Biological Activity of Phyllostine, a Phytotoxic Compound Isolated from the Culture Filtrate of *Phyllosticta* sp.

Ryutaro SAKAI*, Rinzo SATO**, Jiro ITO***
and Sadao SAKAMURA***

酒井隆太郎*・佐藤倫造**・伊藤滋郎***・坂村貞雄***: *Phyllosticta* sp. の
培養濾液から分離した毒物質 Phyllostine の生理活性

Abstract

1. Phyllostine has been isolated from the culture filtrate of *Phyllosticta* sp., a pathogenic fungus of red clover, as the principal toxic compound causing wilting and dark discoloration of leafy stem cuttings of red clover and it is closely related structurally to phyllosinol.
2. Phyllostine stimulated growth of red clover, lettuce and rice seedlings at a concentration range from 10^{-8} to 10^{-5} M. These promoting effects of phyllostine on plant growth were similar to that of phyllosinol.
3. Phyllostine at concentrations between 10^{-6} to 2.5×10^{-4} M promoted adventitious root formation in epicotyls of Azukia cuttings. The promoting effect of phyllostine, however, was slightly higher than phyllosinol at the same concentration level.
4. IAA-induced elongation of isolated *Avena* coleoptile sections was inhibited by phyllostine at a concentration range from 10^{-6} to 5×10^{-4} M.
5. GA₃-induced elongation of lettuce hypocotyls was slightly inhibited by phyllostine at a concentration range from 5×10^{-5} to 10^{-4} M.
6. Phyllostine has antibiotic activity. The activity of phyllostine, on the whole, was about more than four times as high as that of phyllosinol.

(Received November 24, 1971)

Introduction

A new phytotoxic metabolite "phyllostine" has been isolated from *Phyllosticta* sp., a pathogenic fungus of red clover, as the principal toxic compound causing dark discoloration of leafy stem cuttings of red clover, and its chemical structure has been established^{5,7,8)} (Fig 1). Phyllostine is closely related to phyllosinol in structure, and it has similar phytotoxicity as phyllosinol. These similarities led us to study the biological effects of phyllostine on higher plants and microorganisms in comparison with phyllosinol.

Materials and methods

Preparation of phyllostine Phyllostine was isolated in a crystalline state from the culture filtrate of *Phyllosticta* sp. as presented in a preceding paper⁸⁾ (Fig. 2). Re-crystallization from chloroform

* National Institute of Agricultural Sciences, Kita-ku, Tokyo, Japan. 農業技術研究所

** Hokkaido National Agricultural Experiment Station, Sapporo, Japan. 北海道農業試験場

*** Department of Agricultural Chemistry, Hokkaido University, Sapporo, Japan. 北海道大学農学部

Fig. 1. Structures of phyllostine (I) and phyllosinol (II).

Fig. 2. Isolation procedure of the metabolites.

(I) Phyllosinol, (II) Phyllostine.

yielded pale yellow plates, mp 56°C, $[\alpha]_D^{20}$ -105.6 ($c=1$ in ethanol), which was assigned the molecular formula $C_7H_6O_4$ by mass spectra and elementary analysis.

Seedling growth test The seedling growth test was performed according to the procedures described in a previous paper⁶⁾.

Red clover and lettuce Seed of *Trifolium pratense* L. Lakeland and of *Lactuca sativa*, cv, Greatlake were soaked in tap water overnight and allowed to germinate at 23°C for 24 hr. Then, seedlings with roots of uniform size were transferred onto filter paper in a 6 cm Petri dishes and placed under continuous illumination in the growth cabinet at 27°C. The Petri dish was supplied with 5 ml of test solution (0.02 M phosphate buffer, pH 5.6), containing phyllostine at various concentrations for the two sheets of filterpaper. The light intensity in the growth cabinet was about 4,000 lux, supplied by fluorescent tubes (combination of "Natural Day Light", Toshiba Co., and "Vitalux" for plant culture, Shinnihon-Denki Co.).

After four days, lengths of hypocotyls and roots were measured.

