Original Article

4-Allyl-2-azetidinone and *Penicillium simplicissimum* Cooperate to Control Soilborne Fusarium Diseases

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When 4-allyl-2-azetidinone (B-143), without antifungal activity was applied to non-sterilized Kureha-soil (a commercial soil for gardening) artificially infested with *Fusarium oxysporum* f. sp. *cucumerinum*, occurrence of Fusarium wilt disease of cucumber (*Cucumis sativus*) was apparently reduced. However, when the soil was sterilized by autoclaving and infested with the pathogen, no effect of B-143 treatment was observed. These phenomena suggest that the disease suppression with B-143 may depend on the existence of some organisms in Kureha-soil. When *Penicillium simplicissimum* No. 8, isolated from the non-sterilized Kureha-soil, was re-introduced to sterilized Kureha-soil or some other kinds of soil, the effect of B-143 treatment was reproduced. The effect was also observed in the control of other Fusarium diseases such as tomato Fusarium wilt, cabbage yellowing, and stock Fusarium wilt. The results suggested that B-143 together with *P. simplicissimum* No. 8 generally exerted the reduction of soilborne Fusarium diseases.

INTRODUCTION

Occurrence of Fusarium diseases has been reported in most areas of temperate and tropical climate often with severe losses of crop production. Because the pathogen can persist indefinitely in infested soil, the diseases, once established, can pose serious dislocation in land use.¹⁾

For chemical control of soilborne Fusarium diseases, two types of fungicides have been used. One is biocidal fumigants such as chloropicrin, methyl bromide and D-D (a mixture of 1, 3-dichloropropene, 1, 2-dichloropropane, and related hydrocarbons).¹⁾ Captan²⁾ and captafol (Difolatan) are contained in the other type of fungicidal chemicals effective to fusaria.

The former group of chemicals work effectively if the infested soils are treated well. However, these kind of chemicals kill most microorganisms including those keeping the buffer action in the soil, and once a pathogen invades the soil, it multiplies so fast and the disease incidence gets very high. The latter may work specifically to the pathogen, but the efficacy is usually low because of their strong adsorption on the soil particles.¹⁾

Therefore, development of other methods to control Fusarium diseases is desired. Biological control has been focused to control Fusarium diseases: Louvet *et al.*³⁾ added as little as 1% suppressive soil and supplements of organic matters to cultivation soil to control

Fusarium diseases; Marois & Mitchell^{4,5)} used fungal antagonists against crown and root rot of tomato; Yuen *et al.*⁶⁾ controlled carnation wilt by antagonistic bacteria; Homma & Ohta,⁷⁾ Ogawa & Komada⁸⁾ and Yamaguchi *et al.*⁹⁾ showed that non-pathogenic *Fusarium oxysporum* prevented Fusarium wilt of sweet potato; Arie *et al.*¹⁰⁾ and Kijima *et al.*¹¹⁾ showed that *Pseudomonas gladioli* M-2196 controlled Fusarium wilts of bottle gourd and tomato when the rhizoplane bacterium was introduced together with *Allium* spp. to infested soil; Kijima & Gouma¹²⁾ showed that the hypocotyl cuttings of tomato inoculated with some non-pathogenic bacteria showed resistance to *F. oxysporum*; Phae *et al.*¹³⁾ showed that *Bacillus* sp. controlled crown and root rot of tomato when the bacterium was introduced into infested soil with compost.

The effects on biological control of Fusarium diseases, however, have not reached the desirable level due to the difficulty in fixation of the biocontrol agents in the field.¹⁰⁾ Thus, more investigations must be done to find a way to fix the microbial agents.

This paper deals with the possibility of a chemical substance which has no antifungal activity to cooperate with a fungal agent for the control of Fusarium diseases.

