Plant & Cell Physiol., **10**, 183–192 (1969)

Biological activities of rishitin, an antifungal compound isolated from diseased potato tubers, and its derivatives [1]

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(Received August 14, 1968)

Rishitin, a norsesquiterpene alcohol, found in infected, resistant potato-tuber tissue completely inhibited zoospore germination and germtube elongation of *Phytophthora infestans* (MONT.) DE BARY at 10^{-3} M. There was little difference in sensitivity to rishitin among races of *Phytophthora infestans*. IAA-induced elongation of *Avena* coleoptile sections and GA_3-induced elongation of wheat leaf sections were also inhibited by rishitin. The inhibition of IAA-induced elongation of *Avena* coleoptiles was relieved to some extent by increasing IAA concentration. However, little relief of the inhibition of GA_3-induced elongation of wheat leaf sections was obtained by increasing GA_3 concentration. No plant injury was observed at this concentration of rishitin (10^{-3} M).

Examination of a series of rishitin derivatives indicated that the hydroxyl group at C-3 is indispensable for antifungal activity. This activity was intensified by saturating the double bond between the rings of rishitin and/or that of the isopropenyl group at C-7, though activity decreased when oxygenated functional groups were introduced into the side chain.

Aromatization of the A ring did not lower biological activities. The antifungal activities of most rishitin derivatives almost paralleled their activities as plant growth retardants. However, some compounds without antifungal activity were active as growth retardants.

A new antifungal compound "rishitin" (Fig. 1, VI a) has been isolated from potato tubers (*1*, *2*) and tomato plants (*3*) infected with an incompatible race of *Phytophthora infestans*. Rishitin, in a broad sense, is probably a phytoalexin (*1*, *4*) based on MÜLLER's definition (*5*) that such compounds are increased strikingly or are newly produced in plants in response to infection. He presumed that "phytoalexin" plays an extremely important role in disease resistance. However, as pointed out in a previous paper (*1*), it is necessary that phytoalexin be demonstrated in and isolated from many plant species, and that the biological properties of such compounds be investigated before the role of phytoalexin in host-parasite physiology and disease resistance can be fully understood. Here we report some biological activities of rishitin and its derivatives.

Abbreviations : IAA, indole-3-acetic acid ; GA_3, gibberellin A_3.

[1] Studies on the phytoalexins (5).

Material and methods

Preparation of rishitin and its derivatives

Rishitin was isolated from potato tubers infected by an incompatible race of *Phytophthora infestans*. Final purification was effected by crystallizing the di-3,5-dinitrobenzoate (*1*), followed by hydrolysis of the benzoate and distillation *in vacuo*. Some of the rishitin derivatives used were known while others are new (Fig. 1); their preparation will be published elswhere (MURAI *et al.* unpublished data).

Antifungal activity assay

The compounds were dissolved in 10 μl of ethanol (for the compounds I a–I e, II, V, VI a–VI c and VII a), or benzene (VI d) or acetone (III, IV a, IV b and VII b). Phosphate buffer (0.01 M, pH 6.0) containing agar (final conc.: 0.8%) was then added and the contents of the tubes poured into stainless steel planchets. A zoospore suspension (0.02 ml) of *Phytophthora infestans* was placed on the medium, and the planchets kept at 25°C for 4.5 hr. Germination percentage and the growth of germtubes were determined after the preparations were fixed with Bouin reagent. The races of *Phytophthora infestans* used were stock isolates from our laboratory, and their pathogenicity was tested just prior to use.

