

Puccinia coronata var. avenae夏胞子の自己発芽抑制物質の生理的特性

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Physiological Characterization of Germination Self-Inhibitor in Uredospores of *Puccinia coronata* var. *avenae*

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鶴嶋 鉄*・真山滋志**・谷 利一**・上野民夫*・深海 浩* : *Puccinia coronata*
var. *avenae* 夏胞子の自己発芽抑制物質の生理的特性

Abstract

Two self-inhibitors were found in germination fluids of uredospores of *Puccinia coronata* var. *avenae*. Major one identified in the previous paper as methyl *cis*-3,4-dimethoxycinnamate (MDC) was released in the germination fluid as soon as the uredospores were floated on water and mostly liberated from uredospores within 40 min, before protrusion of germ tubes. On the other hand, a minor inhibitor (unidentified) became detectable in the germination fluid when germ tubes had mostly germinated, and increased thereafter. MDC strongly inhibited the germination, but exhibited little effect on the germ tube growth and differentiation of infection structures at the concentration giving complete inhibition of germination. MDC decreased its activity in the presence of sodium pelargonate. Characteristic behaviors of germination and infection structure formation of *P. coronata* var. *avenae* were different in various respects from those of rust fungi producing MDC, but rather resembled those of *Puccinia graminis* var. *tritici* which produced another self-inhibitor, methyl *cis*-ferulate. It is proposed that the hypothesis that rust species producing the same self-inhibitors are closely related to each other in the characteristics of germination and differentiation should be reexamined.

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Key Words : self-inhibitor, uredospore, *Puccinia coronata* var. *avenae*, germination.

Introduction

The self-inhibition of fungal spore germination is a well established phenomenon, especially for the germination of uredospores of various rust species, as reviewed first by Allen¹⁾ in 1965 and recently by Macko³⁾ and Wolf¹⁵⁾. The chemical principles of rust self-inhibitors have been identified to be mainly composed of two compounds, methyl *cis*-3, 4-dimethoxycinnamate (MDC) and methyl *cis*-ferulate (MF). The former was

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found in uredospores of *Uromyces phaseoli*, *Puccinia herianthi*, *Puccinia antirrhini*, *Puccinia sorghi*, and *Puccinia arachidin*^{6,7)}, and the latter was in *Puccinia graminis* var. *tritici*⁸⁾. Physiological behaviors of the self-inhibitors have been discussed by Macko and Staples⁴⁾ in relation to the characteristics of uredospore germination and infection structure formation of various rust species. They stressed that the rust species could be classified into two groups based on the biological features of uredospores, and that the rust species belonging to the same group produced the same self-inhibitor (Cf. Table 3).

The presence of self-inhibitor in *Puccinia coronata* var. *avenae* had been indicated in 1959 by Naito *et al.*¹³⁾. Recently, we¹⁴⁾ isolated a self-inhibitor from uredospores of this fungus and determined its chemical structure as MDC. In this paper, some biological behaviors of MDC are described in relation to the germination and differentiation of infection structures of *P. coronata* var. *avenae*. The results are compared with those of other rust fungi to discuss the hypothesis of Macko and Staples⁴⁾.

Materials and Methods

Rust fungi. Rust fungi used in this study were *Puccinia coronata* Cda. var. *avenae* Fraser & Led. race 226, *Puccinia coronata* Cda. var. *festucae* Eriks. & Henn., and *Puccinia graminis* (Pers.) var. *tritici* Eriks. & Henn. Fresh uredospores of each rust fungus were obtained by incubating previously inoculated mature leaves in a moist chamber at 20–25 C for 48 hr. When necessary uredospores were stored at 5 C in darkness until the use.