Rice plant Seeds of *Oryza sativa* L., Norin 20 were soaked in tap water at 28°C for two days. Germinated seeds were selected for uniformity, and planted on cloth nets, which were set up on cylindrical bottles 5.7 cm in diameter and 7.5 cm in height. These bottles were placed in a 200 ml beaker filled with the test solution dissolved in 0.02 M phosphate buffer at pH 5.6.

They were then incubated for 11 days in the growth cabinet at 27°C.

During this period, distilled water was added to maintain the test solution at the original level. After 11 days, lengths of the shoots and roots were measured.

Azuki rooting test The rooting test was performed according to the procedures described in a previous paper⁶⁾. The cutting which consisted of 5 cm epicotyl, a pair of primary leaves and a small bud was taken from the light-grown (3000 lux at plant level, 27°C) 10 day old *Azuki angularis* “Wase-tairyu” seedling. After treating the basal parts with a test solution for 16 hr, the cuttings were transferred to distilled water and kept under about 4000 lux at 25°C for 7 days, and the number of roots protruded and length of rooting zone on the epicotyl were measured.

Avena straight growth test The Avena straight growth test was the same as described in the previous papers⁶⁾. From the coleoptile of 3 day old etiolated seedlings of *Avena sativa* “Victory” 5 mm long sections were excised 2 mm below the physiological tips. Twelve of such sections were floated on a test solution and their lengths were measured after 18 hr of dark incubation at 27°C. The basic test medium contained 0.02 M phosphate buffer (pH 5.3), 2% sucrose and IAA. Phyllostine dissolved in 0.02 M phosphate buffer (pH 5.3) was added to the basic medium.

Lettuce cotyledon test Seeds of *Lactuca sativa*, cv. Greatlake were sown on filter paper moistened with tap water. After 24 hr incubation, germinated seeds of uniform shape and size were transferred to Petri dishes. Seeds were supplied with culture medium and incubated for 72 hr in the growth cabinet (lux 3000) at 27°C. The culture medium consisted of GA₃ and phyllostine dissolved in half strength Hoagland solution.

Antibiotic activity assay Antimicrobial activity of phyllostine was studied with the agar streak dilution method using potato-glucose agar medium for fungi, and peptone-glucose agar medium for bacteria.

Phyllostine and other test chemicals in 0.02 M phosphate buffer at pH 5.6 and added to the medium at each concentration. The effects of sulfhydryl compounds on the antifungal action of phyllostine was determined as follows.

The required quantity (one or two molecular proportions) of the sulfhydryl compounds were dissolved in 0.02 M phosphate buffer at pH 6.0 and phyllostine was added to a final concentration of 2.5×10^{-4} M. The mixture was kept at 27°C for 20 hr, and tested for antifungal activity, using the agar plate streak method.

Results and discussion

Effect on growth of plant seedling

Phyllostine promoted elongation of red clover, lettuce and rice roots at a concentration range from 10^{-8} to 10^{-5} M and inhibited it at a higher concentration, 10^{-4} M (Table 1 and 2). The growth promoting effects of phyllostine were clearly observed in roots than in shoots. These promoting effects of phyllostine on plant growth were similar to that of phyllosinol⁶⁾. In the preceding paper, we suggested that phyllosinol and unsaturated lactones may behave in the same manner in root growth. The similarity between phyllostine and phyllosinol in structure leads us to speculate that

Table 1. *Effect of phyllostine on growth of plant seedlings*

Concentrations of phyllostine	Plant part length (mm)			
	Red clover		Lettuce	
	Shoot	Root	Shoot	Root
Control	21.2 ± 0.45 (100)	23.6 ± 1.00 (100)	10.3 ± 0.09 (100)	26.3 ± 0.35 (100)
10 ⁻⁸ M	21.0 ± 0.36 (99)	27.9 ± 0.31 (118)	11.5 ± 0.58 (111)	31.7 ± 0.47 (120)
10 ⁻⁷ M	22.3 ± 0.22 (105)	27.1 ± 1.34 (115)	11.4 ± 0.29 (109)	32.1 ± 0.67 (122)
10 ⁻⁶ M	22.0 ± 0.66 (104)	31.0 ± 1.61 (131)	11.3 ± 0.68 (109)	35.4 ± 0.36 (134)
10 ⁻⁵ M	21.1 ± 1.02 (101)	29.9 ± 0.21 (126)	11.1 ± 0.28 (107)	35.0 ± 0.39 (133)
10 ⁻⁴ M	20.8 ± 1.08 (98)	22.0 ± 1.01 (93)	10.0 ± 1.01 (97)	24.7 ± 0.72 (94)

Mean length of 40 seedlings with standard error.

