# ポリオキシン耐性リンゴ斑点落葉病菌に対するポリベリン(R)成分のポリオキシンBとイミノクタジン酢酸塩の共力作用機構


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Original Article

# Synergistic Mechanism of Polyoxin B and Iminoctadine Triacetate in Polybelin® on Polyoxin-Resistant *Alternaria alternata* Apple Pathotype*

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Polybelin (a combined-fungicide containing three parts of polyoxin B and one part of iminoctadine triacetate) was found to cause abnormal germ tube swelling on polyoxin-resistant *Alternaria alternata* apple pathotype. Polybelin at 33.3 $\mu$g/ml ($ED_{50}$ for mycelial growth of a polyoxin-resistant isolate) caused electrolyte leakage and inhibited the chitin biosynthesis on polyoxin-resistant isolate. Polyoxin B at 25 $\mu$g/ml did neither cause electrolyte leakage nor inhibit chitin biosynthesis on polyoxin-resistant isolate. Iminoctadine triacetate at 8.3 $\mu$g/ml showed almost the same electrolyte leakage activity as that of polybelin at 33.3 $\mu$g/ml, but it did not inhibit chitin biosynthesis on polyoxin-resistant isolate. The synergistic effect of polybelin on polyoxin-resistant *A. alternata* apple pathotype may possibly cause polyoxin B incorporation into mycelial cells to increase, by inhibiting cell membrane functions through the action of iminoctadine triacetate.

## INTRODUCTION

Polyoxin B[1] is applied for crop and fruit protection against various plant diseases, including gray mold, powdery mildew, and *Alternaria* diseases such as pear black spot and apple leaf spot. In 1971, polyoxin-resistant strains of *A. alternata* Japanese pear pathotype (formerly, *A. kikuchiana*) were first discovered in Tottori prefecture, Japan,[2] and polyoxin-resistant strains of *A. alternata* apple pathotype (formerly, *A. mali*) were discovered in 1972.[3] Hori *et al.*[4] showed that polyoxin-resistance in *A. alternata* Japanese pear pathotype is due to decrease in polyoxins incorporation into mycelial cells of resistant strains.

Polybelin® (a mixture of 15% polyoxin B and 5% iminoctadine triacetate) was developed in 1984 as a combined-fungicide which is widely applicable for plant diseases.[5] Polybelin has effectiveness against some commercially important plant pathogens resistant to a fungicide, such as triadimefon-resistant powdery mildew and benzimidazole- and/or dicarboxyimide-resistant gray mold.[6]

The authors examined the synergistic effect of polybelin to prevent apple leaf spot in former report.[7] The present paper reports the synergistic mode of action of polybelin toward polyoxin-resistant *A. alternata* apple pathotype.

* A part of this paper was presented at the Annual Meeting of the Pesticide Science Society of Japan, Tokyo, March, 1995.

## MATERIALS AND METHODS

### 1. Chemicals

Polyoxin B (80%) and iminoctadine triacetate (40% aqueous solution) were obtained from the Chemistry Institute in Kumiai Chemical Industry Co. Polybelin was prepared as a mixture of three parts polyoxin B and one part iminoctadine triacetate. Nystatin was purchased from Sigma Co., and glycylglycine was obtained from Wako Pure Chemicals Co. D-[1-$^{14}$C]Glucosamine (HCl salt, 2.0 GBq/mmol) was purchased from New England Nuclear.

### 2. Organisms

Wild type strain of *A. alternata* apple pathotype designated as isolate KU48S (sensitive to polyoxin) and isolate KU16R (resistant to polyoxin)[7] were used, which were isolated from lesion part of apple leaf spot-diseased leaves collected in orchards (Aomori prefecture, Japan).