Detection of inhibitors in germination fluid. Uredospores (50 mg) of race 226 were floated on 50 ml of water in a square box (surface area 475 cm²) and incubated at 20 C in darkness. At 20 min intervals of incubation, germination fluids were taken and replaced with fresh water. Germination fluids thus obtained were extracted with diethyl ether as reported previously¹⁴⁾. Detection of self-inhibitors in the extract was carried out by the modified method of the TLC plate bioassay which had been established for the phytoalexin study⁹⁾. A portion of the concentrated extract was spotted onto a thin-layer (0.3 mm) plate of silica gel (Merck GF₂₅₄) and developed with benzene–diethyl ether (8:2, v/v). MDC and MF were spotted as standards. The plate was thoroughly air-dried to remove the solvent. After moistening the developed plate by spraying water, uredospores of race 226 were brushed onto the plate to make a uniform dense layer. The plate was then sprayed with a mist of water and incubated at 20 C in darkness for 12–16 hr in a moist chamber. Inhibitory activity on the TLC plate was recognized as yellow spots against the fainter color of the background where the original color of uredospores was lost after germination.

Effect of MDC on germination and infection structure formation. Pure preparation of the self-inhibitor, MDC, used in this study was obtained from uredospores of race 226 by the method reported earlier¹⁴⁾. For estimation of inhibitory activity to uredospore germination, uredospores of race 226 and *P. coronata* var. *festucae* were floated on water droplets and at 10 min intervals of incubation the spores were transferred onto the droplets containing MDC (20–80 pg/ml). Germination percent was estimated

3 hr after floating on the water.

The effect of MDC on differentiation of infection structures was examined by the following method. Uredospores of race 226 and *P. coronata* var. *festucae* were dispersed on the upper surface of the stripped lower epidermis of 7-day-old primary leaves of oat cv. *Pc 38*. The inoculated epidermal strip was placed on wet filter papers and incubated in a Petri dish for 8 hr. The epidermis was then floated on aqueous solutions of MDC (20–80 pg/ml) with mesophyll side down for another 8 hr, and placed again on wet filter papers. At 40 hr after inoculation, formation of substomatal vesicles and infection hyphae were observed. The experiments were conducted at 20 C in darkness.

Effect of Sodium pelargonate on MDC activity. Uredospores (5 mg) were floated on MDC solution (40 pg/ml) in a Petri dish (9 cm diam.). After incubation at 20 C for 6 hr in darkness, sodium pelargonate was added to the solution to give final concentrations of 10^{-1} – 10^{-5} M. Germination percent was counted after additional 6 hr incubation under the same condition.

Effect of factors affecting germination and differentiation. The cold treatment for dormancy of uredospores was examined by the method of Bromfield²⁾. Uredospores of race 226 were put into a glass tube and placed in a refrigerator at –20 C for 192 hr. A portion of the spores was transferred to an incubator at either 40 C for 5 min or 20 C for 20 hr. The treated spores were sprayed onto 1% water agar and the percentage of germination was estimated after incubation at 20 C in darkness for 16 hr. Uredospores of *P. graminis* var. *tritici* were also used for the experiment as a reference.

Negative phototropism of emergence of germ tubes was examined by the method of Naito *et al.*¹¹⁾

Induction of infection structure formation by heat-shock was examined by two methods according to the descriptions of Maheshwari *et al.*⁸⁾ and Mendgen¹⁰⁾. Uredospores of race 226 were floated on a water droplet in the former method or dispersed onto the surface of stripped epidermis of oat coleoptile (cv. *Pc 38*) in the latter method. They were kept at 20 C for 2 hr, heat-shocked at 35 C for 2 hr, then incubated at 20 C for another 12 hr in darkness.

The effect of volatile fraction on the formation of infection structures on collodion oil membrane was examined with uredospores of race 226 as reported by Maheshwari *et al.*⁸⁾. For the preparation of the volatile fraction, 1 g of uredospores of race 226 was shaken in 100 ml of 10 mM phosphate buffer (pH 6.5) at 20 C for 12 hr and the eluate was steam-distilled. Differentiation of substomatal vesicles was observed 16 hr after incubation at 20 C in darkness.

Other materials and methods are described in the result section.

