Inductive Effect of Inosine on Formation of Secondary Appressoria from Conidia of *Botrytis cinerea*

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阿久津克己*・小林裕美子*・渡部忠一**・黄 耿堂*・見里朝正*：灰色かび
病菌の第2次付着器形成に対するイノシンの誘導作用について

Abstract

Purine-related compounds, cyclic AMP, 5'-AMP, 5'-IMP, adenosine and inosine, induced formation of secondary appressoria of *Botrytis cinerea* on slide glass in the presence of glucose. Among these purine-related compounds, formation of the secondary appressoria was remarkable in inosine. Formycin B, which is an analogue to inosine, reduced inductive effects of the secondary appressoria with inosine, and competed with inosine in forming the secondary appressoria. The administration of inosine to mycelia of *B. cinerea* resulted in an increase of syntheses of both DNA and RNA, followed by an increase of protein synthesis. When the mycelia incorporated ¹⁴C-inosine were fractionated, radioactivity was detected in RNA fraction at early time after incubation, and afterward in DNA fraction. Formycin B did not inhibit biosynthesis of cellular macromolecules in the mycelia, but inhibited DNA synthesis stimulated with inosine. It was shown that formycin B competed with inosine for DNA synthesis. These results indicated that inosine stimulated DNA synthesis, followed by an increase in RNA and protein syntheses in the mycelia, resulting in induction of the secondary appressoria.

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Introduction

The author *et al.*¹³⁾ reported that the purine-related compound solutions containing glucose stimulated the infection by conidia of *Botrytis cinerea* on cucumber leaves, and the stimulative effect was most remarkable in inosine solution among the solutions. The conidia formed secondary appressoria after elongation of superficial hyphae, and invasion by them was observed in the purine-related compound solutions containing glucose¹⁾. Also, it is suggested that glucose might be of use for the conidial germination, formation of the primary appressoria and elongation of the superficial hyphae, and that inosine might play a significant role in formation of the secondary appressoria¹⁾.

On the plant surface, several factors have been known to stimulate or inhibit the appressorium formation^{2, 3, 4, 7, 15, 21, 22)}, so we attempted to determine whether or not the

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purine-related compounds induce formation of the secondary appressoria of *B. cinerea* on slide glass.

We investigated also on the biochemical effects of inosine on metabolism of cellular macromolecules, and effects of formycin B, an analogue compound of inosine, on formation of the secondary appressoria and metabolism of cellular macromolecules.

Materials and Methods

Fungus. *Botrytis cinerea* was subcultured on potato-dextrose agar at 20 C in dark. The conidia were collected by the method described previously¹⁾.

Plant. The seedlings of cucumber, cultivar Sagami Hanjiro, which was grown under the condition described previously were used¹⁾. The primary leaves were cut into disks of 15 mm diameter with a cork borer and placed on moistened filter papers in sterile Petri dishes.

Chemicals. The purine-related compounds used in this study were cyclic adenosine-5'-monophosphate (cyclic AMP), adenosine-5'-monophosphate (5'-AMP), inosine-5'-monophosphate (5'-IMP), adenosine and inosine. Formycin B, 3-[β -D-Ribofuranosyl]-pyrazolo-[4, 3-d] 6 H-7-pyrimidone, was obtained from P-L Biochemical Inc., 6-Benzyladenine-HCl (Kyowa Hakkō Co.), kinetin (Wako Pure Chemical Co.) and t-zeatin (Sigma Chemical Co.) were employed as cytokinins. All radioactive compounds labelled uniformly were purchased from the RCC in England.

Inoculation. Conidial suspension in the compound solution adjusted to 1×10^5 conidia/ml as inocula on cucumber leaf disks and that to 2×10^4 conidia/ml on slide glass were used. The concentration of each compound was adjusted to 1×10^{-3} M, and these compound solutions contained 5 % glucose. A 5 μ l droplet of each conidial suspension was placed on leaf disks or slide glass, and incubated at the condition described previously¹⁾.

