# Germination of Coffee Rust Uredospores and Their Inhibition by Cinnamic Acid Derivatives

Mark A. Stahmann, Maria Raphaela Musumeci, and Walkyria B. C. Moraes

Professor of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison 53706 (M.A.S.) and Fellows of Conselho Nacional de Pequisas, Instituto Biológico, São Paulo, 01000, Brazil, S.A., respectively.

The work was carried out in part during a visit by M.A.S. while a consultant to the Instituto Biológico for the Food and Agriculture Organization of the United Nations. We thank V. Macko and R. C. Staples for compounds used in this study and for helpful discussions.

Accepted for publication 12 November 1975.

ABSTRACT

STAHMANN, M. A., M. R. MUSUMECI, and W. B. C. MORAES. 1976. Germination of coffee rust uredospores and their inhibition by cinnamic acid derivatives. Phytopathology 66: 765-769

Germination of uredospores of the coffee rust fungus (*Hemileia vastatrix*) was influenced by the ratio of the spore concentration to the volume of Tween-20 solution on which the spores were floated. Uredospore clumps readily formed a film in which spores floated and spread on the surface. The possible role of this film in the infection process is discussed. Twenty-four cinnamic acid derivatives were assayed for uredospore germination inhibition. The similarity in uredospore germination inhibition and chromatographic properties of the coffee rust germination self-inhibitor and that of some methoxycinnamic acids suggest that the coffee rust self-inhibitor may be a cinnamic acid derivative.

*Additional key words:* uredospore germination, coffee rust, cinnamic acid derivatives.

Two germination self-inhibitors from rust fungus uredospores are now known. The germination self-inhibitor of the bean rust, sunflower rust, corn rust, and snapdragon rust fungi was identified as methyl *cis* 3,4-dimethoxycinnamate (3, 4), and from wheat stem rust uredospores methyl *cis* 4-hydroxy-3-methoxycinnamate was identified (2). The germination self-inhibitor in uredospores of the coffee rust fungus (*Hemileia vastatrix*) was reported to be different from the above compounds (6, 7), and its properties suggested that it might be a free organic acid. Neither of the two known rust uredospore self-inhibitors was active against coffee rust uredospores although cinnamic acid and 3,4-dimethoxycinnamic acid were slightly effective (7). The formation of a film when uredospores of the wheat rust fungus contact a water surface and the role of this film in controlling spore germination has been described by Woodbury and Stahmann (9).

The present report is concerned with a study of some factors that influence the germination of coffee rust uredospores, the formation of a film when the uredospores contact a water surface and the effect of 24 derivatives of cinnamic acid on the germination of coffee rust uredospores.

## MATERIALS AND METHODS

**Collection of uredospores.**—Uredospores of the coffee rust fungus (*Hemileia vastatrix,* race 2) were collected from naturally infected trees by gentle scraping using the edge of a 10 ml celluloid centrifuge tube. The spores were stored in gelatin capsules at 5 C after having been conditioned for 48 hours at 50% relative humidity and 5 C as recommended by Zambolim and Chaves (10). The spores were used within 2 months of harvest and showed from 50 to 70% germination.

**Germination assay.**—Solutions of the cinnamic acid derivatives (1 mg/ml) for assay were prepared in absolute ethanol. From 5 to 10 ml of these solutions were irradiated for 1 hour with both the short (254 nm) and long (366 nm) wavelength lamps in open petri dishes in an ultraviolet (UV) chromatography viewing cabinet (Ultra Violet Products Inc., San Gabriel, CA) before assay to assure formation of both *cis* and *trans* isomers. However, the *cis:trans* ratio in these preparations was not determined. The first solution for assay was made by diluting 0.5 ml of the irradiated ethanol stock solutions with 4.5 ml of 0.5% aqueous Tween-20 solution. Serial dilutions were then made with 0.5% Tween-20.