Parenthesized figures, % of control.

Table 2. *Effects of phyllostine on growth of rice seedlings^a*

Concentrations of phyllostine	Shoot		Root		Length of 2nd leaf sheath (cm)
	Length of 2nd leaf (cm)	Dry wt. (mg)	Length of root ^b (cm)	Dry wt. (mg)	
Control	12.75 ± 0.19	(7.0)	5.01 ± 0.72	(3.1)	3.93 ± 0.02
10 ⁻⁸ M	12.71 ± 0.26	(7.7)	7.25 ± 0.60	(3.9)	4.34 ± 0.01
10 ⁻⁷ M	13.44 ± 0.28	(8.5)	9.92 ± 0.24	(4.1)	4.44 ± 0.01
10 ⁻⁶ M	13.15 ± 0.11	(8.8)	7.40 ± 0.17	(4.6)	4.38 ± 0.01
10 ⁻⁵ M	13.23 ± 0.11	(8.1)	7.85 ± 0.14	(4.4)	4.34 ± 0.14
2.5 × 10 ⁻⁵ M	12.99 ± 0.18	(7.2)	5.94 ± 0.15	(3.7)	3.48 ± 0.14

^a Average of 20 seedlings.

^b Length of main root.

Table 3. *Effects of phyllostine and phyllosinol on adventitious root formation from Azukia cuttings^a*

Concentration of chemicals (M)	Root formation		Region of root formation	
	Number of roots	% of control	Length of rooting zone (mm)	% of control
Control	9.5 ± 2.0	100	3.8 ± 0.32	100
Phyllostine 1 × 10 ⁻⁶	10.2 ± 3.6	107	5.1 ± 1.82	132
” 1 × 10 ⁻⁵	10.2 ± 3.6	107	4.7 ± 0.62	121
” 1 × 10 ⁻⁴	16.5 ± 3.5	173	13.1 ± 1.18	337
” 2.5 × 10 ⁻⁴	18.8 ± 5.1	197	25.3 ± 0.61	652
Phyllosinol 1 × 10 ⁻⁴	14.2 ± 2.1	147	16.3 ± 1.27	420
” 2.5 × 10 ⁻⁴	15.2 ± 2.3	160	30.2 ± 0.13	778

^a Average of twelve seedlings.

Fig. 3. A. Promotion of adventitious root formation from Azukia cuttings by phyllostine. From left, Control; 2.5×10^{-4} , 1×10^{-4} , 1×10^{-5} , 1×10^{-6} M of phyllostine.
 B. Comparative effects of phyllostine and phyllosinol at 2.5×10^{-4} M. From left, Control, phyllostine, phyllosinol.

phyllostine may behave in the similar manner as the unsaturated lacton compounds behave in plant growth^{2,3,4}.

Effect on adventitious root formation

Phyllostine at a concentration range from 10^{-6} to 2.5×10^{-4} M promoted adventitious root formation in epicotyls of Azukia cuttings and was the most effective at 2.5×10^{-4} M (Table 3, Fig. 3, A). These results were similar to those obtained with phyllosinol. Phyllostine, however, was slightly more active than phyllosinol at the same concentration level (Fig. 3, B).

Effect on IAA-induced growth of Avena coleoptile and GA₃-induced growth of lettuce hypocotyl

Figure 4 shows that IAA-induced elongation of isolated *Avena* coleoptile sections was inhibited by phyllostine at a concentration range from 10^{-6} to 5×10^{-4} M. This growth inhibition was not relieved by increasing the IAA concentrations. It indicates that phyllostine did not act as an anti-auxin on the promoting effects on plant growth.