Light microscopical observation. The inoculated leaf disks were fixed in FAA (Formalin : Ethanol : Acetic acid = 1 : 1 : 1) solution 12, 24 and 48 hr after inoculation, stained with cotton blue solution, and observed under a light microscope. The conidia on slide glass were observed 3, 6, 12, 24, 48 and 72 hr after incubation using a Nomarski interference apparatus.

Mycelial preparation. The conidia (1×10^6 conidia/ml) were grown in PS (potato-sucrose) medium with shaking at 20 C for 18 hr. The cultured mycelia were washed with 0.01 M Tris-HCl buffer (pH 7.0) and suspended in the Tris-HCl buffer containing 1 % glucose for tracer experiments.

Incorporation of ^{14}C -amino acid mixture into protein. The reaction mixture consisted of 4.5 ml of mycelial suspension containing 1 % glucose and 0.5 ml of the aqueous solution of inosine or formycin B to make 1×10^{-5} , 1×10^{-4} and 1×10^{-3} M in final concentration. The mixture was preincubated for 1 hr at 20 C on a reciprocal shaker, and incubated with addition of 0.1 $\mu\text{Ci/ml}$ of ^{14}C -amino acid mixture (57 mCi/mAtom). One ml of the reacted mixtures at fixed time of incubation was withdrawn and the reaction was stopped by the addition of 1 ml of 10 % perchloric acid.

Mycelial proteins were fractionated by the method of Ogur and Rosen¹⁹⁾, and hydrolyzed overnight in 1 N NH_4OH at room temperature. The alkaline soluble protein

was separated from the residue by centrifugation at $2700 \times g$ for 15 min., and its content was determined by the method of Lowry¹⁶⁾. Radioactivity was measured by a liquid scintillation spectrophotometer (Packard TRI-CARB).

Incorporation of ^{14}C -uridine and ^{14}C -thymidine. The procedure of radioisotope incorporation into the mycelia was the same as above. One-tenth $\mu\text{Ci}/\text{ml}$ of ^{14}C -uridine (490 mCi/mmol) and ^{14}C -thymidine (56.7 mCi/mmol) in final radioactivity were used for incorporation to RNA and DNA, respectively. RNA and DNA in mycelia were fractionated by the method of Ogur and Rosen¹⁹⁾, and the radioactivity of them was counted.

Incorporation of ^{14}C -acetate. To determine the incorporation of ^{14}C -acetate into lipid fraction, 0.1 $\mu\text{Ci}/\text{ml}$ of ^{14}C -acetate (58 mCi/mmol) was used in final radioactivity. The lipid fraction was prepared by the method of Bligh and Dyer⁵⁾ and the radioactivity of the lipid fraction was counted as cpm per total lipids.

Incorporation of ^{14}C -glucosamine. Incorporation was performed by the same procedure as described above. The final radioactivity of ^{14}C -glucosamine (286 mCi/mmol) was 0.1 $\mu\text{Ci}/\text{ml}$. The chitin fraction was prepared by the method of Blumenthal and Roseman⁶⁾ and the radioactivity incorporated into chitin was counted as the total cpm per ml of the sample.

Incorporation of ^{14}C -inosine. To determine the incorporation of ^{14}C -inosine into the mycelia, the reaction mixture consisting of 4.5 ml of mycelial suspension containing 1 % glucose and 0.5 ml of the aqueous solution of inosine or formycin B to make 1×10^{-4} M in final concentration was prepared. The mixtures were incubated with addition of 0.2 $\mu\text{Ci}/\text{ml}$ of ^{14}C -inosine (575 mCi/mmol). One ml of the reaction mixtures at fixed time of incubation was withdrawn and the reaction was terminated by the addition of 1 ml of 10 % perchloric acid. The mycelia were fractionated by the method of Huang *et al.*¹¹⁾ and the radioactivity of 1 ml of each macromolecular fraction was counted.