Using a micro spatula, a small quantity of uredospores (about 0.2 to 0.3 mg) was placed by means of a micro spatula in the cavity of a dry concave slide within a petri dish above 2.5 ml of water. Then 0.1 ml of a 0.5% Tween-20 solution or 0.1 ml of each serial dilution of the compound for assay in 0.5% Tween-20 solution was carefully added to each slide from a 0.1-ml syringe. The dry spores floated and spread on the surface. Counts of the number of spores with germ tubes were made after 6 hours of incubation in the dark at 20 to 23 C. From 100 to 200 spores were counted on each slide and $ED_{50}$ concentrations were computed by probit analysis (1).

**Ratio of amount of uredospores to volume.**—The effect of varying the volume of the Tween-20 solution was studied by using a micro spatula to place about the same small quantity of spores as used in the germination assay into flat bottom glass cups (11 × 22 mm). Then increasing volumes of 0.5% Tween-20 solution were added. The spores floated and spread on the surface.

A study of the effect of varying the quantity of spores on the germination within a fixed volume of water was studied by placing increasing quantities of uredospores from a micro spatula in the dry glass cups and then adding

2.5 ml of 0.5% Tween-20 solution. The spores floated and spread on the surface.

**Formation of films and spreading of uredospores.**—The release of a film from coffee rust uredospores was shown by sprinkling 1 mg of spores onto a clean distilled water surface in a plastic dish (11 × 11 × 3 cm) on which was floating a Parafilm barrier (6.5 × 10.8 cm) (9). The barrier was first pushed to one side to enclose an area 0.5 × 11 cm (6.5 $cm^2$) into which the spores were sprinkled.

The spreading of uredospores with formation of a film was also studied by observing under the microscope the behavior of clumps of dry spores on glass slides or in pustules on the under side of leaf surfaces as drops of water were made to slowly flow around or over them.

**Surface area.**—The effect of surface area on germination was investigated by sprinkling 1 mg of spores onto clean water surfaces of 2, 25, and 64 $cm^2$ formed by holes cut into Parafilm sheets floated on the surface of 400 ml of distilled water contained in 18-cm diameter petri dishes. After incubation, drops containing the floating spores were placed on slides for estimation of the degree of germination.

**Thin-layer chromatography.**—Ten microliters of the UV-irradiated ethanol solutions (1 mg/ml) of the cinnamic acid derivatives were spotted on aluminum sheets precoated with silica gel (Merck) and developed with a mixture of benzene-ethyl ether (80:20, v/v). The position of the compounds was determined by observation under UV light after evaporation of the solvents used for development.

## RESULTS AND DISCUSSION

**Factors that influence germination.**—*Floating of spores.*—The germination assay was developed for use in isolation studies in which the amount of self-inhibitor and viable spores may be very small. Germination counts in assays using floating spores showed much less variation than similar counts from spore suspensions in which many spores would sink. The percentage of spores with germ tubes was similar in both types of assay only when fields near the edge, which showed the highest germination in the suspension assays, were counted for spores that were submerged showed low germination. In contrast, the floating spores were spread much more uniformly than the suspended spores, were easier to count, and showed very little variation in percentage of spores with germ tubes in different fields.

*Tween-20 concentration.*—Spores that showed 24% germination on water, increased in germination to 55% on 0.12% Tween-20. Maximum germination of 70% was observed on 0.5% Tween-20. Germination was reduced as the Tween-20 concentration was increased, falling to 48% with 4% Tween-20. Spore germination was not reduced when as much as 10% ethanol was added to the Tween-20 solution, but 20% ethanol caused some reduction in germination.

*Ratio of uredospores to volume.*—The effect of varying the volume of 0.5% Tween-20 solution on which a fixed quantity of spores were floated in a constant surface area is shown in Table 1. These data show that germination was greatest when the volume was lowest (only 0.1 ml) and decreased to zero as the volume was increased. The length of the germ tubes also decreased as the volume increased. These results differ from those with *Puccinia graminis* spores for which germination was independent of the volume, but increased as the surface area was increased (9). The decrease in germination and germ tube growth with increasing volume suggests that some factor essential for germination may leak out of the spores and germ tubes. As the volume increased, the concentration of this germination factor would decrease; and hence, the percent germination and growth of the germ tubes decreased. The release of an esterase when coffee rust uredospores were stirred with water has been observed (5).