### 3. Culture Conditions

Fungi were grown on potato-dextrose agar medium (Nissui Seiyaku Co.) at 27°C. To prepare spores, the

fungi were cultured on apricot-agar medium containing 15 g agar in a liter of dried-apricot fruit (25 g) decoction in the dark at 27°C for 8 to 10 days. To prepare mycelial cells, the fungi were cultured in liquid medium containing 4 g yeast extract (Difco) and 20 g glucose in a liter of distilled water with reciprocal shaking (100 rpm) at 27°C for 3 days. The mycelia was harvested by filtration and washed thoroughly with distilled water for use in subsequent experiments.

*4. Spore Germination Assay*

This assay was conducted by the method of Eguchi *et al.* with some modifications.[8] Spores of *A. alternata* apple pathotype on apricot-agar medium were suspended in sterile distilled water and adjusted to 10–15 spores per a field under a microscope (×150). Chemicals were added to the spore suspensions. After incubation at 27 °C for 12 hr, the percentages of non-germinating or swelling spores were determined by microscopic observation.

*5. Determination of Antifungal Activity toward Mycelial Growth in Liquid Culture*

Washed mycelia (150 mg wet weight) were suspended in 5 ml fresh potato-dextrose liquid medium (containing 20 g glucose in a liter of 200 g potato tuber decoction) with or without the test chemicals. After 16 hr incubation with reciprocal shaking (100 rpm) at 27°C, the mycelia was harvested by filtration on a glass filter (51G3, Shibata Scientific Technology Co.) and washed with distilled water. The harvested mycelia were dried at 80°C for 3 hr, and dry weight was measured.

*6. Measurement of Electrolyte Leakage from Mycelia*

Washed mycelia (300 mg wet weight) were suspended in 10 ml distilled water with or without the test chemicals. After incubation at 27°C for 3 hr, the suspension was centrifuged at 1200×*g* for 5 min, and conductivity in the supernatant was measured with a conductivity meter (Horiba DS-14).

*7. Inhibitory Activity of Chitin Biosynthesis*

This parameter on *A. alternata* apple pathotype by polyoxin B and polybelin was determined by the modified method of Hori *et al.*[4] using *A. alternata* Japanese pear pathotype. Washed mycelia (150 mg wet weight) were incubated at 27°C for 90 min in 3 ml 0.066 M phosphate buffer (pH 6.8) containing D-[1-$^{14}$C]-glucosamine (11.1 kBq) with or without the test chemicals. The reaction was terminated by adding 2.5 ml 20% trichloroacetic acid. The mycelia were collected by centrifugation (1200×*g*, 5 min) and washed twice with 5 ml distilled water, successively one time with 5 ml ethanol. Then mycelia was extracted with 5 ml chloroform–methanol (3 : 1) at 70°C for 1 hr and finally treated with 2.5 ml 2 N sodium hydroxide at room temperature overnight to remove lipids and protein. The residue was washed three times with 5 ml water, and suspended in 2.5 ml 0.066 M phosphate buffer (pH 5.9) containing 0.1 unit of chitinase from *Streptomyces griseus* (Sigma Co.). The suspension was incubated at 40°C for 20 hr and the supernatant was collected by centrifugation. A 100 μl-aliquot of the supernatant was mixed with 10 ml scintillation cocktail (Aquasol-2, Du Pont Co.), and radioactivity in it was measured with a liquid scintillation counter (Beckman LS 6000TA).

## RESULTS

*1. Swelling Activity in Germ Tube*

This parameter, *i.e.*, the specific morphological effect of polyoxins, on *A. alternata* apple pathotype, is shown in Table 1. One microgram/ml of polyoxin B caused germ tube swelling of isolate KU48S (sensitive to polyoxin) spores, but scarcely caused germ tube swelling against isolate KU16R (resistant to polyoxin). Polybelin at 1.3 μg/ml (containing 1 μg/ml of polyoxin B and 0.3 μg/ml of iminoctadine triacetate) caused abnormal swelling of the germ tubes of isolates KU48S and KU16R. Iminoctadine triacetate had no effect on both isolates at 0.3 μg/ml.

