Cytological and physiological studies of the IBP effects on *Pyicularia oryzae* Cav. (I) The effects on hyphal mass*

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Summary

The IBP effects on hyphal mass of *Pyricularia oryzae* Cav. were studied. Preliminary to the exploration of the IBP effects, the IBP-tolerant isolate, designated as PT83, was obtained through the selection of the tolerant colony which had originated from protoplasts of an IBP-sensitive isolate P2. The growth of hyphal mass of P2 was perfectly inhibited on PDA and in Vogel liquid medium, respectively, at 40 and 20 ppm, whereas that of PT83 was not even at 40 ppm. The appressorium formation of P2 conidia grown on cellophane film was perfectly inhibited at 5 ppm, while that of PT83 was not perfectly even at 50 ppm although it was suppressed more with the increased concentrations of IBP. When 10 ppm IBP was added to the liquid media, where young hyphal colonies of P2 were incubated, at the onset of incubation, the dry weight of colonies never increased during the 96 hr incubation. However, addition of 10 ppm IBP 24 hr after the onset of incubation resulted in prolongation of the lag phase. On the other hand, addition of IBP at 48 and 72 hr did not affect the growth of hyphal mass at all. The initial 4 hr treatment of hyphal mass of P2 with 10 ppm during incubation suppressed the increase of dry weight of hyphae prominently. It was also found that young hyphae obtained from the colony margin of P2 were more sensitive to IBP than those obtained from the intermediate and central region of the colony. All these data suggest that the IBP effects on *P. oryzae* might be largely dependent on ages of hyphae, young hyphae being highly sensitive to IBP.

Introduction

Various modes of action have been reported on IBP (S-benzyl 0, 0-diisopropyl phosphorothioate: commercial name Kitazin P). Maeda *et al.*[6] found that 25–200 ppm of IBP inhibited the incorporation of glucosamine-1-^{14}C into the cell wall of *Pyricularia oryzae* and considered that a mode of action of IBP might be the inhibition of chitin synthesis. Akatsuka *et al.*[1] presented the idea that the control of *P. oryzae* by IBP resulted in the inhibition of phosphatidylcholine biosynthesis, based on their finding that 50 ppm IBP markedly inhibited the incorporation of methionine-methyl-^{14}C into phospholipids, especially phosphatidylcholine. Similarly, Kodama *et al.*[4] reported that the specific inhibition of conversion from phosphatidylethanolamine to phosphatidylcholine by the transmethylation of s-adenosylmethionine might be regarded as one of the modes of action of IBP. Köller *et al.*[5] concluded that the inhbition of cutinase which was significant in the penetration process might be closely associated with the IBP effect.

According to Araki and Miyagi[2], mycelium growth and spore germination of *P. oryzae* was inhibited, respectively, at 10 and 20 ppm IBP. They also showed that 2 ppm IBP inhibited almost perfectly the penetration of *P. oryzae* on cellophane films and rice sheaths.

Primarily we wondered why the effective concentrations of IBP to *P. oryzae* were so different between the data obtained by biological and biochemical experiments. Thus, we designed a series of experiments

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to elucidate the IBP effects on *P. oryzae* by cytological and physiological means in more detail. First, a series of the experiments was done to compare the IBP effects at the levels of hyphal mass, single hypha and protoplast isolated from hyphae. In this paper, the effects of IBP on the hyphal mass are described as the first step.

Materials and methods

Test fungus and preparation of hypha and conidium suspension: *Pyricularia oryzae* Cav. isolate P2 (sensitive to IBP) was used throughout the experiments. The test fungus was grown on PDA slants at 25°C for 7–10 days. The hypha suspension was prepared by adding 5 ml of sterilized deionized water to the slant, followed by stirring the surface of mycelial mat with a sterilized platinum loop. To allow the fungus to form conidia, 0.5 m*l* of the above hypha suspension was added to the V–8 juice-agar medium (V–8 juice, 250 m*l*; $CaCO_3$, 3 g; deionized water, 750 m*l*; agar, 15 g) in Petri dishes and expanded uniformly on the medium surface, followed by the incubation at 25°C for 5 days. Ten m*l* of sterilized water were added to the medium and the mycelial mat was stirred with a brush to release conidia. The conidium suspension was prepared by the filtration of the above suspension of conidia and hyphae with 3 sheets of cheese cloth to eliminate fragmented hyphae from the suspension. The density of conidia in the suspension was adjusted at approximately 4×10^5 conidia/m*l* with sterilized water.