The effect of increasing the spore load while the volume of Tween-20 solution was held constant is shown in Table 2. These data also show that when the quantity of spores is small there was no germination. When a large amount of spores was used, germination was good. At the highest spore load the spores were very crowded yet germination was high. It is not entirely clear why the germination self-inhibitor was less active against floating spores than against spores that were suspended in 1% Tween-20 (7). However, one hypothesis is that the inhibitor will distribute between the spores, the floating film and the Tween-20 solution. If the distribution is such that very much more inhibitor is in the Tween-20 solution than in the film, then an effective concentration of the inhibitor may not contact those spores that float on the film; we observed high germination with floating spores and low

TABLE 1. Effect of volume[a] on germination of uredospores of *Hemileia vastatrix*

| Volume (ml) | Germination (%) | Remarks |
|---|---|---|
| 0.1 | 69 | Long germ tubes |
| 0.2 | 68 | Long germ tubes |
| 0.4 | 60 | Long germ tubes |
| 0.8 | 26 | Mostly short germ tubes |
| 1.6 | 5 | Very short, thin germ tubes |
| 3.2 | 2 | Very short, thin germ tubes |
| 4.8 | 0 | No germ tubes |

[a]Increasing quantities of 0.5% aqueous Tween-20 solution was added to about 0.3 mg dry uredospores in flat-bottom glass cups (20 × 11 mm). The per cent of floating spores showing germ tubes was counted after 6 hours incubation at 20-23 C in darkness.

TABLE 2. Effect of *Hemileia vastatrix* uredospore quantity[a] on germination

| Quantity of uredospores (mg) | Germination (%) | Remarks |
|---|---|---|
| 0.7 | 0 | No germ tubes |
| 1.5 | 8 | Very short, thin germ tubes |
| 3 | 11 | Short germ tubes |
| 6 | 19 | Some short germ tubes |
| 12 | 53 | Crowded yet long germ tubes |
| 24 | 53 | Crowded yet long germ tubes |

[a]Increasing quantities of dry uredospores were placed in flat-bottom cups (20 × 11 mm) and 2.5 ml of 0.5% aqueous Tween-20 solution added. The per cent of floating spores showing germ tubes was counted after 6 hours of incubation at 20-23 C in darkness.

germination in submerged spores. However, spores that were submerged in and wet by the Tween-20 solution may then contact a much higher inhibitor concentration and therefore not germinate. This hypothesis also suggests that the increased germination caused by Tween-20 may be due to its ability to extract and bind the germination self-inhibitor. Germination is a complex process that may be influenced by factors from the spores that both stimulate and inhibit germination.

**Formation of films and spreading of spores.**—The formation of films was suggested by observations made with the microscope that groups of spores floating on the surface would move together while the spores maintained their relative positions as if they were supported on a transparent film. The formation of a film was demonstrated by sprinkling 1 mg of uredospores onto a water surface of 5.5 $cm^2$ which was restricted on one side by the floating barrier. By careful viewing, a film was seen to spread rapidly from the floating spores. Within 6 minutes, this film pushed the floating barrier until the film from 1 mg of spores covered an area of about 33 $cm^2$.

Uredospores sprinkled upon a restricted surface over 400 ml of water spread and formed a film which covered the surface within a few minutes. However, germination was very low, less than 1% when the surface was 2 $cm^2$. The few germ tubes were very thin and short. When the surface area was 25 or 64 $cm^2$, no germ tubes were formed. These results contrast with those for wheat rust uredospores. In that case the uredospore germination increased as the surface area increased and was high when the surface area was large (9). These results likewise suggest that some factor necessary for germ tube development leaks from the spores. When the volume of water is large, the concentration of this factor is too low to allow germination.

Clumps of spores on a dry slide were examined under the microscope as they contacted the surface of droplets of water or Tween-20 solutions. Most individual spores in the clumps did not penetrate the surface of the droplet but were dispersed on its surface either as individual spores or in groups usually one spore thick.