Figure 5 shows that GA₃-induced elongation of lettuce hypocotyls was slightly inhibited by phyllostine at a concentration range from 5×10^{-5} to 10^{-4} M and the inhibition of growth was not relieved by increasing the concentration of GA₃. Generally, at concentrations above 10^{-4} M phyllostine

Fig. 4. Effect of phyllistine on elongation of *Avena* coleoptile sections induced by 1 ppm and 10 ppm IAA. Initial length of section, 5.0 mm. Each point indicates an average of 12 sections.

Fig. 5. Effect of phyllistine on GA₃-induced elongation of lettuce hypocotyl. Each point indicates an average of 12 plants.

Table 4. Comparison of antimicrobial activity of phyllistine and phyllosinol by agar dilution method

Test organisms	Minimum inhibitory concentration (ppm)	
	Phyllistine	Phyllosinol
<i>Colletotrichum lindemuthianum</i>	100	>100
<i>Phytophthora parasitica</i>	50	50<, <100
<i>Helminthosporium turcicum</i>	50	50<, <100
<i>Alternaria kikuchiana</i>	50	50<, <100
<i>Phytophthora capsici</i>	25	50<, <100
<i>Sclerotinia sclerotiorum</i>	25	50<, <100
<i>Pythium debaryanum</i>	25	25<, < 50
<i>Cercospora beticola</i>	12.5	100
<i>Helminthosporium sigmoideum</i>	12.5	100
<i>Sclerotinia mali</i>	12.5	50
<i>Pseudomonas ovalis</i>	50	100
<i>P. tabaci</i>	50	100
<i>Agrobacterium tumefaciens</i>	50	100
<i>Escherichia coli</i>	50	50
<i>Xanthomonas oryzae</i>	50	12.5<, < 25
<i>Bacillus subtilis</i>	25	100
<i>Pseudomonas coronafaciens</i>	25	50
<i>P. fluorescens</i>	12.5	50

Table 5. Comparison of the inhibitory effects of phyllostine and phyllosinol on growth of *Phytophthora capsici*, *P. parasitica*, *Sclerotinia sclerotiorum* and *Alternaria kikuchiana*.

Test organisms	Substance	Inhibition (% of control)		
		12.5 ppm	25 ppm	50 ppm
<i>Phytophthora capsici</i>	Phyllostine	46	66	90
	Phyllosinol	15	25	37
<i>P. parasitica</i>	Phyllostine	76	87	92
	Phyllosinol	7	12	30
<i>Sclerotinia sclerotiorum</i>	Phyllostine	68	74	93
	Phyllosinol	0	12	46
<i>Alternaria kikuchiana</i>	Phyllostine	27	36	50
	Phyllosinol	8	17	25

causes damage on tissues, while as reported in previous paper phyllosinol shows no injurious effect at the same concentration.

The inhibitory effect of phyllostine is more powerful than that of phyllosinol at the same concentration level.

Antifungal and antibacterial activity

In Table 4 and 5, antimicrobial activities of phyllostine were compared with those of phyllosinol. The activity of phyllostine, on the whole, was about more than four times as high as that of phyllosinol except those against *Escherichia coli* and *Xanthomonas oryzae*.

The effects of a sulfhydryl compounds upon the fungistatic properties of phyllostine and phyllosinol were shown in Table 6. At 2.5×10^{-4} M phyllostine inhibited growth of *Phytophthora capsici* by 90 per cent.

This effects were diminished slightly by one equivalent of sulfhydryl compounds tested and two equivalent led to diminished activity by 35 to 50 per cent. Whereas, the activity of phyllosinol was diminished by one equivalent of sulfhydryl compounds by 6 to 29 per cent.

In previous paper⁶⁾, we suggested that the antimicrobial activity of phyllosinol is ascribable, at least partly, to the α , β -unsaturated ketone group. Geiger and Conn³⁾ reported that unsaturated ketone

Table 6. Effect of sulfhydryl compounds on antifungal activity of phyllostine against *Phytophthora capsici*.