Test chemical: IBP of technical grade (Kumiai Chemicals, 95% purity) was used in this study. IBP, acetone and Tween 20 were mixed at the volume ratio of 6: 5: 1 to prepare the 50% IBP stock solution.

Selection of the IBP-tolerant isolate: To attempt to obtain the IBP-tolerant isolate, protoplasts were prepared from hyphae of the P2 isolate by employing the method of Kobayashi *et al*[4]. The protoplast suspension was adjusted to include approximately 4×10^5 protoplasts/m*l*, and 0.1 m*l* of the suspension was added to PDA including 10 ppm IBP and 0.6 M KCl in Petri dishes, then expanded on the surface with a sterilized glass lod. After the 10-day incubation at 25°C, mycelial colonies which had originated from isolated, single protoplasts were transferred onto PDA including no IBP. The IBP tolerance of the respective isolates was tested in terms of colony diameter, dry weight of mycelia and conidium germination in the presence of various concentrations of IBP, as described below.

Evaluation of the IBP sensitivity of test isolates:

i) **Colony diameter**. The isolates of P2 and PT83 (an IBP-tolerant isolate as described below) were grown on PDA at 25°C for 8 days. Mycelial discs of 6 mm diameter were obtained from the marginal region of the colony of the respective isolates, and placed each on the center of PDA medium including 0, 2, 4, 10, 20 or 40 ppm IBP. The colony diameter was measured every 2 days after the start of incubation at 25°C.

ii) **Dry weight of mycleia**. The hypha suspension of the respective isolates prepared as above was added to 100 m*l* Vogel liquid medium in 200 m*l* flasks and incubated on a rotary shaker at 25°C for 48 hr. Small colonies of less than 1 mm diameter were collected with a micropipette and resuspended in Vogel medium. One half m*l* of the colony suspension was added to 10 m*l* of Vogel medium in L-shaped glass tubes and IBP was added to the incubation mixture at a final concentration of 0, 1, 2, 4, 10 and 20 ppm at the start of incubation. After the 24 and 48 hr incubation on a reciprocal shaker at 25°C, mycelia in the respective glass tubes were collected with glass filters, and dried at 65°C for 12 hr. Subsequently the dry weight of mycelia was determined.

iii) **Conidium germination and appressorium formation**. Pieces of filter paper, 1×1 cm^2, were immersed in 0, 5, 10, 20 or 50 ppm IBP solution. Each of them was placed on a watch glass in a moist Petri dish. A cellophane sheet, 1×1 cm^2, which had been boiled previously in deionized water, then dried, was placed on the respective pieces of filter paper. The conidium suspension was sprayed over the cellophane sheets and incubated in moist Petri dishes at 25°C for 24 hr. The rates of germination, appressorium formation and appressorium pigmentation were determined by light microscopy using the following equations.

$$\text{conidium germination rate} = \frac{\text{No. of germinated conidia}}{\text{Total No. of conidia observed}} \times 100\ (\%)$$

$$\text{appressorium formation rate} = \frac{\text{No. of appressoria}}{\text{No. of germinated conidia}} \times 100\ (\%)$$

$$\text{appressorium pigmentation rate} = \frac{\text{No. of pigmented appressoria}}{\text{No. of appressoria observed}} \times 100\ (\%)$$

Effects of the timing of IBP treatments on the mycleial growth: In order to explore some of the parameters of the IBP effects on the mycelial growth, IBP was added to the incubation mixture during shaking culture at a final concentration of 10 ppm every 24 hr after the onset of incubation, as shown in Fig. 1. After the total 96 hr incubation, mycelia were collected and thier dry weight was determined as described above. In another experiment the mycelia were grown in Vogel liquid medium including 10 ppm IBP for initial periods of 0.5–4.0 hr after the onset of incubation on a rotary shaker. After the periods of initial treatment, growing mycelia were collected by centrifugation at ×400 g using fresh Vogel liquid medium to eliminate IBP from the mycelia. After the washing was repeated three times, the whole mycelia were resuspended in 4 m*l* of fresh Vogel liquid medium, and 0.5 m*l* of this suspension was added to 5 m*l* Vogel medium in L-shaped glass tubes, followed by incubation at 25°C for a further 24 or 48 hr. The dry weight of mycelium was determined as above.