Wheat leaf test

From two-day-old etiolated leaves of *Triticum aestivum*, 'Norin-75', 5 mm sections were excised 2 mm from the base of the leaf. These sections were floated on 1 ml of 0.02 M phosphate buffer, pH 5.0 containing GA_3 and rishitin in the presence of 5×10^{-6} M IAA. Rishitin was dissolved in ethanol and added to the test medium

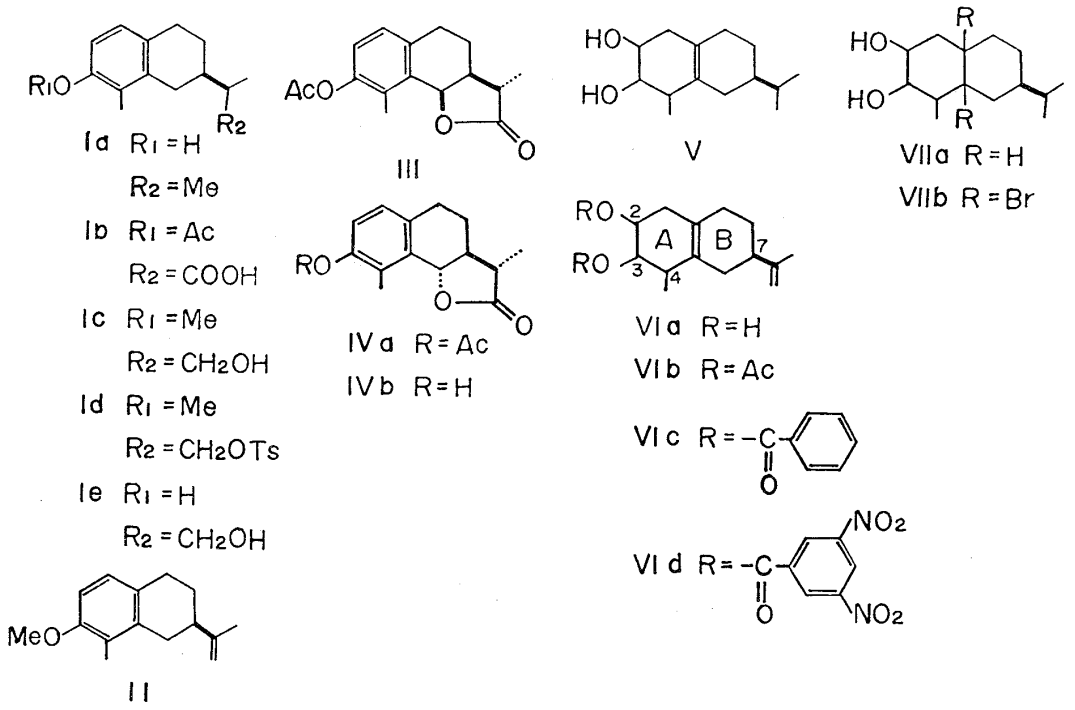


Fig. 1. Structures of rishitin and its derivatives (VI a, rishitin).

so that the final concentration of ethanol was 0.5%. The length of the sections were measured after 20 hr incubation (*6*)

Dwarf maize test

Seeds of the d_5 maize mutant were sown in a mixture of soil and vermiculite. Before the unfolding of the second leaf, a 0.1 ml chemical solution was placed in the folded second leaf according to the procedure of PHINNEY (*7*). The compounds were dissolved in 25% acetone containing 0.025% Tween 20. The lengths of the first and second leaf sheaths were measured 10 days after treatment. These experiments were conducted in a green house (10–21°C)

Avena *straight growth test*

Seeds of *Avena sativa* 'Victory' were sown in sawdust and kept at 25°C under red light for a day. They were then covered 3 cm thick with sawdust and kept in the dark for additional 2 days. The tip 1.0–1.5 mm of coleoptile was eliminated, and endogenus auxin was consumed by placing them in the dark for 2 hr. From the coleoptiles, 5.0 mm long sections were excised 2 mm below the physiological tip. The sections were then floated on a test medium. The basic test medium contained 0.02 M phosphate buffer, pH 5.0, 2% sucrose and IAA (*8*). Rishitin and its derivatives (I a–I e, II, V, VI b, VI c and VII a) dissolved in ethanol (final conc. of ethanol: 0.5%) were added to the basic medium containing 0.6% agar just prior to use. The compound VI d was dissolved in benzene (final conc. of benzene: 0.5%) and the solution was suspended in the basic medium containing 0.6% agar. The compounds III, IV a, IV b and VII b were dissolved in acetone (final conc. of acetone 0.5%) and the solutions added to the basic medium containing 0.6% agar.