Results

Effects of purine-related compounds on the conidial growth of Botrytis cinerea on slide glass

The percentages of conidial germination in each purine-related compound solution containing glucose and glucose solution were about 100 % 12 hr after incubation and higher than in distilled water (Table 1). The germinated conidia formed primary appressoria within about 12 hr after incubation (Plate I-1). Nearly 100 % of germinated conidia formed the primary appressoria in all solutions except control (Table 1). In glucose solution, hyphae elongated from the primary appressoria, and many hyphal branches were formed within 72 hr after incubation on slide glass, but the formation of secondary appressoria was scarcely observed (Table 1, and Plates I-4 and 5). However, in purine-related compound solutions, the hyphae elongated from the primary appressoria and formed multicellular appressoria (secondary appressoria) within 72 hr after incubation (Table 1, and Plates I-6 and 7). Among the purine-related compound solutions, formation of the secondary appressoria was remarkable in inosine solution (Table 1).

Table 1. Germination and formation of primary and secondary appressoria from conidia of *Botrytis cinerea* in the solutions of purine-related compounds on slide glass^{a)}

Compounds ^{b)}	Germination (%) ^{c)}	Formation of primary appressoria (%) ^{d)}	Formation of secondary appressoria ^{e)}
Cyclic AMP	97.3	98.6	13
5'-AMP	98.4	95.2	18
5'-IMP	96.3	96.1	23
Adenosine	97.8	97.4	27
Inosine	98.0	96.5	31
Glucose	95.0	95.7	2
Control (distilled water)	53.4	53.1	0

a) The mean of three trials. Conidia on slide glass were incubated at 20 C under 12 hr-illumination of fluorescent light per day and observed 48 hr after incubation.

b) 1×10^{-3} M purine-related compound solutions containing 5 % glucose

c) Percentage of germinated conidia to total conidia

d) Percentage of primary appressorium-forming conidia to germinated conidia

e) Numbers of secondary appressoria per 1×10^2 conidia

Competitive relation between inosine and formycin B to formation of secondary appressoria and lesion

In 1×10^{-3} M formycin B containing only glucose, the conidia formed small lesions on cucumber leaf disks 48 hr after inoculation (Table 2). The germinated conidia formed primary appressoria and elongated superficial hyphae, but no secondary appressoria were formed. Aggressive lesions were formed by application of inosine into conidial suspension on the leaf disks in the presence of formycin B. In these aggressive lesions, the secondary appressoria were formed and the numbers increased in proportion to the increase of inosine concentration.

On slide glass, formation of the secondary appressoria in 5 μ l droplet was increased

Table 2. Competitive relation between inosine and formycin B to formation of secondary appressoria and lesions by conidia of *Botrytis cinerea* on cucumber leaves^{a)}

Composition of conidial suspension			Formation of secondary appressoria ^{b)}	Diameter of lesions (mm) ^{c)}
Concentrations of inosine(M)	5 % glucose	1×10^{-3} M formycin B		
0	+	+	0	5
1×10^{-6}	+	+	5	11
1×10^{-5}	+	+	11	13
1×10^{-4}	+	+	19	15
1×10^{-3}	+	+	25	13
1×10^{-3}	+	—	41	15
0	+	—	0	0

a) The mean of three trials

b) Numbers of secondary appressoria per lesion were counted 48 hr after inoculation.

c) Diameters were measured 48 hr after inoculation.

Table 3. Competitive relation between inosine and formycin B to formation of secondary appressoria from germinated conidia of *Botrytis cinerea* on slide glass^{a)}

Composition of conidial suspension		Formation of secondary appressoria ^{b)}	
Concentrations of inosine (M)	5 % glucose	Concentrations of formycin B (M)	
		1×10^{-4}	1×10^{-5}
0	+	4	6
1×10^{-6}	+	9	16
1×10^{-5}	+	14	18
1×10^{-4}	+	20	28
1×10^{-3}	+	24	37
1×10^{-4}	+	31 (without formycin B)	
0	+	5 (without formycin B)	

a) The mean of three trials

b) Numbers of secondary appressoria per 1×10^2 conidia were counted 72 hr after incubation.