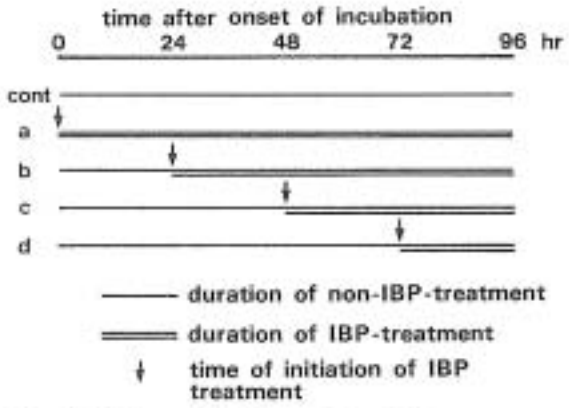

Fig. 1. Timing of initiation of the IBP treatment during culture of *P. oryzae* isolate P2 in Vogel liquid medium.

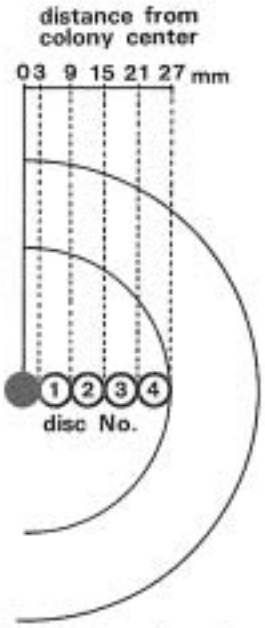

Fig. 2. A schematic figure of sampling mycelial discs of various ages from a colony of the IBP-sensitive isolate of *P. oryzae* growing on potato-dextrose-agar medium.

Sensitivity of mycelia of various ages to IBP: In order to find differences in the sensitivity of mycelia of various ages to IBP, mycelial discs of 6 mm diameter were obtained from the center to margin of the mycelial colony of P2, as shown in Fig. 2. The respective discs were placed on PDA including 0, 10 or 20 ppm IBP and incubated at 25°C for 10 days. On the 5th and 10th days, the diameter of colony developing from the disc was measured.

Results

Selection of the IBP-torelant isolate

Several colonies which had originated from single protoplasts grew on PDA including 10 ppm IBP. However, only one isolate formed abundant conidia on V-8 juice-agar medium, similarly as P2. Various visible characters of its colony such as color and growth rate were all similar to those of P2. Moreover, this isolate showed a similar pathogenicity to a rice cultivar, Jukkoku, as P2. While mycelia of this isolate were transferred onto fresh PDA repeatedly, twenty times, the above characters of this isolate were not changed at all. As far as we tested in the laboratory, this isolate was different from P2 only in the IBP-tolerance. Some of the data showing such differences between two isolates are shown below. Thus, this isolate was designated as PT83 and used in the present study. However, it is not certain whether PT83 is a type of IBP-tolerant strain which causes a serious problem by showing the IBP tolerance at the field levels, since we have not done any field test of PT83.