*2. Inhibition of Mycelial Growth*

$ED_{50}$ and $ED_{90}$ of polybelin, polyoxin B, and iminoctadine triacetate on mycelial growth of *A. alternata* apple pathotype are shown in Table 2. Polyoxin B strongly inhibited the mycelial growth of polyoxin-sensitive isolate KU48S, while $ED_{50}$ and $ED_{90}$ in the polyoxin-resistant isolate KU16R were above 1000 μg/ml. Polybelin strongly inhibited the mycelial growth of polyoxin-resistant isolate KU16R. Semi-quantitative analysis by the method of Gisi *et al.*,[9] using $ED_{90}$ of polyoxin B, iminoctadine triacetate, and polybelin, indicated the joint action of polybelin toward isolate KU16R is synergistic.

*3. Electrolyte Leakage from Mycelial Cells*

This examination was conducted in $ED_{50}$ (described in Table 2) or near $ED_{50}$ value on mycelial growth of each chemical. As shown in Fig. 1, polybelin at 33.3 μg/ml (25 μg/ml of polyoxin B+8.3 μg/ml of iminoctadine triacetate) caused electrolyte leakage for *A. alternata*

Table 1 Ratio of swelling spores on *Alternaria alternata* apple pathotype.

| Chemicals | Swelling spores (%) | |
|---|---|---|
| | KU48S | KU16R |
| Polyoxin B 1 μg/ml | 99.9 | 13.0 |
| Polybelin 1.3 μg/ml | 99.9 | 90.3 |
| ICTA 0.3 μg/ml | 0.0 | 0.0 |

ICTA: iminoctadine triacetate.

Table 2 Effect on the mycelial growth of *Alternaria alternata* apple pathotype.

| Chemicals | $ED_{50}$ ($\mu$g/ml) | | $ED_{90}$ ($\mu$g/ml) | | *R* value of polybelin | |
|---|---|---|---|---|---|---|
| | KU48S | KU16R | KU48S | KU16R | KU48S | KU16R |
| Polyoxin B | 22.0 | >1000 | 114.4 | >1000 | — | — |
| ICTA[a)] | 10.8 | 12.6 | 19.4 | 26.4 | — | — |
| Polybelin | 28.8 | 33.3 | 61.1 | 64.7 | 0.84 | >1.51 |

[a)] ICTA: iminoctadine triacetate.

*R* value was calculated according to the method of Gisi *et al.*[9] as the following equation: $R=[\{4/(3/EC_{90}$ of polyoxin B+1/$EC_{90}$ of ICTA)$\}/EC_{90}$ of polybelin]. *R* value reveals the joint action of the mixture as antagonistic ($R<0.5$), additive ($0.5<R<1.5$), or synergistic ($R>1.5$).

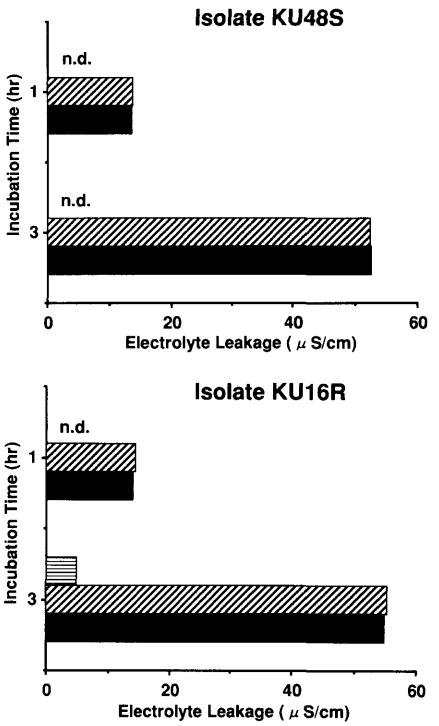


Fig. 1 Electrolyte leakage from mycelial cells of *Alternaria alternata* apple pathotype.

▤: polyoxin B; 25 $\mu$g/ml, ▨: iminoctadine triacetate; 8.3 $\mu$g/ml, ■: polybelin; 33.3 $\mu$g/ml. n.d.: not detected.