MATERIALS AND METHODS

1. Compound and Measurement of the Antifungal Activity

4-Allyl-2-azetidinone (the code number, B-143) (Fig. 1) was synthesized as a candidate in the development of new agrochemicals. Antifungal activity of B-143 was tested *in vitro* by mycelial disk assay against *F. oxysporum* f. sp. *cucumerinum* RIfc1 and other plant pathogenic fungi in the laboratory. The list of the fungal isolates tested is in Table 1. On potato dextrose agar (PDA) plates containing 50 or 100 $\mu\text{g/ml}$ of B-143, mycelial disk (4 mm in diameter) was placed and incubated at 27°C for 8 days. After 8 days of incubation, the diameter of mycelial mat (*A*) was measured and mycelial growth inhibition was calculated by comparison with the diameter of mycelial mat of non-treated control (*B*), by the following equation:

$$\text{Mycelial growth inhibition (\%)} = (B - A) / B \times 100$$

For comparison, 50 and 100 $\mu\text{g/ml}$ of benomyl (Benlate 50WP; DuPont Japan, Tokyo, Japan) was applied as positive control.

2. Fungal Isolates and Incubation for Inoculation

F. oxysporum Schlechtend.: Fr. ff. sp. *cucumerinum* Owen RIfc1, *lycopersici* (Saccardo) Snyder et Hansen race 2 860621a-1, *conglutinans* (Wollenweber) Snyder et Hansen 851108a-1, and *conglutinans* 8801210h, which are the pathogens of cucumber Fusarium wilt, tomato Fusarium wilt, cabbage yellows, and stock wilt, respectively, were used as pathogens. The pathogens were inoculated on potato dextrose broth (PDB) medium (100 ml in a 300 ml Erlenmeyer flask) and were incubated at 25°C for 7 days with shaking (110 rpm). The spores formed in the culture were collected by centrifugation and suspended in 0.1 M phosphate buffer (pH 7.0) up to 3×10^6 spores/ml for inoculation.

3. Plants

Cucumber (*Cucumis sativus* L. cv. Suvo, Yamato Seeds, Yokohama, Japan), tomato (*Lycopersicon esculentum* Mill. cv. Momotaro, Takii Seeds, Kyoto, Japan), cabbage (*Brassica oleracea* L. capitata group cv. Shikidori-kanran, Yamato Seeds) and stock (*Matthiola incana* R. Br. cv. Tokoharu, Dai-Ichi Seed, Tokyo, Japan) were used. One or two seeds were sown to each pot and were cultivated for 7, 21, 14 and 40 days, respectively.

Fig. 1 The structure of 4-allyl-2-azetidinone (B-143).

4. Soils

For isolation of soil-associated microbes and also for raising plants, Kureha-soil (Kureha Chemical Industry Co., Tokyo, Japan), a commercial soil for gardening, was used. Kureha-soil was sterilized by autoclaving at 121°C for 40 min, when necessary. In order to check the consistency of the disease suppressive effect, other types of soils such as lime soil mined in Okinawa (Coral reef rock, Nissho Iwai Chemical, Tokyo, Japan), volcanic sand of Mt. Fuji (Fujizuna), pumice sand (Karuishi), vermiculite, alluvial soil in Saitama and diluvium soil in Tochigi were also used. Vinyl chloride pots (60 mm in diameter) were filled with about 100 g of these soils, and were prepared for the treatment.

5. Isolation and Re-inoculation of Microorganisms in Soil

Microorganisms in Kureha-soil were subjected to isolation by soil plate method.¹⁴⁾ About 500 mg of Kureha-soil was incorporated into PD agar (10 ml). The plates were incubated at 28°C for 2 days and the fungi or bacteria growing were transferred and stored on PDA slants. For re-inoculation, the sterilized soils in the pots were inoculated with the microbes by pouring microbial suspension (50 ml), and incubated at 28°C for 5 days.

