity of greenhouse-grown uredospores to methyl cis-ferulate*

Harvest date	Age at testing (days)	ED_{50} (nM)
12/13/71	23	125
4/7/72	13	11
7/28/71	5	8
7/20/72	4	15
10/5/71	15	65
10/5/71	41	25
10/5/71	116	4

DISCUSSION

Differences in biological activity between *cis*- and *trans*-isomers are common, and the wheat and bean rust inhibitors are no exception to this general rule. A clear demonstration of the difference is dependent upon the rigorous exclusion of radiation during experimental manipulations. For this reason reports of activity by both the *cis*- and *trans*-isomers cannot be accepted unless adequate precautions have been taken to prevent interconversion of the isomers. The report of Bonner and Galston that both the *cis*- and *trans*-isomers of cinnamic acid were active as growth inhibitors of guayule (12) may have rested on insufficient exclusion of radiation during the experiments. This also appears to account for the initial reports that both stereoisomers of synthetic bean and stem rust inhibitors were active (1, 2). There appears to be sufficient short-wave radiation in ordinary laboratory light to cause significant conversion of isomers exposed for appreciable periods.

Methyl ferulate isolated from the uredospores is predominantly in the *cis*-form (2, 13). Preparations obtained from the uredospores can, therefore, only decrease in activity as a result of irradiation. This accounts for the decay in activity previously reported, particularly in the period immediately after extraction (6, 10). With continued irradiation, an equilibrium mixture of *cis*- and *trans*-isomers would be approached, accounting also for the observed incomplete decay of activity in natural inhibitor preparations (6). Equilibrium mixtures contain about equal amounts of *cis*- and *trans*-isomers (13). The apparent resolution of crude preparations into two inhibitory compounds when chromatograms were developed in water resulted from the reconversion of the separated *trans*-component into biologically active *cis*-isomer (6). Although we do not have detailed information on the effect of visible radiation on synthetic germination inhibitors, neither the inhibitors nor their *trans*-isomers have significant absorption in visible light. Ultraviolet radiation of long wavelength (367 nm), where the extinction coefficient of methyl *trans*-ferulate is only about 30, is nevertheless very effective in the interconversion of isomers, and there is probably sufficient radiation of this quality in laboratory light to account for the observed changes in inhibitory activity. Although spores are exposed to abundant radiation during growth, the *cis*-isomer appears not to be converted to the *trans*-form in the spores. Since germination is strongly inhibited by ultraviolet radiation, it probably does penetrate the dark spore wall, and the stability of the *cis*-isomer is further evidence that it is combined in some way within the spore. The possibility of intracellular conversion should not, however, be excluded from consideration, since side reactions disposing of the *trans*-form would tend to preserve a high proportion of the *cis*-form, particularly if it is not in equilibrium with the *trans*-form because of cellular binding.

Fluctuations in the sensitivity of spores harvested under different conditions and stored for different periods may be very great, so that it is not possible to fix an absolute level for the inhibitory activity against a given species or race. Environmental effects on different species may also be different, since the greatest inhibitory activity with *Puccinia triticina* was reported from winter rather than summer spores (11). We have no information to show what factor of the external environment is responsible for the differences in sensitivity of *Puccinia graminis* spores. Low sensitivity is, however, experimentally induced by the addition of germination stimulants. Low sensitivity of winter spores may, therefore, be related to a higher content of germination stimulants, and the gradual loss of volatile endogenous stimulants, which are known to occur in stem rust uredospores (10), could then account for the increase in sensitivity during storage.

Although seasonal differences in sensitivity within a species are appreciable, there are greater differences in sensitivity of different rust fungi to a given inhibitor (13) and a high degree of structural specificity amongst closely related methylated derivatives of dihydroxycinnamate. Besides the methyl ester group itself, the 3-methoxy-, but not the 4-methoxy, group is crucial for activity against stem rust uredospores.

Because they function as naturally occurring regulators of germination, and because they are active at extremely low concentrations, these compounds should be regarded as fungal hormones.

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