Results

Release of self-inhibitors from uredospores

Fig. 1 demonstrates self-inhibition of uredospores of *P. coronata* var. *avenae*. Five mg of uredospores dispersed well on water surface in a Petri dish of 9 cm diam. and germinated regardless whether the spores were pre-washed or not. When 40 mg of ure-

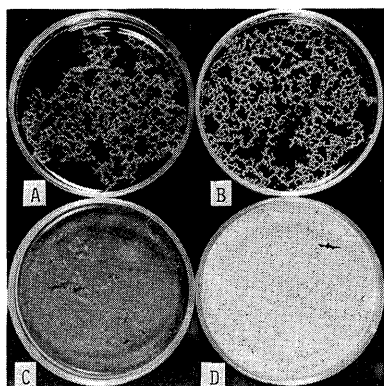


Fig. 1. Effect of pre-washing on self-inhibition in uredospore germination of *Puccinia coronata* var. *avenae*. Five mg of spores with (B) or without (A) pre-washing were floated on water in 9 cm-diam. Petri dishes; the spores germinated in both. Forty mg of spores with (D) or without (C) pre-washing were floated on water in the Petri dishes of the same size; spores were self-inhibited in (C), but germinated in (D).

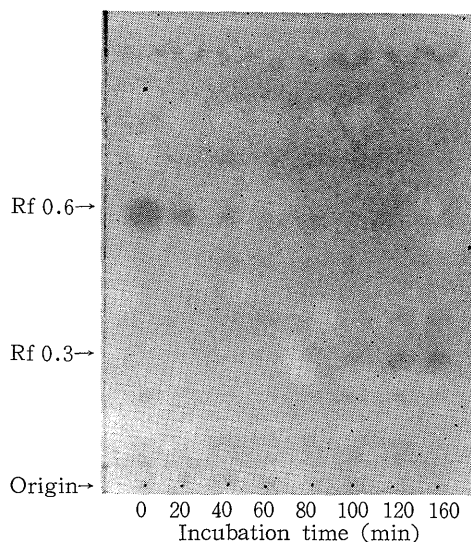


Fig. 2. Self-inhibitors in germination fluids of uredospores of *Puccinia coronata* var. *avenae*. The inhibitory spots detected by the TLC bioassay at Rf 0.6 and 0.3 correspond to methyl *cis*-3,4-dimethoxycinnamate and unidentified compound, respectively.

dospores were floated on the same surface, however, uredospores produced a film covering whole surface of water and did not germinate, and no self-inhibitor was detected in the water. On the other hand, the same amount of spores germinated well in the same size of Petri dish if the spores had previously been washed with 40 ml of water by stirring for 20 min, and MDC was detected in the wash.

The time of release of the self-inhibitors during germination of race 226 was examined by the TLC plate bioassay (Fig. 2). The major self-inhibitor at Rf 0.6, identified as MDC¹⁴⁾, was detected in the germination fluid soon after floating of uredospores on water and most of the inhibitor was liberated from the uredospores within 40 min, before protrusion of germ tubes. On the other hand, the minor inhibitor at Rf 0.3 was first detected at 60 min after incubation and increased thereafter.

Effect of MDC on germination and differentiation

Germination of uredospores of race 226 was prevented almost completely when MDC was added to the germination fluid at the beginning of incubation (Fig. 3). If MDC was added 20 min after floating, inhibitory activity was decreased; and the decrease was more pronounced as the time of MDC supply was delayed even though the emergence of germ tubes had not yet occurred. Little to no inhibition was observed if MDC was supplied 50 to 90 min after floating, at the time when germination had begun. Neither germ tube elongation nor morphological manifestation was affected by MDC when it was added to the germination fluid after the emergence of germ tubes.

The treatment with MDC (40 pg/ml) just giving complete inhibition of germination of race 226 uredospores did not affect the formation of substomatal vesicles and sub-

Table 1. Effect of methyl *cis*-3,4-dimethoxycinnamate (MDC) on germination and infection structure formation

Concentration of MDC (pg/ml)	<i>P. coronata</i> var. <i>avenae</i>			<i>P. coronata</i> var. <i>festucae</i>		
	Germination(%)	Substomatal vesicle(%) ^{a)}	Infection hypha(%) ^{b)}	Germination(%)	Substomatal vesicle(%) ^{a)}	Infection hypha(%) ^{b)}
0	68	86	80(165)	92	66	88(143)
20	41	84	77(156)	63	70	81(141)
40	1	80	81(154)	8	66	79(136)
80	0	65	60(106)	0	49	59(107)

a) Formation of substomatal vesicles per total appressoria observed.

b) Formation of infection hyphae per total substomatal vesicles observed. The value in parenthesis is the average length (μm) of infection hyphae.