Sulfhydryl compounds (M)		None	Colony diameter (mm)			
			Phyllostine (2.5×10^{-4} M)	Inhibition (% of control)	Phyllosinol (2.5×10^{-4} M)	Inhibition (% of control)
None		52	5	90	22	58
Cysteine	2.5×10^{-4}		7	87	37	29
"	5×10^{-4}		27	48	36	30
Glutathion	2.5×10^{-4}		8	85	30	42
"	5×10^{-4}		26	50	39	35
Thiosulfate	2.5×10^{-4}		14	73	49	6
"	5×10^{-4}		34	35	51	4

P. capsici was grown on potato-glucose agar plate.

Fig. 6. A. Effects of phyllostine, phyllosinol, p-quinone and 2-hydroxymethyl-1, 4-quinone on growth of *Phytophthora capsici* on potato-glucose agar containing 2×10^{-4} M of each chemicals.

Fig. 6. B. Effects of analogs of phyllostine at 2×10^{-4} M on growth of *Phytophthora capsici* in potato-glucose agar.

C, Control, 1, p-quinone, 2, 2-hydroxymethyl-1, 4-quinone, 3, phyllostine, 4, phyllosinol.

reacts specifically with the sulfhydryl groups of certain enzymes. If this should be the mechanism by which phyllosinol inhibit growth of microorganisms, then one might expect that phyllostine, a substance having two carbonyl groups, would be more inhibitory against microorganisms than phyllosinol, a substance having only one carbonyl group. Subsequently, attempts were made to determine the effects of the antifungal activity of analogous compounds, phyllostine, phyllosinol, p-quinone and 2-hydroxymethyl-1, 4-quinone at a concentration of 2×10^{-4} M. Fig. 6, A and B shows that phyllosinol reduced growth considerably but was not as effective as phyllostine.

In addition, phyllostine that has an epoxy group, was more inhibitory than 2-hydroxymethyl-1, 4-quinone that has no epoxy group.

These results indicate that the antimicrobial activity of phyllostine may be attributable to the unsaturated carbonyl groups with some additional effects of the epoxide group.

Literature cited

- 1) Andreae, W. A. (1952). Nature, 170: 83.
- 2) Burstrom, H. (1954). Physiol. Plant., 7: 548-559.
- 3) Geiger, W. B. and J. E. Coon. (1945). J. Amer. Chem. Soc., 67: 112-116.
- 4) Goodwin, R. H. and C. Taves (1950). Amer. J. Bot., 37: 224-231.
- 5) Ichihara, A., Oda, K. and Sakamura, S. (1971). Agr. Biol. Chem., 35: 445-446.
- 6) Sakai, R., Sato, R., Niki, H and Sakamura, S. (1970). Plant & Cell Physiol., 11: 907-920.
- 7) Sakamura, S., Ito, J. and Sakai, R. (1970). Agr. Biol. Chem., 34: 153.
- 8) Sakamura, S., Ito, J. and Sakai, R. (1971). Agr. Biol. Chem., 35: 105-110.

和文摘要

Phyllosticta sp. 菌の培養濾液から分離した
毒物質 phyllostine の生理活性

酒井隆太郎・佐藤倫造・伊藤滋郎・坂村貞雄

アカクローバ罹病葉から分離した *Phyllosticta* sp. 菌の培養濾液から、自然感染葉病斑類似の障害を起こす物質として、既に報告した phyllosinol について新物質 phyllostine を分離した。本物質は phyllosinol について多量に生産され、その化学構造は phyllosinol に類似する。本報告では phyllostine と phyllosinol の植物および微生物に対する生理活性を比較した。phyllostine は低濃度 (10^{-8} ~ 10^{-4} M) で幼植物の生育、特に根部の生育を促進するが高濃度 (10^{-4} M) で阻害する。また小豆 Cutting の下胚軸において不定根の形成を促進する。これらの活性は一般に phyllosinol より高い。また phyllostine は幅広い抗菌スペクトルを有し、阻害は phyllosinol の2~8倍の活性を示した。また抗菌力は各種の SH-化合物により抑制が認められ、また 2-hydroxy-methyl-1, 4-quinone との抗菌力の比較試験等から、phyllostine の抗菌作用は、本物質の有する α , β -不飽和ケントおよびエポキシ基によると考えられる。