

N-(3', 5'-dichlorophenyl)-1, 2-dimethylcyclopropane-1, 2-dicarboximideの灰色かび病菌に対する抗菌作用についての形態学的研究

誌名	日本植物病理學會報 = Annals of the Phytopathological Society of Japan
ISSN	00319473
著者名	久田,芳夫 川瀬,保夫
発行元	日本植物病理學會
巻/号	43巻2号
巻号補足	
掲載ページ	p. 151-158
発行年月	1977年4月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council
Secretariat



Morphological Studies of Antifungal Action of *N*-(3', 5'-dichlorophenyl)-1, 2-dimethylcyclopropane-1, 2-dicarboximide on *Botrytis cinerea*

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久田芳夫・川瀬保夫 : *N*-(3', 5'-dichlorophenyl)-1, 2-dimethylcyclopropane-1, 2-dicarboximide の灰色かび病菌に対する抗菌作用についての形態学的研究

Abstract

N-(3', 5'-dichlorophenyl)-1, 2-dimethylcyclopropane-1, 2-dicarboximide, S-7131 (Sumilex®), inhibited remarkably the mycelial growth and conidial germination of *Botrytis cinerea* in a liquid nutrient medium, causing unusual swelling of hyphae and shortening of their internodes at its sublethal concentrations, and bursting cells at the lethal concentrations. Bursting of cells which was observed 2 to 3 hr after the treatment of the fungicide was the earliest morphological response. This was observed on growing cells but not on resting cells. The hyphal cells treated with S-7131 burst similarly under high osmotic pressure. In the presence of S-7131, protoplasts obtained from the hyphae of *B. cinerea* were stable and regenerated cell wall-like structures, which were observed under a phase-contrast microscope after osmotic shock. The results suggest that S-7131 neither affects the cell wall and the cell membrane by its direct action nor inhibits the synthesis of the cell wall. S-7131 probably causes bursting of cells as an indirect effect.

(Received November 29, 1976)

Introduction

N-(3', 5'-dichlorophenyl)-1, 2-dimethylcyclopropane-1, 2-dicarboximide, S-7131 (Sumilex®), is a recently developed systemic fungicide^{15, 16, 17)}. Effectiveness of this fungicide has been shown in practical control of plant diseases such as gray mould of grape vine and vegetable crops, Sclerotinia rot of kidney bean and vegetable crops, brown rot of peach and Helminthosporium leaf spot of rice. It has been expected that the curative action on disease development and the systemic property in plants of S-7131 may contribute to its high efficacy in disease control. Recently, benomyl-resistant strains have been reported for several plant pathogenic fungi that previously were sensitive, and their development has become a serious problem in agriculture. The use of S-7131 will provide a possibility to prevent the spread of benomyl-resistant strains, because cross-resistance between benomyl and S-7131 was not observed in *Botrytis cinerea*^{17, 28, 31)}. It has been found that some other 3,5-dichlorophenyl cyclic imides also possess high antifungal activity^{4, 7, 8, 9, 14)}. However, the mode of fungitoxic action of these compounds has remained unknown. Knowledge of the mechanism of fungitoxic action as well as the mode of action under the interaction among fungicide, pathogen and host should aid both in more

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efficient usage of the fungicide for controlling the plant disease and in further search for analogues which are more effective in disease control. This study was undertaken as a part of the research concerning the mode of fungitoxic action of S-7131 and relates with its effect on growth, morphogenesis and morphology of *B. cinerea* *in vitro*.

Materials and Methods

Fungus. *B. cinerea* used throughout this work was an isolate from gray mould-infected fruit of mandarin orange. The stock cultures were maintained on a potato-sucrose agar medium. Much production of the conidia was obtained by culturing the fungus on the medium used by Hislop¹⁸⁾. To prepare a conidial suspension, the conidia were washed off with sterile distilled water from 7 to 10 days-old cultures and filtered through four layers of cheese cloth. The resulting suspension was then centrifuged and the precipitates were resuspended in sterile water. This conidial suspension was used as an inoculum. The conidia were inoculated to give a final concentration of 10^6 cells per ml, unless otherwise stated.