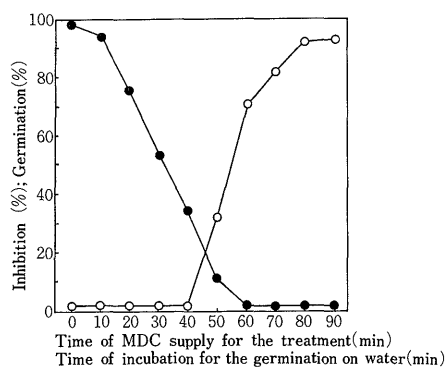


Fig. 3. Effect of the time of treatment with methyl *cis*-3,4-dimethoxycinnamate (MDC; 40pg/ml) on the germination of uredospores of *Puccinia coronata* var. *avenae*. ○—○: Germination on water, ●—●: Inhibition of germination by MDC.

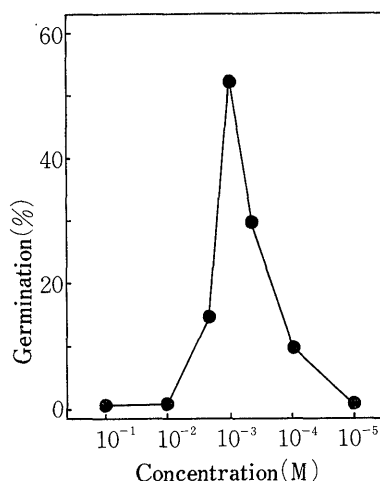


Fig. 4. Effect of sodium pelargonate on the inhibitory activity of methyl *cis*-3,4-dimethoxycinnamate (40pg/ml) to uredospore germination of *Puccinia coronata* var. *avenae*. Germination on water and MDC solution (40 pg/ml) was 85 and 0%, respectively.

sequent growth of infection hyphae (Table 1). Slight inhibition of differentiation of substomatal vesicles and growth of infection hyphae were observed, if the inhibitor concentration was doubled. Similar results were obtained for uredospores of *P. coronata* var. *festucae*.

Effect of sodium pelargonate on MDC activity

It has been reported that sodium pelargonate at concentrations between 2×10^{-3} and 5×10^{-4} M overcomes self-inhibition due to over-crowded population¹²⁾. In the present experiment, addition of sodium pelargonate at 2×10^{-3} to 5×10^{-4} M also prevented the inhibition of uredospore germination of race 226 caused by MDC whose concentration was just enough to the complete inhibition (Fig. 4).

Physiological characteristics of uredospores of *P. coronata* var. *avenae*

Present result on the negative phototropism of uredospore germination confirmed the result of Naito *et al.*¹¹⁾. Germination dormancy of uredospores of *P. coronata* var. *avenae*

Table 2. Effect of cold treatment and following heat shock on germination

Treatment	Germination (%)	
	<i>P. coronata</i> var. <i>avenae</i>	<i>P. graminis</i> var. <i>tritici</i>
Non-treated	94±3	82±10
Cold treatment	1±1	17± 3
Heat shock 1 ^{a)}	12±2	63± 3
Heat shock 2 ^{a)}	11±2	Not examined

a) After the cold treatment, uredospores were exposed at 40 C for 5 min (Heat shock 1) or at 20 C for 20 hr (Heat shock 2), respectively.