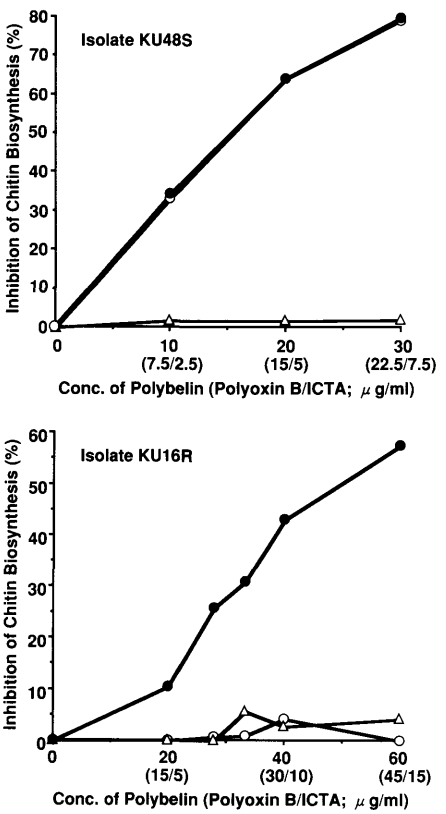


Fig. 2 Inhibition of chitin biosynthesis in the mycelial cells of *Alternaria alternata* apple pathotype.

○: polyoxin B, △: iminoctadine triacetate (ICTA), ●: polybelin.

apple pathotype isolates KU48S and KU16R. Iminoctadine triacetate at 8.3 $\mu$g/ml also caused leakage for isolates KU48S and KU16R, whereas polyoxin B at 25 $\mu$g/ml did not cause leakage for both isolates.

### 4. *Inhibitory Activity of Polybelin on Chitin Biosynthesis*

The values for this parameter of *A. alternata* apple pathotype by polyoxin B, iminoctadine triacetate, and polybelin are shown in Fig. 2. Polyoxin B inhibited the chitin biosynthesis in isolate KU48S at 30 $\mu$g/ml, but had hardly any effect on it in isolate KU16R at 60 $\mu$g/ml. Polybelin at 33.3 $\mu$g/ml inhibited the chitin biosynthesis in both isolates. Iminoctadine triacetate at 8.3 $\mu$g/ml scarcely inhibited chitin biosynthesis in both isolates. However, as shown in Fig. 3, iminoctadine triacetate augmented the inhibitory activity of 25 $\mu$g/ml polyoxin B toward the chitin biosynthesis of isolate KU16R.

### 5. *Effect of Polyoxin B in Combination with Nystatin*

The effect of electrolyte leakage of iminoctadine triacetate seems to be essentially the same as that of nystatin, a polyene antibiotic that attacks the cell membrane.[10] In our examination, nystatin in combination with polyoxin B caused germ tube swelling (data not shown) and inhibited chitin biosynthesis of *A. alternata*

Table 3 Incorporation of $^{14}$C-glucosamine into chitin fraction of isolate KU16R.

| Conc. of polyoxin B ($\mu$g/ml) | Combination with | | |
|---|---|---|---|
| | None | Nys[a] | ICTA[b] |
| No addition | 41,658 | 34,000 | 40,481 |
| 25 $\mu$g/ml | 39,018 | 20,968 | 25,619 |

[a] Nys: nystatin (0.025 $\mu$g/ml). [b] ICTA: imonoctadine triacetate (8.3 $\mu$g/ml).
Data indicates the radioactivity (dpm) of chitin fraction from 150 mg mycelia.

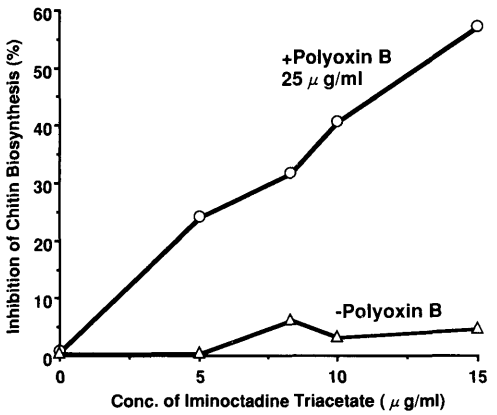


Fig. 3 Effect of polyoxin B in combination with iminoctadine triacetate on the chitin biosynthesis of *Alternaria alternata* apple pathotype isolate KU16R.

apple pathotype isolate KU16R as did polybelin (Table 3).