Fungicide and chemicals. Stock solution of S-7131 were prepared by dissolving the fungicide in methanol and added to cultures at appropriate concentrations to give a final concentration of 0.2% methanol. Nystatin was kindly supplied from National Institute of Health (Japan), and dissolved in methanol just before use. D-Glucose-U-¹⁴C (101 mCi/mmol) was the product of Daiichi Pure Chemicals (Tokyo, Japan). Snail enzyme (Helicase®) and cellulase (Cellulase Onozuka R-10®) were purchased from Industrie Biologique Française (Gennevilliers, France) and Kinki Yakult Manuf. (Nishinomiya, Japan), respectively.

Measurements of fungal growth. Growth was measured by determining increase in mycelial dry weight of the cultures. The conidia of *B. cinerea* were incubated in Sakaguchi flask containing the nutrient medium at 27 C on a reciprocal shaker for 16 hr. From the resulting mycelial suspensions, 5 ml aliquots were taken into petri dishes and at the same time the fungicide or the solvent was added. After further incubation, duplicate samples of treated or control cultures were collected by filtration. After washing with distilled water, the mycelia were dried overnight at 80 C and weighed.

Measurements of inhibition of sporulation and sclerotium formation. *B. cinerea* was inoculated by placing a mycelial disc in the center of an agar plate of the nutrient medium (2% agar). After incubation for 3 days at 20 C, the plates with the hyphae were immersed in the fungicide suspensions for 60 min and then the solutions were removed. They were further incubated for 10 days in light at 25 C for sporulation or in darkness at 20 C for sclerotium formation. The conidia were washed off with water containing one drop of Tween 20 per 20 ml. The number of the conidia was counted under a microscope. The sclerotia were dried overnight at 80 C and weighed.

Observation of morphological effect. S-7131 was added to the nutrient medium in a petri dish immediately or 16 hr after inoculating the conidia to give 10^5 cells per ml. After incubating for appropriate periods, hyphal morphology was observed under a phase-contrast microscope.

Measurements of release of radioactive cellular materials. Sixteen hr-old culture was incubated with 0.5 μ Ci D-glucose-U-¹⁴C per 10 ml at 27 C for 60 min. The mycelia were washed twice with the nutrient medium or distilled water and resuspended in respective solutions. The mycelial suspensions were treated with

S-7131 or nystatin and incubated with shaking. The incubation was terminated by centrifugation at 1800 g for 10 min. The radioactivity contained in the supernatant fluid was quantitatively measured on a liquid scintillation spectrometer¹⁶).

Formation and regeneration of protoplasts. The mycelium was obtained from 20 to 24 hr cultures. Washed mycelium was suspended in a 0.6 M KCl solution of 5 mM citrate-phosphate buffer (pH 5.8) containing snail enzyme (10 mg/ml) and cellulase (20 mg/ml)^{5,29}. After 60 min incubation at 30 C with gentle shaking, the incubation mixture was filtered through a cheese cloth and then glass wool for separation of protoplasts from the rest of the mycelial debris. The filtrate was centrifuged at 600 g for 10 min and precipitated protoplasts were washed with a 0.6 M KCl solution of distilled water or the nutrient medium. Protoplasts free from the digestive enzymes were incubated with S-7131 at a concentration of 20 μ M in a petri dish containing a 0.6 M KCl solution. Samples were periodically examined by a phase-contrast microscopy.

Results

Effect on mycelial growth

Probits of inhibition of mycelial growth was plotted against logarithm of the concentrations of S-7131 (Fig. 1).