Phaseolus *rooting test*

Cuttings were prepared from seedlings of *Phaseolus mungo* and the method followed the description by SHIBAOKA and MITSUHASHI (*9*)

Results

Antifungal activity of rishitin

As shown in Fig. 2 and Table 1, the medium effective dose (ED_{50}) of rishitin required to inhibit the growth of spore germtubes of *Phytophthora infestans* was about 2×10^{-4} M. Germination was completely inhibited at 10^{-3} M. Little difference was observed among the ED_{50} values of rishitin on the inhibition of growth in spore germtubes of various races.

Effect of rishitin on GA_3-induced growth

GA_3-induced elongation of wheat leaf sections were inhibited by rishitin at concentrations higher than 10^{-4} M (Fig. 3). Wheat leaf sections showed no sign of injury at these concentrations. The inhibition of growth was not relieved by increasing the concentration of GA_3 (Fig. 4). GA_3-induced growth of dwarf corn was inhibited by rishitin (Table 2). Tissue where rishitin was applied at concentrations more than 1 mmole/plant lost its green color. In spite of the strong growth

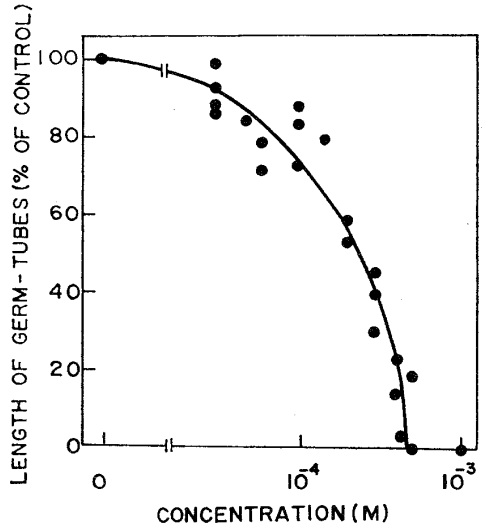


Fig. 2. Effect of rishitin on the growth of zoosporial germtubes in race 0 of *Phytophthora infestans*.

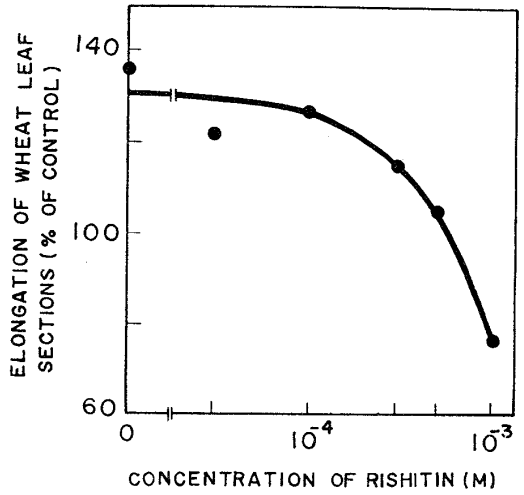


Fig. 3. Effect of rishitin on the elongation of wheat leaf sections induced by GA_3 at 3×10^{-6} M.