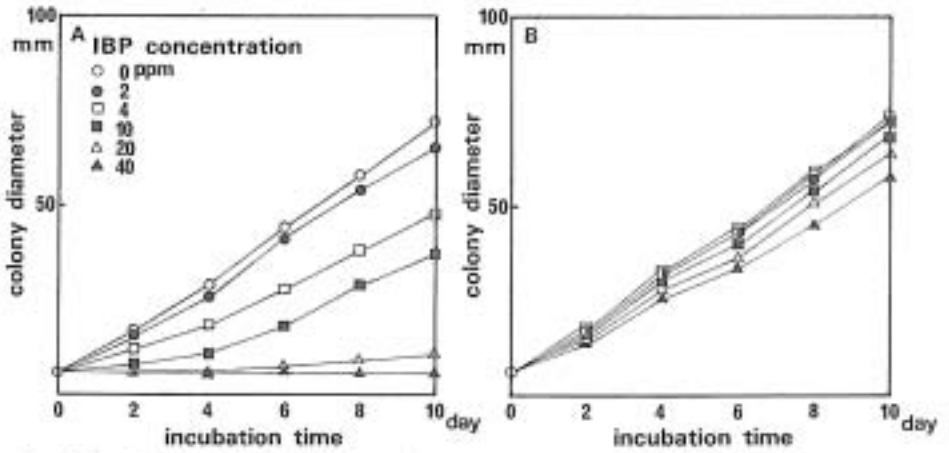

Fig. 3. Effect of various concentrations of IBP on mycelial growth of the IBP-sensitive isolate P 2 (A) and IBP-tolerant isolate PT83 (B) of *P. oryzae* on potato-dextrose-agar medium.

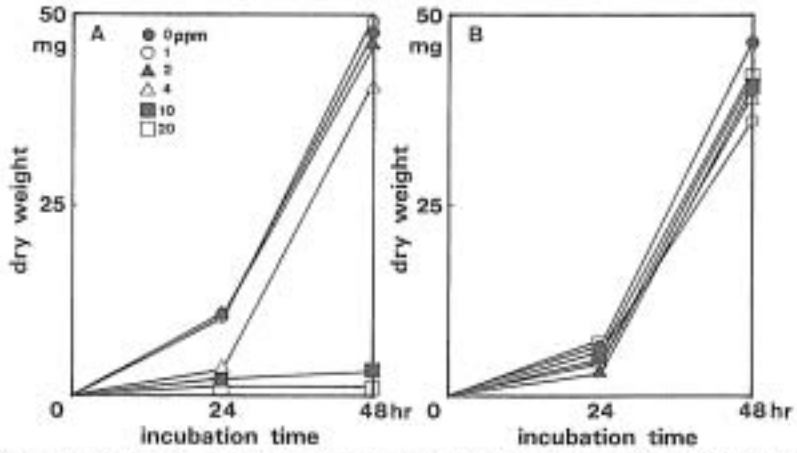

Fig. 4. Effect of various concentrations of IBP on dry weight of the IBP-sensitive isolate P2 (A) and IBP-tolerant isolate PT83 (B) of *P. oryzae* in Vogel liquid medium.

Effect of IBP on mycelial growth and conidium germination

Increase of colony diameter of isolate P2 was remarkably affected by 4 and 10 ppm of IBP and was perfectly suppressed at 40 ppm (Fig. 3A). On the other hand, mycelial growth of PT83 in terms of colony diameter was not prominently affected in the presence of 2–40 ppm IBP (Fig. 3B).

When the IBP effect on mycelial growth was evaluated in terms of dry weight the growth of P2 was delayed by 4 ppm IBP and inhibited by 20 ppm perfectly, while that of PT83 was not affected even by 20 ppm (Fig. 4A, B).

Effects of IBP on coindium germination of P2 became greater with the increased concentrations of IBP, leading to the perfect inhibition at 50 ppm (Fig. 5A). The appressorium formation was inhibited perfectly in the presence of 5 ppm IBP. In contrast to P2, the conidium germination of PT83 was not affected by 50 ppm. However, the appressorium formation was remarkably suppressed at 50 ppm and appressorium pigmentation at 20 ppm (Fig. 5B).

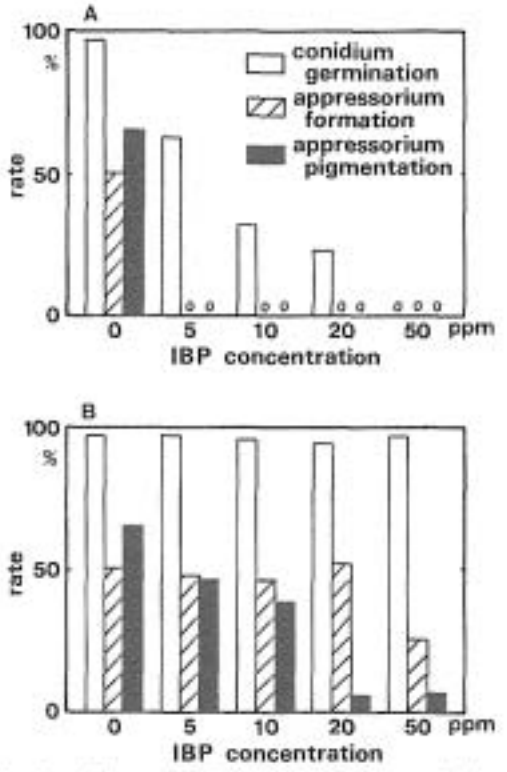

Fig. 5. Effect of IBP on conidium germination, appressorium formation and appressorium pigmentation of the IBP-sensitive isolate P2 (A) and IBP-tolerant isolate PT83 (B) of *P. oryzae* on nitrocellulose films.

