Mode of Action of Hymexazol I) Effect of Hymexazol on Cell Permeability of *Pellicularia sasakii*

Toshiro NAKANISHI*, Yukiyoishi TAKAHI*
and Kazuo TOMITA*

中西逸朗*・高日幸義*・富田和男*: ハイメキサゾールの作用機構 I) *Pellicularia sasakii* の細胞膜透過性におよぼすハイメキサゾールの影響

Abstract

The mode of action of hymexazol against *Pellicularia sasakii* was studied. When the living mycelium of *P. sasakii* was incubated in aqueous solution of hymexazol for 24 hr, a decrease in dry mycelial weight caused. Measurable decrease occurred at 50 ppm, with progressively greater weight losses as the concentration increased. On the other hand, a decrease in the dry weight of heat-treated mycelium was negligible even at 1000 ppm of hymexazol. The decrease in the dry mycelial weight was found to be closely correlated to a leakage of protein from the mycelium. At 250 ppm of hymexazol, the protein leakage increased in almost linear fashion during 8 hr incubation period, and then leveled off. When the mycelium was immersed in hymexazol solution for 1 hr and subsequently incubated in distilled water for 23 hr, a large amount of protein leaked out from the mycelium during the 23 hr incubation period in distilled water, which was in proportion to the concentration of hymexazol. While only a small amount of protein leaked out during the 1 hr incubation period in hymexazol solution. Hymexazol inhibited the uptake of glucose by *P. sasakii*. Hymexazol was readily absorbed by the mycelium from the ambient solution within the first 1 hr, and then the absorption reached to equilibrium. On potato-sucrose agar containing hymexazol at 100 ppm or 10 ppm, the growth of *P. sasakii* was reduced to some extent. At growth-inhibitory concentration of hymexazol, the hyphae appeared swollen parts as the hyphal tips and at various points along the sides of hyphae, and in certain cases the hyphae burst with loss of protoplasm.

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Introduction

Hymexazol (3-hydroxy-5-methylisoxazole, Tachigaren) is highly effective against the soil-borne diseases caused by *Fusarium* spp., *Aphanomyces* sp., *Pythium* spp. and *Cor-ticium* sp.¹⁰⁾. This fungicide has been used extensively for the control of damping-off of rice and sugar beet seedlings.

Recently, Iwahashi *et al.*⁴⁾ found that hymexazol was active against sheath blight of rice plants in paddy fields. They also reported that when hymexazol was sprayed to rice plants before the upward development of the disease, later development of sheath blight was strongly suppressed for a long time. Subsequently, Takahi *et al.*¹¹⁾ showed that *Pellicularia sasakii*, causal fungus of sheath blight of rice plant, previously exposed to hymexazol solution was unable to express the disease symptom on rice

* Agricultural Chemicals Research Laboratories, Sankyo Co., Ltd., Yasu, Shiga, Japan. 三共(株) 農薬研究所

plants but the fungus easily infected on rice plants previously sprayed with hymexazol. This finding indicates that hymexazol directly acts on the pathogen rather than on the host. This paper concerns with the mode of action of hymexazol against *P. sasakii*, especially for its effect on the cell permeability.

Materials and methods

Organism.—A stock culture of *Pellicularia sasakii* (Shir.) S. Ito was cultured on potato-sucrose agar at 26 C for 10–14 days before being used.

Chemical.—Hymexazol-3-¹⁴C and its purification procedures were the same as used by Nakanishi *et al.*⁸⁾.