When small drops of water were either placed upon or allowed slowly to roll over the uredospore clumps of a leaf pustule, the spore mass was not easily wet by the water. Instead, the surface film of the drop would dislodge many individual spores and groups of spores from the uredospore masses in the pustules. These spores would then spread out on the surface film and float there as individual spores or in small groups. This observation may suggest that as dew forms on a pustule, the lipid nature of the spore coat and its resistance to wetting may enable the surface tension of the enlarging drop of dew to detach mature spores from the pustule. The detached spores rapidly release a film on which they float. An esterase is also released (5). The esterase may hydrolyze the lipids from the spores and perhaps from the plant cuticle that could provide nutrients for the growing germ tube. This could assure a continuous supply of nutrients for the growing germ tube. When the germ tube contacts a stomate, it may penetrate to produce infection. Thus, it may be hypothesized that each uredospore carries a support system consisting of a film on which it floats, a

TABLE 3. Coffee rust uredospore germination inhibition by cinnamic acid derivatives[a]

| Compound | Inhibition at 100 μg/ml (%) | $ED_{50}$ (μg/ml) |
|---|---|---|
| *p*-Methoxycinnamic acid | 100 | 15 |
| Cinnamic acid | 100 | 24 |
| 3,4-Dichlorocinnamic acid | 100 | 25 |
| *o*-Methoxycinnamic acid | 100 | 25 |
| *m*-Methoxycinnamic acid | 100 | 28 |
| 3,5-Dimethoxycinnamic acid | 100 | 30 |
| 2,4-Dimethoxycinnamic acid | 100 | 30 |
| 3,4-Dimethoxycinnamic acid | 100 | 35 |
| 2,5-Dimethoxycinnamic acid | 100 | 42 |
| Methyl-m-hydroxycinnamic acid | 81 | ... |
| Methyl-p-hydroxycinnamic acid | 68 | ... |
| 3-Hydroxy-4-methoxycinnamic acid | 62 | ... |
| 4-Hydroxy-3-methoxycinnamic acid | 57 | ... |
| Methyl-2-hydroxy-3-methoxycinnamic acid | 50 | ... |
| 4-Hydroxycoumarin | 49 | ... |
| 3,4-Dihydroxycinnamic acid | 48 | ... |
| Methyl-3,4-dimethoxycinnamic acid | 38 | ... |
| *p*-Hydroxycinnamic acid | 35 | ... |
| 7-Hydroxycoumarin | 20 | ... |
| Coumarin | 18 | ... |
| 2,3,4-Trimethoxycinnamic acid | 18 | ... |
| *o*-Hydroxycinnamic acid | 7 | ... |
| 3,4,5-Trimethoxycinnamic acid | 6 | ... |
| 3,4-Dimethoxycinnamic acid | 6 | ... |
| Cinnamamide | 3 | ... |

[a]0.5 ml of a UV-irradiated ethanol solution of each compound (1.0 mg/ml) was diluted with 4.5 ml of 0.5% aqueous Tween-20, and 0.1 ml added to about 0.2 mg dry uredospores in a concave slide. After incubation over water at 20-23 C for 6 hours in darkness, the inhibition was determined from counts of the percentage of spores showing germ tubes in controls without the cinnamic acid derivative and in those containing the compound under assay. Five serial dilutions in 0.5% Tween-20 were assayed with each compound which showed complete inhibition at 100 μg/ml to determine the $ED_{50}$ (1).

reserve of lipid nutrients and an esterase to hydrolyze the lipid for the nutrition of the germ tube.

Such a concept would predict that spore germination and infection would be highest when the volume of water in the infection droplet is small so that the nutrients would be retained near the growing germ tube. This is consistent with the view that infection occurs at night when dew is formed. It would also suggest that the use of a dew chamber after application of dry spores to leaves or the spraying of a nonaqueous spore suspension in oil would give the highest infection rate.

**Germination inhibition by cinnamic acid derivatives.**—The two known rust uredospore germination self-inhibitors are methyl esters of 3,4-dimethyloxycinnamic acid and 3-hydroxy-4-methoxycinnamic acid (2, 3, 4). Cinnamic acid and 3,4-dimethoxycinnamic acid are slightly effective in inhibiting the germination of coffee rust uredospores (7). The results obtained when cinnamic acid and 24 cinnamic acid derivatives were assayed for their activity in inhibiting the germination is given in Table 3. The data show that cinnamic acid and many substituted cinnamic acids have inhibitory activity toward the germination of the coffee rust uredospores.