Table 1

Inhibition of germtube elongation of the spores of Phytophthora infestans *by rishitin*

Phytophthora infestans	ED_{50}
Race 0	2.1×10^{-4} M
Race 1	2.1×10^{-4} M
Race 3	2.1×10^{-4} M
Race 4	2.2×10^{-4} M
Race 2, 3, 4	2.4×10^{-4} M
Race 1, 2, 3, 4	2.4×10^{-4} M

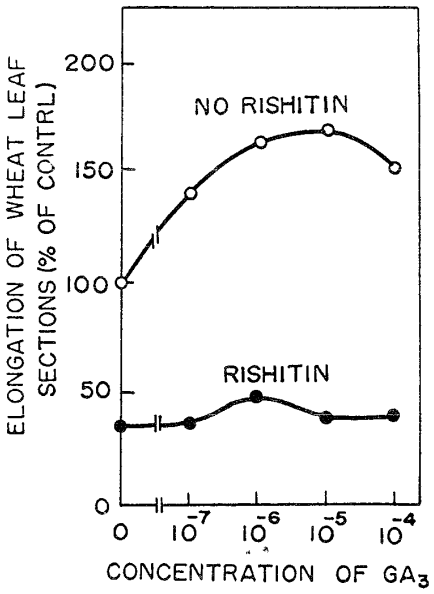


Fig. 4. Effect of various amounts of GA_3 on the inhibition of growth of wheat leaf sections caused by rishitin at 10^{-3} M.

Table 2

Effect of rishitin on GA_3-induced growth of dwarf corn [a]

	$-GA_3$	$+GA_3$ [b]			
		Rishitin concentration (mmoles/plant)			
		0	0.1	1	10
1st Leafsheath (cm)	1.84±0.23	4.56±0.41	5.00±0.54	4.60±0.59	2.77±0.22
2nd Leafsheath (cm)	2.24±0.03	13.15±1.07	11.65±0.89	11.30±0.60	6.05±1.01
Sum (cm)	4.08±0.26	17.71±0.11	16.65±0.13	15.90±0.92	8.82±1.41
(%)	100	434	408	390	206

[a] Average of 5 plants with standard error.
[b] Ten μg of GA_3 was administered to each plant.

inhibition, the leaves of GA_3- and rishitin-treated dwarf corn resumed normal leaf form and did not show the typical dwarf form.

Effect of rishitin on IAA-induced growth of Avena *coleoptile*

As reported in a previous paper (*1*), IAA-induced elongation of isolated *Avena* coleoptiles was completely inhibited by rishitin at 10^{-3} M. *Avena* coleoptiles showed no apparent sign of injury at this concentration. The inhibition of growth was relieved to some extent by increasing IAA concentration. The relief of inhibition, however, was not complete (Fig. 5). Recovery from inhibition due to 10^{-3} M rishitin by increasing IAA concentration was reported in a previous paper (*1*).

THOMAS *et al.* (*10*) reported that the abscisic acid inhibition of IAA-induced growth of *Avena* coleoptile was relieved when GA_3 was added to a medium containing

1 mg per liter of IAA. A similar experiment was carried out with rishitin and a slight reversal of inhibition by GA_3 was observed (Fig. 6).

Promotion of adventitious root formation by rishitin

Rishitin at 10^{-4} M – 5×10^{-4} M promoted adventitious root formation in hypocotyles of *Phaseolus* cuttings (Table 3 and Fig. 7). No sign of injury was observed at these concentrations. However, when plants were treated with rishitin at concentrations greater than 10^{-3} M for 24 hr, injury was observed.

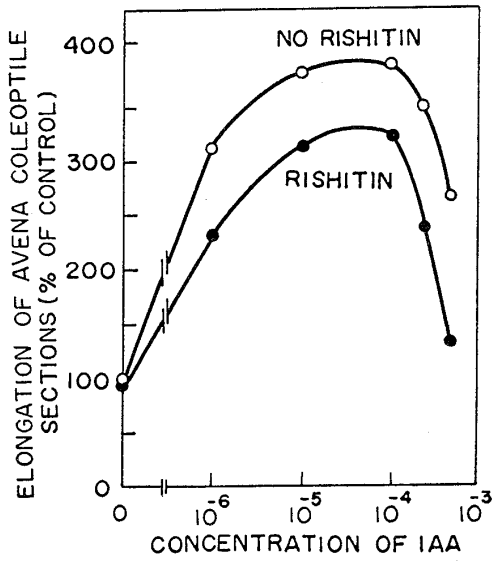


Fig. 5. Effect of various amounts of IAA on the inhibition of elongation of *Avena* coleoptile sections caused by rishitin at 3×10^{-4} M.