Leakage of cellular constituents from the mycelium of *P. sasakii*.—One hundred ml of a modified Czapek medium⁷⁾ in 500 ml flask was inoculated with a mycelial disc of *P. sasakii* and incubated at 26 C for 4 days on a reciprocal shaker. The mycelium was collected in a Buchner funnel and washed with distilled water. Three grams (fresh weight) of living mycelium or heat-treated (for 30 min at 100 C) mycelium was put into 100 ml of an aqueous solution of hymexazol, and incubated at 26 C on a reciprocal shaker. After 24 hr, the mycelium was collected in a Buchner funnel, dried under an infrared lamp, and weighed. The filtrate of each flask was filled up to 100 ml with distilled water and the amount of protein in the filtrate was determined by the method of Lowry *et al.*⁶⁾, calibrating with bovine serum albumin. In order to determine the time course for protein leakage from the mycelium, 3 g (fresh weight) of the mycelium in 100 ml of 250 ppm hymexazol solution was incubated at 26 C on a reciprocal shaker. After 1, 2, 4, 8, or 24 hr, the content was centrifuged at 3000 rpm for 5 min., and the amount of protein in the supernatant was determined. For the purpose of studying the effect of short period treatment with hymexazol on the leakage of cellular constituents, 3 g (fresh weight) of the mycelium was added to 100 ml of hymexazol solution at a desired concentration. After incubation for 1 hr at 26 C on a reciprocal shaker, the mycelium was collected in a Buchner funnel and washed with distilled water. The filtrate of each flask was filled up to 100 ml with distilled water and the amount of protein in the filtrate was determined. The collected mycelium was transferred in 100 ml of distilled water, and incubated for 23 hr at 26 C on a reciprocal shaker. The mycelium was removed by filtration. The filtrate was filled up to 100 ml with distilled water and the amount of protein was determined.

Uptake of glucose by the mycelium of *P. sasakii*.—Three grams (fresh weight) of shake-cultured mycelium of *P. sasakii* was added in 100 ml of 0.2% glucose solution containing hymexazol at a desired concentration, and incubated at 26 C on a reciprocal shaker. After 4 hr, the content was centrifuged at 3000 rpm for 5 min. The amount of glucose in the supernatant was determined by the anthrone method reported by Scott and Melvin⁹⁾. The amount of glucose absorbed by the mycelium was calculated by subtracting that found in the supernatant from total amount initially added.

Uptake of hymexazol by the mycelium of *P. sasakii*.—The uptake of hymexazol by *P. sasakii* was determined by using ¹⁴C-labeled hymexazol. Nonradioactive hymexazol was added to aliquots of a known uniform amount of the radioactive toxicant to give the final concentration of 10, 20, and 100 ppm. Three grams (fresh weight) of shake-

cultured mycelium was added in 100 ml of the aqueous solution of hymexazol- ^{14}C in 500 ml flask, and incubated at 26 C on a reciprocal shaker. After 1, 2, 4, 8 or 24 hr, the content was centrifuged at 3000 rpm for 5 min. Two tenths ml of the supernatant was mixed with 15 ml of a scintillation solution (8 g of PPO and 200 mg of dimethyl POPOP in mixture of 800 ml dioxane and 200 ml toluene) in a counting vial. All measurement of radioactivity were made with a Packard Tri-Carb liquid scintillation spectrometer model 3002. Counting efficiency for each sample was determined by adding reference ^{14}C -toluence. The amount of hymexazol absorbed by the mycelium was calculated by subtracting that found in the supernatant from the total amount initially added.

Morphological observation.—On a plate of 100 ml of potato-sucrose agar containing 200 ppm of rose bengal and a given concentration of hymexazol in a 15 cm Petri dish, a 5 mm disc of mycelial mat of *P. sakakii* (from periphery of 4 days culture on potato-sucrose agar) was placed. The plate was incubated for 2 days at 27 C, and then moved into a 20 C chamber. After incubation for 4 or 10 days, a piece of the mycelium of the periphery of the culture was stripped off with a razor blade, immersed in 0.5% rose bengal solution for 5 min, and then supplied to microscopic observation.