The amount of cinnamic acid required for 50% inhibition in this assay in which the uredospores were floated on the solutions under assay was about three times more than that when the spores were suspended. This apparent lower activity might be due to the higher ratio of spores to solution volume in the present assay or to a lower cinnamic acid uptake by floating spores.

The data show that nine cinnamic acid derivatives caused complete inhibition of germination at 100 $\mu$g/ml. The $ED_{50}$ was determined for these nine compounds. The most active compound was *p*-methoxycinnamic acid which showed an $ED_{50}$ of 15 $\mu$g/ml. Cinnamic acid with an $ED_{50}$ of 24 $\mu$g/ml was slightly less active. The monomethoxy, dimethoxy, and dichloro derivatives of cinnamic acid were the most active compounds tested. They all showed $ED_{50}$'s from 15 to 42 $\mu$g/ml. The methyl esters of the methoxy acids were less active than the acids. Activity was abolished when the carboxyl group of cinnamic acid was converted to the amide group. The introduction of a hydroxyl or a third methoxy group onto the benzene ring lowered the activity.

TABLE 4. Chromatography of cinnamic acid derivatives that were tested for inhibition of uredospore germination in *Hemileia vastatrix*, the coffee rust fungus

| Compound[a] | $R_f$[b] |
|---|---|
| *p*-Methoxycinnamic acid | 0.27, 0.32 |
| Cinnamic acid | 0.17, 0.34 |
| 3,4-Dichlorocinnamic acid | 0.16, 0.21 |
| *o*-Methoxycinnamic acid | 0.22, 0.35 |
| *m*-Methoxycinnamic acid | 0.14, 0.27 |
| 3,5-Dimethoxycinnamic acid | 0.15, 0.29 |
| 2,4-Dimethoxycinnamic acid | 0.25, 0.36 |
| 3,4-Dimethoxycinnamic acid | 0.09, 0.20 |
| 2,5-Dimethoxycinnamic acid | 0.13, 0.29 |

[a]Ten microliters of the ultraviolet UV-irradiated ethanol solution (1 mg/ml) were chromatographed on thin-layer plates of silica gel developed with benzene-ethyl ether (80:20, v/v). The position of the *cis* and *trans* isomers were determined by observation under UV light.

[b]Average of four determinations with range of about ± 0.05. The $R_f$ of the inhibitor from coffee rust uredospores was 0.3 in the same chromatographic system (8).

Twenty-one cinnamic acid derivatives caused significant inhibition of the germination of coffee rust uredospores when tested at 100 $\mu$g/ml. Although the level of activity was only about 1% of that reported for the naturally occurring germination self-inhibitors of other rusts, it is nevertheless highly significant. Only the *cis* isomers of the two known rust uredospore germination self-inhibitors show high germination inhibition activity (4). The cinnamic acid derivatives tested were undoubtedly mixtures of the *cis* and *trans* isomers, and the *cis* isomers alone may be much more inhibitory than these data indicate.

Since extracts from uredospores (6, 7, 8) and some of these cinnamic acid derivatives have shown fairly high activity in inhibiting the germination of coffee rust uredospores, it would seem worthwhile to conduct preliminary laboratory and field trials to determine if the cinnamic acid derivatives can reduce infection by the coffee rust fungus. The most active compound, *p*-methoxycinnamic acid, showed an $ED_{50}$ at 15 $\mu$g/ml. This activity would seem low enough to warrant such studies which are planned.

The spore germination inhibition activity of the methyl ester of *p*-hydroxycinnamic acid was greater than the corresponding acid. The methyl ester of 3,4-dihydroxycinnamic acid was synthesized in Madison after the other experiments were finished, and a single assay with spores that showed 45% germination suggested that this ester was very active; the ester completely inhibited germination at 25 $\mu$g/ml and allowed only 5 and 30% germination at 12.5 and 6 $\mu$g/ml, respectively. These preliminary results suggest that methyl 3,4-dihydroxycinnamic acid also should be further studied in laboratory and field trials.