### 6. *Effect of Glycylglycine on Inhibitory Activity of Chitin Biosynthesis by Polyoxin B*

Hori *et al.*[11] found that dipeptides such as glycylglycine acted as antagonists on the uptake of polyoxins by polyoxin-sensitive *A. alternata* Japanese pear pathotype. We studied the inhibition of chitin biosynthesis by polyoxin B in combination with iminoctadine triacetate or nystatin on polyoxin-sensitive *A. alternata* apple pathotype isolate KU48S with or without glycylglycine in reaction mixture (Fig. 4). The inhibitory activity of 15 $\mu$g/ml polyoxin B on isolate KU48S remarkably disappeared in the presence of 15 mM (1982 $\mu$g/ml) glycylglycine. However, the inhibitory activity of chitin biosynthesis by 15 $\mu$g/ml polyoxin B in combination with 10 $\mu$g/ml iminoctadine triacetate or 0.025 $\mu$g/ml nystatin was scarcely prevented in the presence of 15 mM glycylglycine.

## DISCUSSION

Polybelin caused abnormal swelling of the germ tube, and showed synergistic effect on the mycelial growth of polyoxin-resistant *A. alternata* apple pathotype isolate KU16R (Tables 1, 2). Eguchi *et al.*[8] reported polyoxin

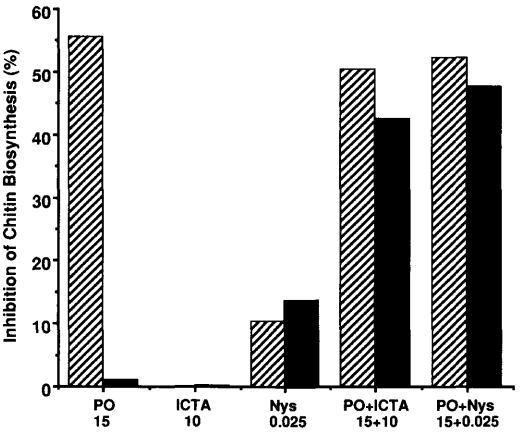


Fig. 4 Effect of polyoxin B in combination with iminoctadine triacetate or nystatin against the competitive action of glycylglycine (isolate KU48S).
▨: no addition, ■: Gly-Gly; 15 mM. PO: polyoxin B ($\mu$g/ml), ICTA: iminoctadine triacetate ($\mu$g/ml), Nys: nystatin ($\mu$g/ml).

B, a component of polybelin, causes germ tube swelling and inhibits mycelial growth of *A. alternata* apple pathotype. Hori *et al.*[12] elucidated the mode of action of polyoxin B on *A. alternata* Japanese pear pathotype to be interference with cell wall chitin biosynthesis. They also showed polyoxin-resistance in *A. alternata* Japanese pear pathotype to be due to decreased polyoxins incorporation into mycelial cells of a resistant strain.[4] Iminoctadine triacetate, another component of polybelin, has been shown to cause leakage of potassium due to its effect on cell membrane permeability.[13] The present report showed iminoctadine triacetate inhibited the mycelial growth of *A. alternata* apple pathotype, but caused no germ tube swelling. From these findings, we supposed that the synergistic mode of action of polybelin on polyoxin-resistant *A. alternata* apple pathotype is resulted from the increment of incorporation of polyoxin B into mycelial cells by the action of iminoctadine triacetate.

Polybelin at 33.3 $\mu$g/ml caused electrolyte leakage from both polyoxin-sensitive and polyoxin-resistant *A. alternata* apple pathotype isolates to nearly the same degree as 8.3 $\mu$g/ml iminoctadine triacetate (Fig. 1). Thus possibly, the interference with cell membrane functions by iminoctadine triacetate may occur in polybelin independently of polyoxin-resistance.