6. B-143 Treatment and Pathogen Inoculation

After growing the seedling, 50 ml of B-143 solution (100 $\mu\text{g/ml}$), water (for negative control) and 125 $\mu\text{g/ml}$ benomyl (for comparison) were separately added to the pots for different treatments. After 24 hr, 3 ml of spore suspension ($2-3 \times 10^6$ spores/ml) of *F. oxysporum* was added to each pot for inoculation. After inoculation, the plants were kept under 28°C, 12 hr light-12 hr dark conditions for 3 to 4 weeks and the disease development was monitored. Five to 10 pots were applied for each treatment.

7. Estimation of Disease Control

After 3 to 4 weeks of incubation, disease occurrence was judged for each plant by the symptoms as follows; blight (death), wilt, yellowing, or discoloration of leaf vessel. Proportion of diseased plants was calculated. Usually, each test was performed more than twice, and the average values of the proportion of diseased plants associated with the standard deviation were shown.

RESULTS

1. Antifungal Activity of 4-Allyl-2-azetidinone (B-143)

B-143 showed little antifungal activity against *F. oxysporum* f. sp. *cucumerinum* RIfc1 at 50 or 100 $\mu\text{g/ml}$ *in vitro* in comparison with benomyl, as well as against other plant pathogenic fungi (Table 1).

Table 1 Antifungal activity of B-143 against fungi on PDA plate.

Tested fungi	Mycelial growth inhibition ^{a)}
<i>Fusarium oxysporum</i> f. sp. <i>cucumerinum</i> ^{b)}	11.4±0.7 (%)
<i>F. oxysporum</i> f. sp. <i>niveum</i>	2.4
<i>F. oxysporum</i> f. sp. <i>raphani</i>	2.4
<i>F. moniliforme</i>	9.4
<i>Pyricularia oryzae</i>	2.3
<i>Cochliobolus miyabeanus</i>	15.9
<i>Helminthosporium sigmoideum</i>	0.0
<i>Botrytis cinerea</i>	3.1
<i>Sclerotinia sclerotiorum</i>	0.0
<i>Colletotrichum lagenarium</i>	3.7
<i>Cercospora beticola</i>	0.0
<i>Cercospora kikuchii</i>	0.0
<i>Alternaria alternata</i> (mali)	1.0
<i>Alternaria alternata</i> (kikuchiana)	0.5
<i>Glomerella cingulata</i>	0.0
<i>Rhizoctonia solani</i>	12.4

^{a)} Diameter of fungal growth (8 days old) in the presence of B-143 (100 µg/ml) was compared with the non-treated control.

^{b)} Growth inhibition was 0.0 and 100.0% with 50 µg/ml of B-143 and 100 µg/ml of benomyl, respectively. Tests were repeated two times with 4 samples for each and the average values are shown with standard deviations.

Fig. 2 Effect of B-143 in non-sterilized and sterilized Kureha-soil.

Non-sterilized Kureha-soil treated with 50 ml of B-143 (100 µg/ml) (A); sterilized (121°C, 40 min) Kureha-soil treated with B-143 (C); B and D were without B-143 treatment of A and C, respectively.

2. Control Effect of B-143 with Non-sterilized and Sterilized Kureha-soil

With non-sterilized Kureha-soil, B-143 treatment (100 µg/ml, 50 ml/pot) was effective for the reduction of cucumber wilt in pot test (Figs. 2 and 3). The efficacy was almost the same as that of benomyl at 125 µg/ml (50 ml/pot). More than ten trials were held, and similar results were obtained.

With an autoclave sterilization (121°C, 40 min) of

Fig. 3 Disease reduction of cucumber wilt (*Fusarium oxysporum* f. sp. *cucumerium*) with B-143.

■ : non-sterilized Kureha-soil, ▨ : sterilized (121°C, 40 min) Kureha-soil. Each treatment was repeated twice with more than 6 plants. Percentage of diseased plants was calculated with standard deviation. The effect of B-143 in non-sterilized Kureha-soil was almost the same as that of benomyl treatment. When the soil was sterilized before B-143 treatment and pathogen inoculation, no disease reduction was observed (pot test, 21 days after sowing).