**Chromatography of cinnamic acid derivatives.**—The unknown germination self-inhibitor from coffee rust uredospores was partially purified by Musumeci et al. (7) by chromatography on thin layer plates of silica gel developed with benzene-ethyl ether. From germination assays of ether extracts of silica gel scraped from the plates, these authors determined that the $R_f$ value of the coffee rust uredospore self-inhibitor was 0.3.

The $R_f$'s of the nine most active cinnamic acid derivatives were determined after UV irradiation using the same chromatography system as used with the partially purified inhibitor from uredospores. In this silica gel, benzene ether (8:2) system, the *cis* isomers show a slightly higher mobility than the *trans* isomers.

The $R_f$'s of the nine most active cinnamic acid derivatives are shown in Table 4. These data show that the $R_f$'s of cinnamic acid, the *o*-, *m*-, and *p*-monomethoxycinnamic acids and the 3,5-, the 2,4-, and the 2,5-dimethoxycinnamic acids all have $R_f$'s for their *cis* isomer between 0.27 and 0.36. These $R_f$'s are within the range of the experimental variation of the method to the $R_f$ 0.3 reported for the germination self-inhibitor purified from coffee rust uredospores (7).

Because the self-inhibitor from the coffee rust fungus has not yet been isolated, we could not do co-chromatography. Nevertheless, the close similarity of the chromatographic properties of the partially purified inhibitor from coffee rust uredospores and that of

cinnamic acid and these methoxycinnamic acid derivatives, together with their common activity in inhibiting the germination of uredospores of the coffee rust fungus, may suggest that the germination self-inhibitor of *Hemileia vastatrix* might also be a cinnamic acid. Studies will continue to further characterize this inhibitor.

## LITERATURE CITED


1. FINNEY, D. J. 1952. Prohibit analysis. 2nd ed. Cambridge University Press, Cambridge, England. 318 p.
2. MACKO, V., R. C. STAPLES, P. J. ALLEN, and J. A. A. RENWICK. 1971. Identification of the germination self-inhibitor from wheat stem rust uredospores. Science 173:835-836.
3. MACKO, V., R. C. STAPLES, H. GERSHON, and J. A. A. RENWICK. 1970. Self-inhibitor of bean rust uredospores: methyl 3,4-dimethoxycinnamate. Science 170:539-540.
4. MACKO, V., R. C. STAPLES, J. A. A. RENWICK, and J. PIRONE. 1972. Germination self-inhibitors of rust uredospores. Physiol. Plant Pathol. 2:347-355.
5. MUSUMECI, M. R., W. B. C. MORAES, R. L. NICHOLSON, and J. KUĆ. 1973. Observations on an esterase activity associated with uredospores of Hemileia vastatrix. J. Coffee Res. 3(2):46-49.
6. MUSUMECI, M. R., W. B. C. MORAES, and R. C. STAPLES. 1973. Evidencia de um auto-inhibidor da germinaçao nos uredospores de Hemileia vastatrix. Lo Congresso Brasileiro Sobre Pragas e Doencas Do Cafeeiro, pg. 5, July 4-6, 1973 (Abstr.).
7. MUSUMECI, M. R., W. B. C. MORAES, and R. C. STAPLES. 1974. A self-inhibitor in uredospores of the coffee rust fungus. Phytopathology 64:71-73.
8. MUSUMECI, M. R., R. L. NICHOLSON, W. B. C. MORAES, and J. KUĆ. 1972. Substancias, liberadas pelos uredospores de Hemileia vastatrix (Berk. et Br.) e inhibidoras da germinaçao des mesmos. Ciencia e Cultura. 24:416 (Abstr.).
9. WOODBURY, W., and M. A. STAHMANN. 1970. Role of surface films in the germination of rust uredospores. Can. J. Bot. 48:499-511.
10. ZAMBOLIM, L., and G. M. CHAVES. 1974. Efeito de baixas temperaturas e do binomio temperatura - umidade relativa sobre a viabilidade dos uredosporos de Hemileia vastatrix (Berk. et Br.) e Uromyces phaseoli typica. Arch. VIIth Congress of the Brazilian Society of Phytopathology, 3-8 Feb., 1974 (Abstr.).