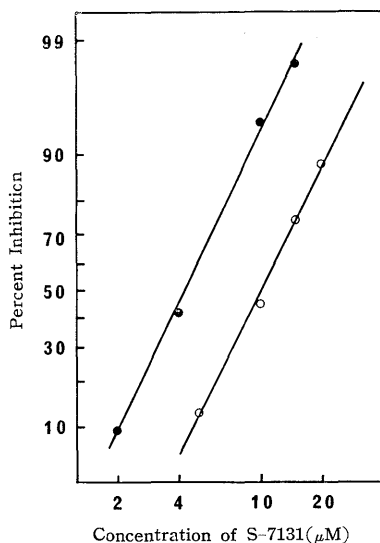


Fig. 1. Mycelial growth of *B. cinerea* treated with various levels of S-7131. Mycelia were grown in the presence of S-7131 at various concentrations for 24 hr. An initial inoculum was 0.40 (—●—) and 2.34 (—○—) mg dry weight per ml, respectively.

When the densities of an initial inoculum were 0.40 and 2.34 mg dry weight per ml, the concentrations of S-7131 for inhibiting 50 % of growth (ED_{50}) were 4.2 and 10.0 μ M, respectively. The inhibiting rate of mycelial growth was considerably influenced by cell density of the inoculum.

Effect on sporulation and sclerotium formation

As represented in Table 1, when the hyphae grown on a solid medium in a petri dish was immersed in the fungicide suspension for 60 min, sporulation was moderately affected at the concentration of 25 μ M. Sclerotium formation was remarkably inhibited by S-7131 at the concentrations higher than 25 μ M. Inhibition of sclerotium formation was enhanced with an increase in the concentration of the fungicide.

Table 1. Effect of S-7131 on sporulation and sclerotium formation of *B. cinerea*

Concentration of S-7131 (μ M)	Number of spore ^a) ($\times 10^4$)	Dry weight of sclerotia ^b) (mg)
50	—	(immatured)
25	90	5.2
10	127	55.6
5	228	74.3
0	183	87.3

a) The plates with hyphae were incubated for 10 days in light at 25 C after the treatment of the fungicide.

b) The plates with hyphae were incubated for 10 days in darkness at 20 C after the treatment of the fungicide.

Morphological effect

When the conidia were incubated in the presence of S-7131 at the concentration as low as $2\ \mu\text{M}$, the elongation of germ tubes was suppressed with shortening of internodes and unusual swelling.

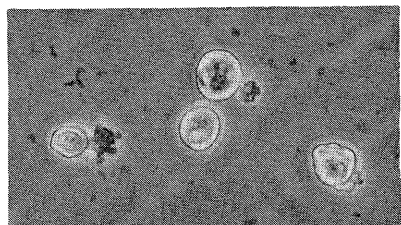


Fig. 2. Bursting of the conidia of *B. cinerea* caused by S-7131. The conidia were incubated in the presence of $7\ \mu\text{M}$ S-7131 for 24 hr and observed under a phase-contrast microscope ($\times 600$).

At the concentrations more than $7\ \mu\text{M}$, most of the conidia burst during the swelling process (Fig. 2). Two to three hr after adding S-7131 at $10\ \mu\text{M}$ to 16 hr-culture, significant swelling of hyphal cells was observed and it was accompanied by cell bursting. At $20\ \mu\text{M}$, the fungicide caused cell bursting to a remarkable extent (Fig. 3-b). The bursting occurred at various points along the hyphae such as apical and basal cells of the hyphae and germinated spores.