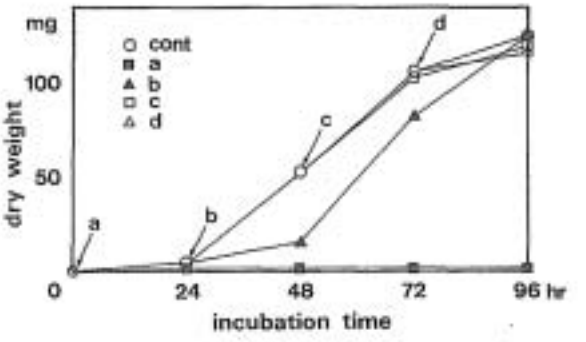

Fig. 6. Effect of timing of terminal 10 ppm IBP treatment on dry weight of mycelia of the IBP-sensitive isolate P2 of *P. oryzae* growing in Vogel liquid medium. For a through d, see Fig. 1.

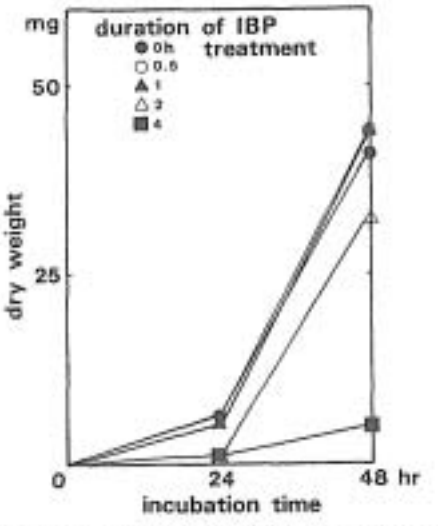

Fig. 7. Effect of timing of initial 10 ppm IBP treatment of various durations followed by incubation in Vogel liquid medium including no IBP on dry weight of mycelia of the IBP-sensitive isolate P2 of *P. oryzae*.

Effects of the timing of IBP treatments on the mycelial growth

When young mycelia were treated with 10 ppm IBP from the start of incubation, their dry weight did not increase at all during the 96 hr incubation (Fig. 6). When the IBP treatment was initiated 24 hr after the onset of incubation, the increase of dry weight was delayed but recovered to the level of control by 96 hr after the onset of incubation. The increase of dry wieght was not affected, when the IBP treatment was initiated at 48 hr or thereafter (Fig. 6).

The 0.5–1.0 hr initial IBP treatment did not affect the subsequent increase of dry weight, while the 2 hr treatment prolonged the lag phase of growth (Fig. 7). On the other hand, the 4 hr initial IBP treatment suppressed the increase of dry weight remarkably (Fig. 7).

Sensitivity of mycelia of different ages to IBP

Mycelia grew similarly from all mycelial discs of various ages in the presence of 10 ppm IBP as well as in control (Fig. 8A, B). However, the growth of only mycelia obtained from the margin of original colony was inhibited perfectly in the presence of 20 ppm IBP but that of others was not (Fig. 8C).