Fig. 1. Lineweaver-Burk plots obtained from relation between inosine and formycin B to the secondary appressorium formation from germinated conidia of *Botrytis cinerea*, ▲, 1×10^{-5} M formycin B; ●, 1×10^{-4} M formycin B.

with the increase of inosine concentration containing 1×10^{-5} and 1×10^{-4} M formycin B (Table 3). The Lineweaver-Burk plots obtained from relation between inosine and formycin B to the secondary appressorium formation showed that formycin B competed with inosine in forming the secondary appressoria (Fig. 1).

Effects of cytokinins on formation of the secondary appressoria

In all cytokinin solutions containing glucose, the formation of secondary appressoria and lesions was not observed on cucumber leaf disks.

Effects of inosine and formycin B on mycelial metabolism

The effects of inosine and formycin B on the incorporation of various labelled

precursors into the macromolecules of mycelial cells were studied (Table 4).

When the mycelial suspension containing 1 % glucose was incubated with ^{14}C -acetate in the presence of inosine or formycin B for 1 hr, there was no remarkable difference in incorporation of ^{14}C -acetate into the mycelial lipid fraction.

By using ^{14}C -glucosamine, the effects of inosine and formycin B on incorporation into chitin of mycelial cell wall was also shown in Table 4. Inosine and formycin B did not show any effects on the incorporation of ^{14}C -glucosamine.

Formycin B did not show remarkable effects on the incorporation of ^{14}C -amino acid mixture into alkaline soluble and insoluble protein fractions. In the presence of inosine,

Table 4. Effects of inosine and formycin B on the mycelial metabolism of *Botrytis cinerea*

Compounds	Incubation times (hr)	Incorporation of the dosed ^{14}C -precursors (cpm) ^{a)}					
		Lipid	Chitin	Alkaline soluble protein	Alkaline insoluble protein	RNA	DNA
Inosine ($1 \times 10^{-4}\text{M}$)	0	1009	68	450	1563	97	75
	1	11942	1517	10076	23400	4188	625
	3	13586	5020	9356	19367	4519	1361
	5	12749	11150	14263	32556	3352	1071
Formycin B ($1 \times 10^{-4}\text{M}$)	0	748	59	883	1154	116	58
	1	13854	1382	17900	26443	2009	437
	3	13072	4722	18338	27940	3117	712
	5	11985	11500	10563	21003	2754	759
Control (1 % glucose)	0	783	62	368	651	92	71
	1	10213	1275	19273	28572	1826	427
	3	12594	5603	20087	31037	2099	732
	5	11370	13029	13972	23950	1894	678

a) The mean of three trials. ^{14}C -Acetate, ^{14}C -glucosamine, ^{14}C -amino acid mixture, ^{14}C -uridine and ^{14}C -thymidine were used as precursors of lipid, chitin, protein, RNA and DNA, respectively.

Fig. 2. Effects of inosine on the incorporation of ^{14}C -thymidine into DNA fraction of mycelia of *Botrytis cinerea*. $\triangle$, $1 \times 10^{-5}\text{M}$; $\square$, $1 \times 10^{-4}\text{M}$; $\bullet$, $1 \times 10^{-3}\text{M}$; $\blacktriangle$, control.

the incorporation of ^{14}C -amino acid mixture into protein fractions decreased at 3 hr after incubation temporarily, but increased at 5 hr after incubation.

On the incorporation of ^{14}C -uridine into RNA fraction of the mycelia, inosine showed remarkably stimulative effect, and formycin B showed somewhat higher than that of control in the incorporation of ^{14}C -uridine into RNA fraction.