Kureha-soil before B-143 treatment and pathogen inoculation, the control efficacy of B-143 on cucumber wilt disappeared (Figs. 2 and 3). This suggests that a factor in Kureha-soil, probably some organisms which act in conjunction with B-143, might have been lost by sterilization.

3. Isolation of Microorganisms in Non-sterilized Kureha-soil

Based on the assumption that there is a possible relationship between some organisms in the Kureha-soil and the disease suppression with B-143 treatment, we tried to isolate microorganisms in the soil. By soil plate method isolation, fungi and a few bacteria were isolated. Among them, *Penicillium* spp. were the most frequently isolated and an isolate named No. 8 was identified as *P. simplicissimum* (oudemans) Thom. which was deposited in National Institute of Bioscience and Human Technology, Tsukuba, Ibaraki with the isolate number FERMP-11347 (March 6, 1991).

4. Re-inoculation of Isolated Microorganisms to Sterilized Kureha-soil

The isolates obtained from non-sterilized Kureha-soil were individually re-inoculated to sterilized Kureha-soil with B-143, and their effects on disease suppression were tested. When *P. simplicissimum* No. 8 (10⁷ spores/ml, 50 ml/pot) was introduced to sterilized Kureha-soil, the effect of B-143 treatment (100 µg/ml) to control cucumber wilt was reproduced (Figs. 4 and 5). Some other isolates of *Penicillium* spp. also showed such activity, but *P. simplicissimum* No. 8 exhibited the highest disease suppression (data not shown). The other isolates in-

Fig. 4 Disease reduction of cucumber wilt with B-143 and *Penicillium simplicissimum* No. 8.

Sterilized (121°C, 40 min) Kureha-soil inoculated with *P. simplicissimum* No. 8 (10^7 spores/ml, 50 ml/pot) and treated with 50 ml of B-143 (100 µg/ml) (upper). The lowers are without B-143.

Fig. 5 Disease reduction of cucumber wilt by introducing *Penicillium simplicissimum* No. 8 and B-143.

Each soil was inoculated with *P. simplicissimum* No. 8 (10^7 spores/ml, 50 ml/pot) (+) or without inoculation (-), and 3 days later, B-143 (100 µg/ml) was poured (■) or water was poured (□). Each experiment was repeated 6 to 18 times. Percentage of diseased plants was calculated with standard deviation. Only when both *P. simplicissimum* No. 8 and B-143 were treated to sterilized soil (the left column), the effect on disease reduction was reproduced (pot test, 21 days after sowing).

cluding bacteria showed no synergistic disease suppression with B-143.

5. Control of *Fusarium* Diseases by *P. simplicissimum* No. 8 and B-143 in Pot Test

When *P. simplicissimum* No. 8 and B-143 (100 µg/ml) were introduced together to sterilized Kureha-soil, cabbage yellows and stock wilt caused by *F. oxysporum* f. sp. *conglutinans* and tomato wilt caused by *F. oxysporum* f. sp. *lycopersici* race 2 were prevented at high rate as that of cucumber wilt. On the other hand, B-143 or *P.*

Fig. 6 Control of *Fusarium* diseases by B-143 in conjunction with *Penicillium simplicissimum* No. 8.

Sterilized Kureha-soil was treated with both B-143 (100 µg/ml) and *P. simplicissimum* No. 8 (10^7 spores/ml, 50 ml/pot) (▨), only with B-143 (■), or only with *P. simplicissimum* No. 8 (□), then inoculated with a pathogen. For each treatment, in the total, 5 to 23 plants were applied. Percentage of diseased plants was calculated with standard deviation. When B-143 and *P. simplicissimum* No. 8 were treated together, good disease suppression was obtained with cabbage, stock and tomato (pot test, 27 days after inoculation).

Fig. 7 Disease reduction of cucumber wilt in several kinds of soil with *Penicillium simplicissimum* No. 8 and B-143.