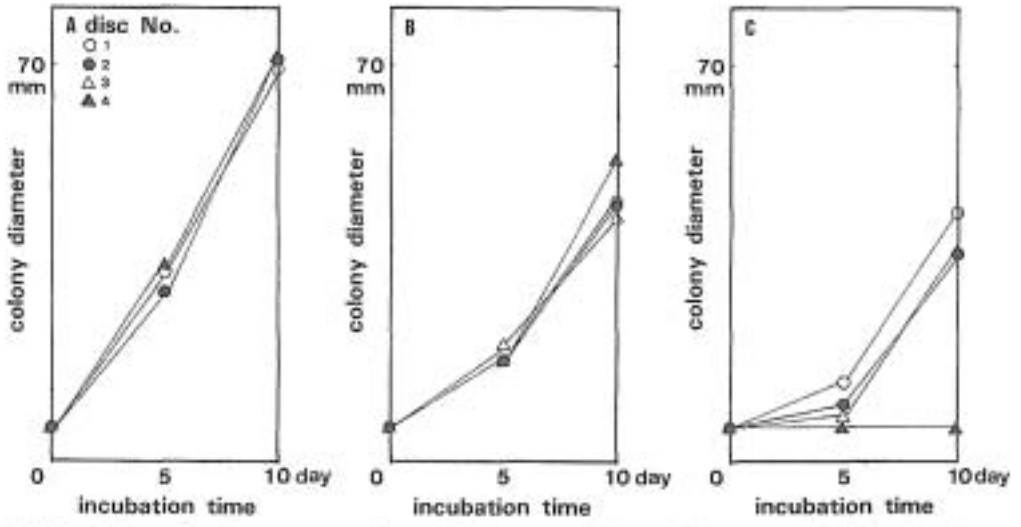

Fig. 8. Effect of various concentrations of IBP on mycelial growth from mycelial discs of various ages obtained from the mycelial colony of the IBP-sensitive isolate P2 of *P. oryzae*. A: 0 ppm, B: 10 ppm, C: 20 ppm. For disc No., see Fig. 2.

Discussion

The mycelial growth of P2 was perfectly inhibited on PDA by 40 ppm IBP, while that of PT83 was not even by 40 ppm IBP. On the other hand, the P2 growth was inhibited in Vogel liquid medium by 20 ppm IBP while the PT83 growth was not by 20 ppm IBP. The data shown in Fig. 5 indicate that appressorium formation of P2 was very sensitive to the IBP treatment at low concentrations. However, the appressorium formation of PT83 occurred even in the presence of 50 ppm, although it was somewhat suppressed at high concentrations. These results are almost consistent with the effective IBP concentrations to *P. oryzae* described by Araki and Miyagi[2] and also with the finding of Yamamoto and Hosoi[11] that the mycelial growth of *P. oryzae* was affected by the type and/or component of the medium and the effective concentrations of IBP largely depended on the medium component. The reason why the inhibitory concentration

of IBP was higher on PDA than in Vogel liquid medium is unknown at present. However, it might be related to the findings of earlier researchers[1-10] that several phosphoric fungicides including IBP were metabolized by *P. oryzae*, diminishing their effects on the fungus during incubation. It is possible that on PDA IBP might be metabolized by hyphae, leading to the localized reduction of IBP concentration around the fungal colony, while in liquid medium the IBP concentration given to the medium might be kept nearly constant around the hyphal colony by continuous shaking. By this reason, it would appear that *P. oryzae* was more insensitive on the hard medium than in the liquid medium. A further study is required to elucidate this point before we make a final conclusion.

The treatment with 20 ppm IBP in Fig. 3A and that with 10 ppm in Fig. 4A allowed the P2 mycelia to grow slowly, respectively, until 10 days and 48 hr after the onset of incubation. These results suggest that the treated mycelia might include some hyphae and/or some cells within single hyphae which were highly insensitive to the given concentrations of IBP. The tolerant isolate PT83 might have originated from protoplasts released from such hyphae or cells included in the P2 mycelia.

As shown in Fig. 6, the incipient growth stages of hyphal colony of P2 was sensitive to IBP, especially until 48 hr after the onset of incubation. When the hyphal colony was treated with 10 ppm IBP for the initial 0.5–2.0 hr after the onset of incubation, the subsequent growth was not prominently affected by the treatment, while it was largely suppressed when the hyphal colony was treated with 10 ppm IBP for the initial 4 hr. These results indicate the possibilities that the incipient growth was specifically sensitive to IBP, suggesting that hyphal apices might be more sensitive to IBP than its bases, and that at least 4 hr are required for IBP to show the inhibitory effect on the incipient growth of mycelia. The first possibility is supported by the results shown in Fig. 8 that hyphae of relatively young ages at the colony margin was perfectly inhibited to grow in the presence of 20 ppm IBP, while those of older ages locating at the colony center was insensitive to 20 ppm IBP. Data in Fig. 3 show that the 20 ppm treatment allowed the P2 mycelia to grow slowly on PDA, although the total growth was suppressed prominently. In considering the results of Fig. 8, such a slow increase of colony diameter was probably dependent on the growth of branched hyphae originating from older hyphae or basal parts of hyphae locating at the colony center.