Results

Effects of hymexazol on the leakage of cellular constituents from the mycelium of *P. sakakii*

When the living mycelium of *P. sakakii* was incubated in an aqueous solution of hymexazol for 24 hr, decrease in the dry mycelial weight was caused (Table 1-A). Measurable decrease in dry mycelial weight occurred at 50 ppm, with progressively greater weight loss as the concentration increased. On the other hand, a decrease in dry weight of heat-treated mycelium was negligible even at 1000 ppm (Table 1-B). As shown in Table 2, protein, one of the cellular constituents, leaked out from the mycelium into the ambient solution as a result of contact with hymexazol. The amount of protein leaked out increased in proportion to the concentration of hymexazol up to 1000 ppm. The result of a time course experiment for protein leakage from the mycelium is shown in Fig. 1. At 250 ppm of hymexazol, the protein leakage increased in almost linear fashion during 8 hr incubation period, and then leveled off. The

Table 1. Effect of hymexazol on the dry mycelial weight of *Pellicularia sakakii*

A) Living mycelium

Hymexazol concentration ($\mu\text{g/ml}$)	Relative dry mycelial weight
0	100
10	99
25	100
50	93
100	89
250	64
500	35
1000	25

B) Heat-treated mycelium

Hymexazol concentration ($\mu\text{g/ml}$)	Relative dry mycelial weight
0	100
100	98
250	99
500	100
1000	98

effect of short period treatment with hymexazol is shown in Fig. 2. Only a small amount of protein leaked out during 1 hr incubation period in aqueous hymexazol solution, though the amount of protein leaked out increased in proportion to the concentration of hymexazol. During subsequent 23 hr incubation period in distilled water, a large amount of protein leaked out from the mycelium, which was also in proportion to the concentration of hymexazol.

Table 2. Effect of hymexazol concentration on protein leakage from *Pellicularia sasakii*

Hymexazol concentration ($\mu\text{g/ml}$)	Protein leaked ($\mu\text{g/ml}$)
0	44.4
10	50.9
25	60.2
50	74.0
100	95.9
250	462.6
500	775.8
1000	997.5

Fig. 1. Time course for protein leakage from hymexazol-treated mycelium of *Pellicularia sasakii*.

Effect of hymexazol on glucose uptake by *P. sasakii*

As shown in Table 3, glucose uptake by *P. sasakii* was strongly inhibited in the pre-

Fig. 2. Protein leakage from the mycelium of *Pellicularia sasakii* previously exposed to hymexazol.

■ The amount of protein leaked out from the mycelium within 1 hr incubation in aqueous hymexazol solution.

□ The amount of protein leaked out from the mycelium, which was previously exposed to hymexazol for 1 hr, within subsequent 23 hr incubation period in distilled water.

Table 3. Effect of hymexazol on glucose uptake by *Pellicularia sasakii*

Hymexazol concentration ($\mu\text{g/ml}$)	Glucose uptake (mg/g dry wt.)	Inhibition (%)
0	175.7	
1	176.0	-0.1
10	136.6	22.2
100	34.1	80.4

sence of hymexazol at 100 ppm, but not inhibited at all at 1 ppm.

Uptake of hymexazol by *P. sasakii*

Uptake of hymexazol by *P. sasakii* is presented in Fig. 3. At all concentrations tested, hymexazol was rapidly absorbed by the mycelium from the ambient solution within the first 1 hr, and then the absorption reached to plateau. The amount of hymexazol absorbed increased in proportion to the concentration of hymexazol.

Effect of hymexazol on growth and morphology of the mycelium of *P. sasakii*

On potato-sucrose agar containing hymexazol at 1000 ppm, the growth of *P. sasakii* was completely inhibited. The growth occurred at 100 and 10 ppm, but it was reduced to some extent. At growth-inhibitory concentrations, the hyphae appeared swollen parts at the hyphal tips and various points along the sides of the hyphae, and in certain cases the hyphae burst with loss of protoplasm (Fig. 4). Number of swollen parts on hyphae grown in the presence of hymexazol gradually increased with time. A few swelling structure was also observed in the hyphae of the untreated control.