Polyoxin B interferes with chitin biosynthesis. Inhibitory activity of polybelin on the chitin biosynthesis of *A. alternata* apple pathotype was determined to examine the synergistic mode of action of polybelin toward polyoxin-resistant *A. alternata* apple pathotype. In polyoxin-sensitive isolate KU48S, polyoxin B and polybelin inhibited chitin biosynthesis. In polyoxin-resistant isolate KU16R, polyoxin B did not inhibit chitin biosynthesis,

but polybelin did so (Fig. 2). Iminoctadine triacetate did not inhibit chitin biosynthesis in either isolate, as also noted for *A. alternata* Japanese pear pathotype.[14] However, iminoctadine triacetate augmented the inhibition of chitin biosynthesis of polyoxin B in polyoxin-resistant isolate KU16R (Fig. 3). The synergistic mode of action of polybelin on polyoxin-resistant *A. alternata* apple pathotype would thus quite likely appear to result from the increased incorporation of polyoxin B into mycelial cells through the effects of iminoctadine triacetate on cell membrane functions.

Nystatin in combination with polyoxin B inhibited the chitin biosynthesis of polyoxin-resistant *A. alternata* apple pathotype isolate KU16R as also noticed for polybelin (Table 3). Cell-membrane attacking compounds, such as iminoctadine triacetate and nystatin, may thus provide protection when applied in combination with polyoxin B from polyoxin-resistant *A. alternata* apple pathotype.

Hori *et al.*[11] considered that the uptakes of polyoxins and the dipeptides through the cell membrane into the cell are mediated by a similar system on polyoxin-sensitive *A. alternata* Japanese pear pathotype. The data in Fig. 4 suggest the incorporation of polyoxin B to increase activation of the usual uptake system and/or a new polyoxin uptake system in cell membranes through the effects of cell membrane-attacking compounds.

The synergistic effects of polybelin on polyoxin-resistant *A. alternata* apple pathotype would thus appear to be possibly due to increased polyoxin B uptake by iminoctadine triacetate. The fungicide resistance of plant pathogens is a serious problem in crop protection. In recent years, several mixture of fungicides, for instance, diethofencarb in combination with thiophanate-methyl, carbendazim, or procymidone, based on negatively correlated cross-resistance,[15-17] are provided to cope with fungicide resistance in the field. Biochemical elucidation of the mechanism, such as negatively correlated cross-resistance and synergism among agrochemicals, toward fungicides-resistance in phytopathogens may contribute to the research and development of novel fungicides.

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## 要　　約

### ポリオキシン耐性リンゴ斑点落葉病菌に対するポリベリン®成分のポリオキシンBとイミノクタジン酢酸塩の共力作用機構

関戸治知，清水　力，三浦一郎
前野真一郎，林　茂，中山　礎

ポリベリン（ポリオキシンBとイミノクタジン酢酸塩を3：1の割合で含む複合殺菌剤）はポリオキシン耐性リンゴ斑点落葉病菌の胞子発芽管を膨潤化した．ポリベリンはポリオキシン耐性菌の菌糸生育を50%阻害する33.3 μg/mlにおいて電解質漏出を誘起し，キチン生合成系阻害活性を示した．ポリベリンの成分の一つであるポリオキシンBは25 μg/mlでポリオキシン耐性菌に対して電解質漏出およびキチン生合成系阻害活性を示さず，またもう一つの成分であるイミノクタジン酢酸塩は8.3 μg/mlでポリベリン8.3 μg/mlとほぼ同等の電解質漏出活性を示したものの，キチン生合成系を阻害しなかった．これらの結果は，ポリオキシン耐性菌に対するポリベリンの共力作用機構がイミノクタジン酢酸塩の細胞膜機能阻害による菌体内へのポリオキシン取込み量の増加に起因することを示唆するものである．