Fig. 4 shows the time course of release of the radioactivity from the hyphal cells that were

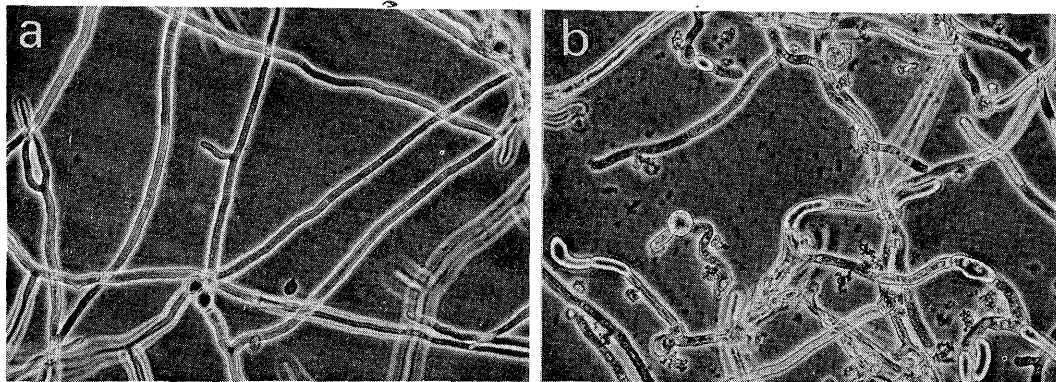


Fig. 3. Effect of S-7131 on hyphal morphology of *B. cinerea*. Hyphal morphology was observed under a phase-contrast microscope ($\times 200$). (a) Normal hyphae. (b) The hyphae incubated with $20\ \mu\text{M}$ S-7131 for 6 hr.

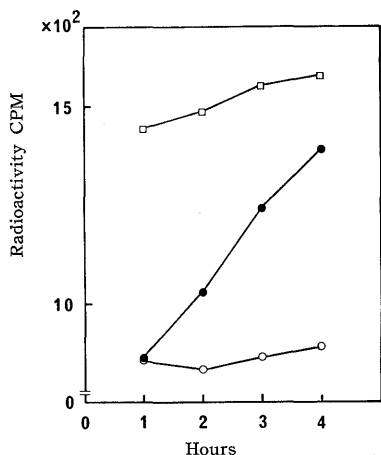


Fig. 4. The time course of release of radioactivity caused by S-7131 from radio-labeled hyphal cells of *B. cinerea*. ○: Untreated control, ●: $20\ \mu\text{M}$ S-7131, □: $5\ \mu\text{g/ml}$ nystatin.

preincubated in the presence of ^{14}C -U-glucose. Release of the radioactivity from the cells became evident after 2 hr incubation with S-7131 at the concentration of $20\ \mu\text{M}$. Thereafter, the radioactivity liberated from the cells almost linearly increased for further 2 hr incubation period. On the other hand, nystatin, a polyene antibiotic which affects the membrane functions by interacting with sterols in the membrane structures and consequently induces leakage of the cellular materials (12, 13, 23), caused faster release of the radioactivity after the treatment. Most of the radioactivity was liberated during as early as one hr. Release of the radioactive cellular materials was very pronounced at the concentrations of S-7131 more than $10\ \mu\text{M}$ (Table 2). These results indicate that release of the radioactive materials from hyphal cells treated with S-7131 can be ascribed to cell burst-

Table 2. Effect of the concentration of S-7131 on release of the radioactivity from radio-labeled hyphal cells of *B. cinerea*

Concentration of S-7131 (μ M)	Radioactivity ^{a)} released (cpm/0.5ml)
20	3,610
10	3,418
5	2,684
2	2,542
0	2,560

a) The mycelial suspensions which were incubated with S-7131 at various concentrations for 4 hr were centrifuged at 1,800 g for 10 min. The radioactivity contained in the supernatant fluid was measured.

Table 3. Release of the radioactivity caused by S-7131 from radio-labeled hyphal cells of *B. cinerea* in the nutrient medium containing 2, 4-dinitrophenol or in distilled water.

Exptl. No.	Incubation medium	Radioactivity released ^{a)} (cpm/0.5ml)	
		Control	S-7131
1	Nutrient medium + 0.2 mM DNP ^{b)}	4,248	7,007
		6,319	7,013
2	Nutrient medium Distilled water ^{c)}	5,097	7,692
		1,041	1,079

a) The mycelial suspensions were centrifuged at 1,800 g for 10 min after incubation with 20 μ M S-7131 for 4 hr. The radioactivity contained in the supernatant fluid was measured.

b) The hyphal cells were preincubated for 2 hr before the treatment of S-7131.

c) The hyphal cells were preincubated for 30 min before the treatment of S-7131.