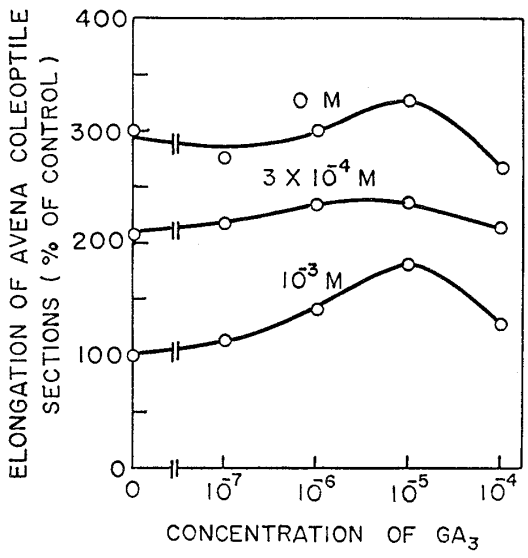


Fig. 6. Interaction between rishitin and GA_3 in the *Avena* coleoptile section test. All sections received IAA at 5×10^{-6} M.

Table 3
Promotion of adventitious root formation by rishitin [a]

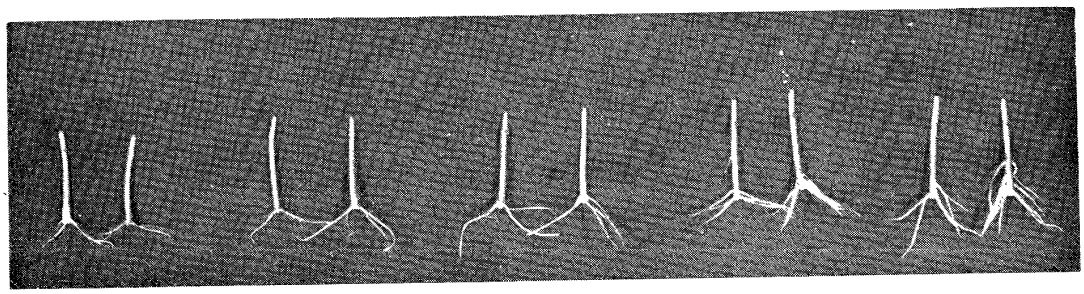
	Concentration of rishitin (M)			
	0	10^{-4}	3×10^{-4}	5×10^{-4}
Number of roots	7.67±0.51	9.43±0.69	9.51±0.89	10.15±0.98
Length of rooting zone (cm)	6.62±3.00	8.07±2.44	8.03±2.92	15.52±1.87

[a] Number of roots, average of 20 cuttings with standard error.

Table 4
Biological activities of rishitin derivatives

Compound	Inhibition of zoosporial germtube [a] growth	Percentage inhibition of zoospores [a] germination	Inhibition of IAA-induced growth of *Avena* coleoptile
I a	++	++	+
I b	±	±	±
I c	±	±	−
I d	−	−	−
I e	±	±	++
II	±	±	±
III	−	−	±
IV a	−	−	+
IV b	±	±	±
V	++	++	++
VI a	+	+	+
VI b	−	++	−
VI c	−	−	−
VI d	−	−	−
VII a	++	++	++
VII b	±	±	±

[a] *Phytophthora infestans.*
++, > rishitin ; +, = rishitin ; ±, < rishitin ; −, no effect.