When the mycelial suspension was incubated with ^{14}C -thymidine, inosine stimulated the incorporation of ^{14}C -thymidine into DNA fraction, but formycin B did not show any effect. The incorporation of ^{14}C -thymidine into the DNA fraction of mycelia increased with the increase of inosine concentration (Fig. 2).

Competitive relation between inosine and formycin B to the incorporation of ^{14}C -thymidine into DNA fraction of mycelia

From the above experiments, it was found that inosine and formycin B showed

Fig. 3. Competitive relation between inosine and formycin B to the incorporation of ¹⁴C-thymidine into DNA fraction of mycelia of *Botrytis cinerea*. ●, 1×10^{-3} M inosine; □, 1×10^{-3} M inosine + 1×10^{-3} M formycin B; ○, 1×10^{-4} M inosine + 1×10^{-3} M formycin B; ■, 1×10^{-5} M inosine + 1×10^{-3} M formycin B; △, 1×10^{-3} M formycin B; ▲, control.

¹⁴C-thymidine into DNA fraction was examined.

By 1×10^{-3} M formycin B, the incorporation of ¹⁴C-thymidine into the DNA fraction increased in proportion to the increase of inosine concentration (Fig. 3).

Incorporation of ¹⁴C-inosine into macromolecule fractions of mycelial cells

Mycelia incubated in the presence of ¹⁴C-inosine were prepared to examine which macromolecule fraction does incorporate inosine. The higher radioactivity was detected in RNA fraction 3 hr after incubation, and in DNA fraction 6 hr after incubation (Fig. 4).

Fig. 4. Incorporation of ¹⁴C-inosine into some macromolecular fractions from mycelia of *Botrytis cinerea*. ■, Lipid fraction; □, Alkaline soluble protein fraction; ▲, Alkaline insoluble protein fraction; △, RNA fraction; ●, DNA fraction.

competitive relation to formation of the secondary appressoria (Tables 2 and 3, Fig. 1). Also, the experiment on the incorporation of labelled compounds showed that inosine stimulated the incorporation of ¹⁴C-thymidine into DNA fraction, but no effect of formycin B in its incorporation (Table 4). Accordingly, relation between inosine and formycin B to the incorporation of

Discussion

It was reported that in infection process by conidia of *Botrytis cinerea* on cucumber leaves, glucose stimulated conidial germination, formation of primary appressoria and elongation of superficial hyphae, and that inosine induced formation of secondary appressoria¹⁾. These findings were obtained *in vivo* by using cucumber leaves, and

several factors on plant surface have been known to stimulate or inhibit formation of appressoria of plant pathogenic fungi^{2,3,4,7,15,17,21,22}. To compare the case on cucumber leaves we investigate, therefore, whether or not the purine-related compounds, especially inosine, induce formation of the secondary appressoria in conidia of *B. cinerea* on slide glass. The present results indicated that the purine-related compounds had an inductive effect on formation of the secondary appressoria in the presence of glucose, and that the effect was most remarkable in inosine. These results support that inosine induces to form the secondary appressoria in *B. cinerea*.

From the biochemical examination, the administration of inosine to mycelia of *B. cinerea* resulted in an increase of syntheses of both DNA and RNA, followed by an increase of protein synthesis (Table 4). Furthermore, when the mycelia which incorporated ¹⁴C-inosine were fractionated, radioactivity was detected in RNA fraction 3 hr after incubation, and in DNA fraction 6 hr after incubation (Fig. 4). These results suggest that inosine may play a role as trigger of DNA synthesis rather than as precursor of DNA synthesis in mycelia of *B. cinerea*. Formycin B did not inhibit biosyntheses of the cellular macromolecules in mycelia of *B. cinerea*, but inhibited the DNA synthesis stimulated with inosine (Table 4 and Fig 3). It was shown that formycin B competed with inosine for formation of the secondary appressoria and the DNA synthesis (Figs. 1 and 3). These results indicated that inosine stimulated *de novo* DNA synthesis, followed by an increase of RNA and protein syntheses in the mycelia, resulting in induction of the secondary appressoria.