Table 4. Effect of extracellular osmotic pressure on release of the radioactivity caused by S-7131 from radio-labeled hyphal cells of *B. cinerea*

Incubation medium	Radioactivity released ^{a)} (cpm/0.5ml)		Growth ^{b)} mg dry weight/ml	
	Control	S-7131	Control	S-7131
Nutrient medium	5,097	7,692	13.70	3.50
Nutrient medium + 0.8 M KCl	4,459	5,831	11.76	2.60
Nutrient medium + 1.6 M KCl	5,148	4,710	4.34	2.76

a) The mycelial suspensions were centrifuged at 1,800 g for 10 min after incubation with 20 μ M S-7131 for 4 hr. The radioactivity contained in the supernatant fluid was measured.

b) Increase in mg dry weight for 24 hr incubation from 2.82 mg/ml of original dry weight was measured.

ing rather than disturbance of membrane permeability followed by leakage of the cellular materials. Table 3 shows the effect of the metabolic inhibitor on release of the radioactivity from hyphal cells. Release of the radioactivity caused by S-7131 was extremely suppressed by 0.2 mM 2,4-dinitrophenol. A similar result was obtained on starved hyphal cells suspended in distilled water. In neither of these cases, the bursting of cells was observed under a microscope even after 6 hr incubation with S-7131. For the examination of osmotic fragility of hyphal cells treated with S-7131, release of the radioactivity was measured in the presence of 0.8 M and 1.6 M KCl as osmotic stabilizers (Table 4). Addition of 0.8 M KCl considerably suppressed release of the radioactivity and 1.6 M KCl perfectly. In the presence of KCl, however, hyphal growth was inhibited and especially in the presence of 1.6 M KCl growth hardly occurred. No protoplast-like or osmotically sensitive structures were formed under this high osmotic pressure.

Effect on protoplasts and their regeneration

Fungal protoplasts are available to study the effect of antifungal agents on the cell membrane and the synthesis of the cell wall³⁰⁾. It may be supposed that damage of the cell membrane and/or inhibition of the cell wall synthesis is responsible for bursting of hyphal cells caused by S-7131. Purified protoplasts (14.7×10^5 /ml) which were suspended in a 0.6 M KCl solution containing 20 μ M S-7131 were entirely stable and normal in morphology without shrinkage, swelling or lysis after 7 hr incubation. It was impossible

to obtain quantitative results of the effect of S-7131 on regeneration of protoplasts because of clumping of protoplasts in the nutrient medium. When protoplasts (3.5 to $4.9 \times 10^5/\text{ml}$) were incubated in the nutrient medium containing 0.6 M KCl , after 2 hr some protoplasts formed buds which later on gave rise to chains of yeast-like cells as observed by Bachman & Bonner²⁾, Garcia Acha *et al.*¹⁰⁾, Peberdy & Gibson²⁶⁾ and Annè *et al.*¹⁾ Protoplasts and abnormal hyphae were osmotically fragile still after 6 hr incubation but they had developed thick-walled structures which remained