Fig. 3. Uptake of hymexazol by *Pellicularia sasakii*.

Fig. 4. Hyphae of *Pellicularia sasakii* grown in the absence of hymexazol (left). Hyphae of *P. sasakii* grown in the presence of hymexazol at 100 ppm (right). S: Swollen part of hyphal tip. B: Burst of hyphae with loss of protoplasm.

Discussion

When mycelium of *P. sasakii* was incubated in aqueous solution of hymexazol, a decrease in the dry mycelial weight caused. This finding indicates that cell membrane of the fungus is affected by hymexazol and the fungus loses in its cellular contents as a result of the permeability change of cell membrane or the burst of fungal cell. Indeed, protein, one of the main cellular constituents, leaked out from the mycelium into the surrounding solution as a result of contact with hymexazol. Hymexazol may induce the permeability change of cell membrane by acting on vital sites at the cell surface, since a decrease in the dry weight of heat-treated mycelium was negligible as a result of contact with hymexazol at 1000 ppm. When the mycelium immersed in hymexazol solution for 1 hr was subsequently incubated in distilled water for 23 hr, a large amount of protein leaked out from the mycelium during 23 hr incubation period in distilled water, which was in proportion to the concentration of hymexazol. While, only a small amount of protein leaked out from the mycelium during 1 hr incubation period in hymexazol solution. This result indicates that hymexazol is rapidly absorbed by the mycelium and gradually affects permeability of the cell membrane. This assumption would be supported by the fact that hymexazol was readily absorbed by the mycelium from the ambient solution within the first 1 hr, and then the absorption reached to plateau.

Many reports on permeability change in cell membrane by chemicals have been published. A polyene antibiotic, filipin, induces permeability change in cell membrane of sensitive fungi, because this antibiotic combines with sterols in the cell membrane⁴⁾. Dodine (*N*-dodecylguanidine acetate) damages the cell membrane of *Saccharomyces cerevisiae* to result in a loss of vital constituents¹⁾. This fungicide is a cationic surfactant, which blocks the vital anionic sites on the cell surface. Kitazin (*O*, *O*-diethyl-*S*-benzyl phosphorothioate), which is effective against rice blast and is surface active, also induces leakage of cellular substances from the mycelium of *Pyricularia oryzae*⁵⁾.

The disruption of cellular permeability also prevents the uptake of essential metabolites. Higher alkylphenols inhibit uptake of glucose and glutamate into the cells of *Pyricularia oryzae* and remove ninhydrin-positive substances from the cells¹²⁾. Tetrin A, a polyene antibiotic, induces permeability changes in the cell membrane of *Saccharomyces cerevisiae* and also prevents the uptake of glucose, glycine, and uracil by the yeast¹³⁾. Hymexazol inhibited the uptake of glucose by *P. sasakii*. The inhibition of glucose uptake was observed at lower concentrations of hymexazol than those with which protein leakage occurred.

As reported previously¹¹⁾, *P. sasakii* forms the mycelial mats having only a few hyphae on potato-sucrose agar containing hymexazol. In such case, sclerotium formation of the fungus is strongly suppressed in spite of sufficient radial growth. This phenomenon is similar to the growth status of *P. sasakii* on agar. It is possible that the fungus is on the verge of starvation as a result of permeability change and its growth is consequently inhibited.

Some fungicides, which induce permeability change in cell membrane, have also been shown to produce morphological changes in sensitive fungi. In the presence of filipin, swollen parts appear on the hyphae of *Aspergillus flavus* and subsequently burst

with loss of protoplasm²⁾. Kitazin also forms swollen parts on the hyphal tips of *Pyricularia oryzae*⁵⁾. Hymexazol was also found to produce a morphological change in *P. sasakii*. Appearance of swollen parts on the hyphae and subsequent burst of the hyphae points conclusively that hymexazol acts on cell membrane of the fungus. Further studies are under way in hope of elucidating the mode of action of hymexazol.