It has been reported that cyclic AMP plays an important role as an intracellular inducer in morphogenesis, for example, formation of fruiting bodies in the higher Basidiomycetes^{8,18,20} and formation of conidia^{14,23,24}, zoosporangia^{26,27} and sclerotia⁹ in the plant pathogenic fungi. Cyclic AMP induced formation of the secondary appressoria in *B. cinerea*, but the inductive effect of cyclic AMP was lower than inosine, 5'-AMP, 5'-IMP and adenosine¹. 5'-AMP, 5'-ADP and 5'-ATP were not effective in inducing fruiting bodies¹², sclerotia⁹ and zoosporangia²⁶, but these nucleotides were effective in inducing the secondary appressoria¹. These facts suggest that the morphogenesis of the secondary appressoria as infection structure formed in only pathogenic fungi is different from that of fruiting bodies, sclerotia and zoosporangia. Also, 5'-ATP and cyclic AMP were immediately transformed into 5'-AMP and inosine in cell free system of *B. cinerea* mycelia¹³. It has been known that cyclic AMP incorporated into animal cells is converted to AMP, ADP, ATP and inosine¹⁰. Uno and Ishikawa²⁵ indicated that incorporated cyclic AMP might have been immediately converted by the action of phosphodiesterase to 5'-AMP. From these facts, the purine-related compounds, which are effective in forming the secondary appressoria, may be postulated to act through inosine in the stage of induction of the secondary appressoria of *B. cinerea*.

Some cytokinins, which take part in morphogenesis of higher plants, showed no inductive effect in formation of the secondary appressoria. However, it is interesting that inosine is common to cytokinins in the chemical structure.

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和 文 摘 要

灰色かび病菌の第2次付着器形成に対するイノシンの誘導作用について

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灰色かび病菌の第2次付着器形成に対する核酸塩基プリン関連化合物 (cyclic AMP, 5'-AMP, 5'-IMP, adenosine, inosine) の誘導効果をスライドグラス上で検討した。これらの物質はキュウリ葉上の場合と同様に第2次付着器形成を誘導するが、中でもイノシンが最も顕著な効果を示した。化学構造上イノシンの類似物質であるホルマイシンBをイノシン溶液に添加すると、第2次付着器形成の誘導効果が抑えられ、両者間に競合関係のあることが明らかになった。つぎにイノシン処理菌体では、RNA, DNA 両画分への¹⁴C-ウリジン、¹⁴C-チミジンの取り込み量が1～3時間に促進され、続いてたんぱく画分への¹⁴C-アミノ酸混合物の取り込みが促進された。一方、ホルマイシンBはDNA画分への¹⁴C-チミジンの取り込みに対して、イノシンと競合することを示したが、他の画分では競合関係はみられなかった。以上のことから、イノシンによる第2次付着器形成誘導には、DNA合成が関与し、そのDNAに基づくRNA、たんぱく合成など一連の生合成系が関与すると考えられる。

Explanation of Plate

Plate I. Light micrographs of the growth process in conidia of *Botrytis cinerea* in the presence of inosine on slide glass

1. Growth process in conidia of *B. cinerea* in the presence of inosine solution containing glucose on slide glass, 12 hr after incubation
2. Growth process in conidia of *B. cinerea* in the presence of distilled water on slide glass, 48 hr after incubation
3. High magnification of 2
4. Hyphae elongated from primary appressoria in the presence of glucose on slide glass, 48 hr after incubation
5. High magnification of 4
6. Secondary appressoria formed in the presence of inosine solution containing glucose on slide glass, 48 hr after incubation
7. High magnification of 6

All scales: 10 μ m

Abbreviation

c, conidium; gt, germ tube; h, hypha; pa, primary appressorium; s, septum; sa, secondary appressorium.

Plate I