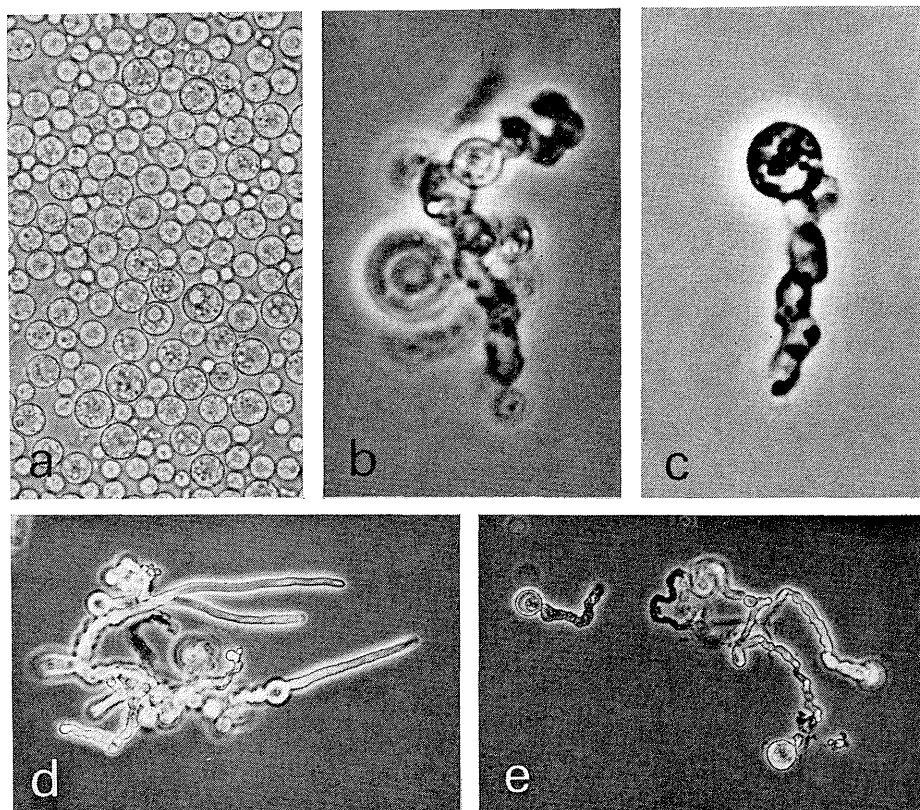


Fig. 5. Effect of S-7131 on regeneration of protoplasts obtained from hyphal cells of *B. cinerea*. Regenerating hyphae were observed under a phase-contrast microscope. (a) Purified protoplasts ($\times 600$). (b) Wall regeneration of protoplast after 6 hr incubation. It was observed after osmotic shock ($\times 900$). (c) Wall regeneration of protoplast in the presence of $20 \mu\text{M}$ S-7131 after 6 hr incubation ($\times 900$). (d) Regeneration into new normal hyphae of protoplast after 12 hr incubation ($\times 400$). (e) Regeneration of protoplast in the presence of $20 \mu\text{M}$ S-7131 after 24 hr incubation ($\times 400$).

visible after osmotic shock (Fig. 5-b). Emergence of normal hyphae was observed after 12 hr incubation (Fig. 5-d). In the presence of $20 \mu\text{M}$ S-7131, protoplasts regenerated new yeast-like cells and cell wall identical to that of untreated control, but any normal hyphae did not develop even after 24 hr incubation (Fig. 5-c, e).

Discussion

S-7131 is highly effective to inhibit mycelial growth of *B. cinerea* as well as its

spore germination in a liquid nutrient culture. ED_{50} values of inhibition of mycelial growth depended on the size of the inoculum used in the growth test, suggesting that there was a considerable absorption of the fungicide by fungi. Inhibition of sclerotium formation by S-7131 is probably ascribed to indirect action rather than specific interference with some biochemical processes for sclerotium formation, because the concentrations of the fungicide required for inhibition of sclerotium formation was also inhibitory to mycelial growth.