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Literature cited

- 1) Brown, I. F. and Sisler, H. D. (1960). Phytopathology 50: 830-839.
- 2) Gottlieb, D., Carter, H. E., Wu, L. and Sloneker, J. H. (1960). Phytopathology 50: 594-603.
- 3) Gottlieb, D., Carter, H. E., Sloneker, J. H., Wu, L. and Gaudy, E. (1961). Phytopathology 51: 321-330.
- 4) Iwahashi, T., Okada, H. and Goto, S. (1971). Proc. Assoc. Pl. prot. Kyushu 17: 47-50.
- 5) Kakiki, K., Maeda, T., Abe, H. and Misato, T. (1969). J. Agr. Chem. Soc. Japan 43: 37-44.
- 6) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). J. Biol. Chem. 193: 265-275.
- 7) Nakanishi, T. and Oku, H. (1969). Ann. Phytopath. Soc. Japan 35: 339-346.
- 8) Nakanishi, T., Takahi, Y. and Tomita, K. (1974). Ann. Phytopath. Soc. Japan. 40: 383-391.
- 9) Scott, T. A. and Melvin, E. H. (1953). Anal. Chem. 25: 1656-1661.
- 10) Takahi, Y., Nakanishi, T., Tomita, K. and Kamimura, S. (1974). Ann. Phytopath. Soc. Japan 40: 354-361.
- 11) Takahi, Y., Nakanishi, T. and Nakagami, K. (1974). Proc. Kansai Pl. Prot. Soc. 16: 52-58.
- 12) Uesugi, Y. and Fukunaga, K. (1967). Ann. Phytopath. Soc. Japan 33: 168-175.
- 13) Van Etten, J. L. and Gottlieb, D. (1967). J. gen. Microbiol. 46: 377-387.

和文摘要

ハイメキサゾールの作用機構: *Pellicularia sasakii* の細胞膜透過性におよぼすハイメキサゾールの影響

中西逸朗・高日幸義・富田和男

Pellicularia sasakii (イネ紋枯病菌) に対するハイメキサゾールの作用機構について検討した。*P. sasakii* の生菌体をハイメキサゾール溶液に24時間浸漬すると菌体の乾燥重量が減少した。乾燥菌体重の減少度はハイメキサゾール50 ppm以上の濃度で濃度が高くなるにつれて顕著であった。一方、加熱処理した菌体をハイメキサゾール溶液に浸漬しても乾燥菌体重の減少は認められなかった。ハイメキサゾールとの接触によって菌体から漏出する蛋白量はハイメキサゾールの処理濃度が高くなるにつれて増加し、乾燥菌体重の減少と密接な関係にあった。ハイメキサゾール250 ppm溶液に菌体を浸漬し、蛋白の漏出量を経時的に観察した結果、菌体から漏出する蛋白量は処理後8時間まで直線的に増加し、それ以後24時間までほとんど増加しなかった。また、菌体をハイメキサゾール溶液に1時間浸漬し、その後引きつづいて蒸溜水に23時間浸漬した場合、菌体がハイメキサゾールと直接接触している最初の1時間内に菌体から漏出する蛋白量は少ないが、処理菌体を蒸溜水に移した後は、前処理したハイメキサゾールの濃度に応じて多量の蛋白が菌体から漏出した。さらに、ハイメキサゾールは乾燥菌体重の減少を起こすよりも低濃度で、菌体によるグルコース摂取を阻害した。ハイメキサゾールは *P. sasakii* により急速に吸収され、1時間以内にほぼ平衡状態に達した。ハイメキサゾールを含有したショ糖加用ジャガイモ寒天培地に *P. sasakii* を培養すると、10 ppm, 100 ppmで菌糸の伸長がある程度抑制された。この場合、*P. sasakii* の菌糸に多数の膨化した部分が誘起され、ある場合には菌糸が破裂して細胞内容物が細胞外へ漏出しているのが観察された。