Table 3. Germination characteristics of three rust uredospores

Treatment	<i>P. coronata</i> var. <i>avenae</i>	<i>Uromyces</i> <i>phaseoli</i> ^{a)}	<i>P. graminis</i> var. <i>tritici</i> ^{a)}
Native self-inhibitor	MDC ^{b)}	MDC ^{b)}	MF ^{b)}
Suppression of self-inhibition by sodium pelargonate	+	—	+
Negative phototropism of germ tube emergence	+	—	+
Dormancy induced by cold	+	—	+
Induction of infection structures by heat shock	— ^{c)}	—	+
on collodion oil membrane	+	+	—
by volatile fraction	—	—	+

+ and — : positive and negative responses, respectively.

a) Data are compiled from Bromfield²⁾, Maheshwari *et al.*⁸⁾, and Macko and Staples⁴⁾, respectively.

b) MDC : methyl *cis*-3,4-dimethoxycinnamate, MF : methyl *cis*-ferulate.

c) Differentiation was induced by the heat shock of uredospores placed on the epidermal layers of oat primary leaves.

was induced by the cold treatment to large extent, as compared with *P. graminis* var. *tritici*, while the recovery of germination was relatively low (Table 2). The heat shock did not induce infection structures of *P. coronata* var. *avenae* when uredospores were germinated on water, but induced at considerable frequency (about 20% of germinated spores) when they were germinated on the epidermal layers stripped from oat primary leaves. About 80% of germinated uredospores of *P. coronata* var. *avenae* produced infection structures on collodion membrane, but no appressorium formation was induced in the Petri dish incubated with the volatile fraction.

Discussion

Two germination inhibitors were found in the germination fluids of *P. coronata* var. *avenae* uredospores. The compound detected at Rf 0.6 on the TLC plate is MDC¹⁴⁾ and considered to be fully responsible for the self-inhibition. The compound at Rf 0.3 was not detected in the resting uredospores and found at about the time when the germ

tubes had mostly germinated; thus it does not appear to function to self-inhibition. MDC seems to be characterized as a compound active mainly to the emergence of germ tubes; no inhibition of germ tube growth and infection structure formation occurred at the concentration just giving complete inhibition of germination.

Macko and Staples⁴⁾ proposed that there are two groups of species within rust fungi; one group is those producing MDC as a natural self-inhibitor and rust species belonging to this group are more closely related to each other in the behaviors of germination and infection structure formation and more distantly related to rust species which contain the other self-inhibitor, MF. The present results on the behaviors of germination and infection structure formation of *P. coronata* var. *avenae* are summarized and compared with those of two rust fungi; one is *Uromyces phaseoli* producing MDC and the other is *Puccinia graminis* var. *tritici* producing MF (Table 3). It is quite clear that the behaviors of *P. coronata* var. *avenae* are different from those of *U. phaseoli* in the response to sodium-pelargonate, cold treatment, and light irradiation, and probably to heat shock, but rather resemble those of *P. graminis* var. *tritici* in various respects. We may thus point out that the proposal by Macko and Staples⁴⁾ should be reexamined. Much additional experiments with various rust species are required before any conclusive remark is made.

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和 文 摘 要

Puccinia coronata var. *avenae* 夏胞子の自己発芽
抑制物質の生理的特性

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エンバク冠さび菌 *Puccinia coronata* var. *avenae* の夏胞子には2種の自己発芽抑制物質が存在する。主要物質は既報の methyl *cis*-3,4-dimethoxycinnamate (MDC) で、夏胞子を水面に浮かべると、その直後から発芽液中に溶出しはじめ、発芽管出現以前(40分後)までに殆んどが溶離した。他の物質(未同定)は発芽管の出現後に発芽液中に検出された。MDC は発芽をほぼ完全に阻害する濃度(40 pg/ml)では発芽管伸長、気孔下のう形成および感染菌糸伸長に影響を示さなかったので、発芽に対して特に阻害力が強いものと思われる。MDC は sodium pelargonate 存在では活性が低下した。*P. coronata* var. *avenae* の発芽および感染構造体形成における特性は MDC を生成する他のさび菌類と種々の点で異なり、むしろ、他の自己発芽抑制物質 methyl *cis*-ferulate を生成する *Puccinia graminis* var. *tritici* に類似していた。したがって、同じ自己発芽抑制物質を産生する菌種間では発芽および分化の特性が類似しているとの仮説は再検討されるべきである。