Swelling and bursting of the cells were the most remarkable morphological abnormality which was produced in *B. cinerea* by S-7131 at appropriate growth-inhibitory concentrations. Measurement of release of ^{14}C -labeled materials from hyphal cells quantitatively determined the rate of cell bursting. Bursting of the cells was observed on actively growing cells in the nutrient medium, but not in resting cells in distilled water and the cells treated with the metabolic inhibitor. If S-7131 have a softening effect on preformed cell walls, it would affect similarly both to growing and non-growing cells. Therefore, this is not the case presumably. Antimicrobial agents such as 2-deoxy-D-glucose^{19, 25)} and polyoxin D⁶⁾ which interfere with the synthesis of the cell wall caused bursting of the cells of yeasts and filamentous fungi, respectively. However, S-7131 seems not to inhibit the synthesis of the cell wall, because it was shown by microscopic observations that hyphal protoplasts could regenerate new wall in the presence of S-7131. Besides, the bursting was observed at random on the hyphae, and was never frequent at hyphal tip cells where the hyphae extend by synthesizing new wall^{3, 11, 21)}. It is not plausible that S-7131 cause an elevation of the osmotic pressure of the cells by altering cellular metabolism or disturbing the permeability of the cell membrane. The reason is that bursting of hyphal cells was not observed when the hyphae treated with S-7131 under relative high osmotic pressure (1.6 M KCl) were transferred to distilled water. Complete suppression of bursting of the cells in the nutrient medium containing 1.6 M KCl does not probably reflect the decrease in osmotic fragility, since mycelial growth was remarkably reduced under this condition. This is conceivable from the fact that on bursting of the cells occur in non-growing cells. Kinsky reported that the polyene antibiotics nystatin, amphotericin B and filipin induced shrinkage of *Neurospora crassa* protoplasts which was followed by swelling and lysis²²⁾. Protoplasts were stable in the presence of S-7131 without any morphological abnormality. This indicates that S-7131 does not probably affect cell membrane by its direct action. Autolytic enzymes hydrolyze polymers in the cell wall and are considered to function in hyphal extension and branching^{24, 27)}. Autolysis in an isotonic solution sometimes leads to the formation of protoplasts. Hyphal cells treated with S-7131 burst even under high osmotic pressure and form no protoplast-like structures. It seems that bursting of the cells caused by S-7131 was not due to autolysis as a result of alteration of cellular metabolism or damage of the cell wall.

Though bursting of hyphal cells is a prominent morphological abnormality caused by S-7131 and the earliest response, it is likely that this is not the direct result of the fungicide action. Interpretations of the grounds of cell bursting are complicated by few possibility of the primary action of S-7131 to the cell wall or its synthetic process. Probably, S-7131 would affect the cell membrane and the cell wall of *B. cinerea* secondarily as a result of interference with some cellular metabolic process. We are attempting to investigate metabolic effect of S-7131 on hyphal cells of *B. cinerea*.

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和 文 摘 要

N-(3',5'-dichlorophenyl)-1,2-dimethylcyclopropane-1,2-dicarboximide
の灰色かび病菌に対する抗菌作用についての形態学的研究

久 田 芳 夫・川 瀬 保 夫

S-7131 (スミレックス®) は、液体培地中で灰色かび病菌 (*Botrytis cinerea*) の菌糸生育および分生孢子発芽を著しく抑制した。致死濃度で処理したとき、孢子および菌糸細胞の多くが破裂し、細胞内容物を吐出した。低濃度では、菌糸の節間が短縮し、細胞は異常な膨潤を示した。この細胞の破裂は、薬剤処理 2~3 時間後からひき起されるきわめて初期の形態変化であった。この現象は、生育菌糸で認められたが休止菌糸または 2,4-ジニトロフェノールで処理した菌糸では認められなかった。また、S-7131 処理した菌糸細胞は、高張液中でも同様に破裂した。*B. cinerea* 菌糸のプロトプラストは、S-7131 の存在下で形態的に安定であり、また浸透圧ショック後位相差顕微鏡下で観察したところ、細胞壁様の構造物を再生していた。以上の結果から、S-7131 は細胞膜や細胞壁に直接的に作用するのではなく、また細胞壁合成を阻害するのではないことが示唆される。したがって、S-7131 は、おそらく何らかの代謝阻害の 2 次的な結果として細胞の破裂をひき起していると推察される。