All of these data suggest that the IBP effects on *P. oryzae* might be dependent on the ages of hyphae or cells in single hyphae and/or their phsyiological states associated with aging. To elucidate these points might be helpful to understand why appressorium formation is inhibited by relatively low concentrations of IBP. A further study is required to observe responses of single hyphae to IBP and to elucidate how the present observations are associated with modes of action of IBP which have been proposed by biochemical experiments employing high concentrations of IBP, 25–50 ppm[1,4,8].

Literature cited

1) Akatsuka, T., O. Kodama and H. Yamada. 1977. Agr. Biol. Chem. **41**: 2111–2112.
2) Araki, F. and Y. Miyagi. 1977. J. Pestic. Sci. **2**: 457–461.
3) Kobayashi, I., T. Ohkuma, H. Ishizaki and H. Kunoh. 1985. Exp. Mycol. **9**: 161–169.
4) Kodama, O., H. Yamada and T. Akatsuka. 1979. Agr. Biol. Chem. **43**: 1719–1725.
5) Köller, W., C. R. Allan and P. E. Kolattukudy. 1982. Phytopathology **72**: 1425–1430.
6) Maeda, T., H. Abe, K. Kakiki and T. Misato. 1970. Agr. Biol. Chem. **34**: 700–709.
7) Tomizawa, C. and Y. Uesugi. 1972. Agr. Biol. Chem. **36**: 294–300.
8) Uesugi, Y., O. Kodama and T. Akatsuka. 1978. Agr. Biol. Chem. **42**: 2181–2183.
9) Uesugi, Y. and H. D. Sisler. 1978. Pestic. Biochem. Physiol. **9**: 247–254.
10) Uesugi, Y. and C. Tomizawa. 1971. Agr. Biol. Chem. **36**: 313–317.
11) Yamamoto, H. and T. Hosoi. 1982. Ann. Phytopath. Soc. Japan **48**: 378 (Abst. in Japanese).

摘　　要

イネいもち病菌 *Pyricularia oryzae* Cav. に及ぼす IBP の影響
（1）菌叢に及ぼす影響

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Pyricularia oryzae Cav. の菌叢に及ぼす IBP（キタジンP）の影響を調べた。供試菌として IBP 感受性の分離株 P2 及び耐性の PT83 を用いた。前者の菌糸から調製したプロトプラストを 10 ppm IBP 含有の PDA 培地に接種し，その上で伸長してきたコロニーを選抜して後者の株を得た。P2 の菌叢の伸長は PDA 上及び Vogel 液体培地中でそれぞれ 40, 20 ppm IBP によってほぼ完全に阻害された。一方いずれの培地を用いても PT83 の菌叢の伸長は 40 ppm 存在下でも阻害されなかった。セロファン膜上の P2 分生胞子の付着器形成は 5 ppm IBP で完全に阻害されたが，PT83 の付着器形成は 50 ppm IBP でも完全には阻害されなかった。P2 の若い菌糸を 10 ppm IBP 含有液体培地で培養するとその後の菌体重の増加は見られなかった。また，培養開始24時間後に IBP を添加すると，対数増殖期の始まりが遅くなった。しかし，培養開始後48時間以降に IBP を添加しても菌体重の増加には全く影響が現われなかった。一方，P2 の菌叢を培養初期 4 時間のみ 10 ppm IBP で処理し，IBP を十分に洗浄した後に培養を続けると菌糸の伸長は顕著に抑制された。それ以下の短時間 IBP 処理では菌糸伸長に影響は現われなかった。次に平面培地上で生育する P2 の菌叢周縁部から中央部にかけて直径 6 mm の菌叢小円板をコルクボーラーで打ち抜き，それぞれ齢の異なる菌叢小円板を得た。これらを 20 ppm IBP 含有の平面培地に移植して培養したところ，菌叢周縁部から得た小円板からのみ菌叢の発達が認められなかった。以上の結果は，*P. oryzae* に及ぼす IBP の影響は菌叢を構成する菌糸の齢に関係しており，若い菌糸は IBP 感受性が著しく高いことを示唆している。