0 10^{-5} 10^{-4} 3×10^{-4} 5×10^{-4}
Concentration of rishitin (M)

Fig. 7. Promotion of root formation in *Phaseolus* cuttings by rishitin.

Biological activities of rishitin derivatives

The antibiotic and plant-growth retarding properties of rishitin derivatives are shown in Table 4. Compounds I a, V, VI b and VII a were more active than rishitin in inhibiting zoospore germination of *Phytophthora infestans*. Although compound VI b had little effect on the elongation of zoosporial germtubes, it significantly reduced the percentage germination of zoospores.

The degree of inhibition of IAA-induced growth of *Avena* coleoptile by the derivatives almost paralleled that of their antifungal effect. However, compound IV a which showed no antifungal activity was as active as rishitin as a growth retardant.

Discussion

Rishitin inhibited the elongation of zoospore germtubes of *Phytophthora infestans*. CRUICKSHANK (*11*), UEHARA (*12*) and NONAKA (*13*) stated that fungi which are pathogenic to pea tissue are relatively insensitive to pisatin, a kind of phytoalexin, while, in general, fungi which are nonpathogenic to this host are highly sensitive to the pisatin. NONAKA (*14*) reported that ipomeamarone, another phytoalexin, was more toxic to fungi which are nonpathogenic to sweet potato than to those which are pathogenic to sweet potato. Our results indicate that there was little difference among the sensitivities of the races of *Phytophthora infestans* to rishitin, although the pathogenicity of the races is quite different from each other.

Our experiments indicate that rishitin acts also as a plant-growth retardant. Inhibition of GA_3-induced growth of wheat leaf sections by rishitin was not relieved by increasing the concentration of GA_3. At rishitin concentrations where growth inhibition occurred, no sign of injury was observed. The degree of inhibition of section growth caused by a definite amount of the inhibitor was about the same regardless of the amount of IAA supplied. Accordingly, the inhibition of IAA-induced growth by rishitin seems to resemble that by 2,4-dichloroanisole (*15*) and heliangine (*16*).

Some sesquiterpenoids; *e.g.*, abscisic acid and heliangine, are known to have plant-growth retarding activities. The above facts show that rishitin, a new sesquiterpenoid, also has biological activities, which are plant-growth retardant. Because of this, it was interesting to investigate the relationship between biological activity and chemical structure. The antifungal activity of rishitin and 15 closely related compounds suggests that for compounds to exhibit antifungal activity, a hydroxyl group at C-3 is indispensable, that saturation of the double bond between the two rings and/or that of the isopropenyl group does not reduce antifungal activity, that introduction of oxygenated functions into the side chain decreases activity, and that aromatization of the A ring does not reduce antifungal activity.

With few exceptions the inhibition of zoospore germination by most rishitin derivatives paralleled their effects on zoosporial germtube elongation and *Avena* coleoptile growth. Acetylation of the C-3 hydroxyl group did not reduce inhibition of zoospore germination, but it abolished inhibition of germtube elongation and *Avena* coleoptile growth. Compound IV a had almost the same *Avena*-growth retarding activity as rishitin, but had little antifungal activity.

Altered plant metabolism and formation of a barricade tissue zone are known to be caused by wound or infection (*17*). Rishitin is present in tuber tissue adjacent to brown lesions caused by infection (*1*) in amounts sufficient to act as a plant-growth retardant. Accordingly, rishitin can reasonably be supposed to play a role in the host plant metabolism, as well as in the inhibition of fungal development in diseased tissue. Further investigation into the mechanism of the biological activity of rishitin is needed to clarify the mechanism of disease resistance of potato plants to parasites.

The authors thank Dr. H. ABUMIYA, Chief of Dept. of Plant Pathology and Entomology of Hokkaido Agric. Experiment Sta. for promoting this research series, Dr. B. O. PHINNEY, University of California, for his generous supply of d_5 maize seeds and Dr. R. D. DURBIN, University of Wisconsin for his help in preparing the manuscript.

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