Each sterilized soil was inoculated with *P. simplicissimum* No. 8 (10^7 spores/ml, 50 ml/pot) and 3 days later, B-143 (100 µg/ml) (■) or water (□) was poured. Four to 8 plants were tested for each treatment and experiment was repeated twice. Percentage of diseased plants was calculated with standard deviation. In lime soil, volcanic sand, pumice soil, and mixture of vermiculite and Kureha-soil (3:1), B-143 showed disease reduction, as well as Kureha-soil, but less in diluvium soil and alluvial soil (pot test, 21 days after seeding).

simplicissimum No. 8 showed no significant effect by individual treatment (Fig. 6).

6. Effects of *P. simplicissimum* No. 8 and B-143 in Different Kinds of Soils

In order to test the efficacy of B-143 and *P. simplicissimum* No. 8 in different types of soils other than Kureha-soil, we applied both agents to several kinds of sterilized soils inoculated with *F. oxysporum*. Cucum-

ber wilt was controlled in volcanic sands, pumice sand, lime soil, and also in the mixture of vermiculite and Kureha-soil (3 : 1), but less in diluvium and alluvial soils (Fig. 7).

DISCUSSION

B-143, a non-antifungal substance, and *P. simplicissimum* No. 8 showed disease reduction when they were applied to soils simultaneously. *P. simplicissimum* No. 8 showed no disease control activity by itself. Likewise, when B-143 was introduced to *P. simplicissimum* No. 8 free soils, we could not observe any significant effect on the control of Fusarium disease. These results suggest that B-143 may work in conjunction with the fungus (probably as a potential biocontrol agent) to control Fusarium diseases.

Recently, a combination of microbial agents such as *Streptomyces corchorusii* and herbicides e.g. pendimethalin was presented as an effective control method for Fusarium wilt of tomato.¹⁵⁾ In this case, both agents, microorganisms and herbicides, were reported to have antifungal activity against *F. oxysporum* f. sp. *lycopersici*, and the final effect was culminative of both the agents.

However, in the case of B-143 and *P. simplicissimum*, both agents do not show antifungal activity independently as mentioned above. This suggested that B-143 is a substance classified to a new category that has no antifungal activity but still has capability to control Fusarium diseases in conjunction with *P. simplicissimum*.

Twenty-three days after the treatment with *P. simplicissimum* No. 8 to sterilized Kureha-soil, about 2×10^7 cfu/g soil of *P. simplicissimum* was detected from both B-143 treated and non-treated samples. The population decreased to around 2×10^6 cfu/g soil in diluvium, alluvial, volcanic soils, and the mixture of vermiculite and Kureha-soil. However, the number was almost the same in both B-143 treated and non-treated samples.

Although the control mechanism of Fusarium diseases by B-143 and *P. simplicissimum* No. 8 is not known yet, it is possible that some metabolites of B-143 by *P. simplicissimum* No. 8 are playing roles in the biocontrol system. Thus the screening of antifungal or elicitable substances from the metabolites of B-143 was performed and the 10 and 25 days-culture supernatant of *P. simplicissimum* No. 8 in PDB containing 100 µg/ml B-143 showed some antifungal activity against *F. oxysporum* on a PDA plate. The phenomenon, however, was not reproducible and we could not reach a definitive conclusion yet.

In this study, we applied Kureha-soil as a primitive source for isolation of microorganisms. Although we used several lots of the soil which have been produced at different periods, the disease suppression of B-143 treatment was reproduced with every lot. Further, *Penicillium* spp. mycologically similar to *P. simplicissimum* No.

8 were successfully isolated from all the lots tested.

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要 約

4-Allyl-2-azetidinone と *Penicillium simplicissimum* による土壌伝染性フザリウム病の発病抑制

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4-Allyl-2-azetidinone (B-143) は抗菌活性を示さない物質であるが, 滅菌していない培土 (クレハ園芸培土) に灌注 (100 µg/ml, 0.5 mg/g 土) すると *Fusarium oxysporum* f. sp. *cucumerinum* によるキュウリ (*Cucumis sativus*) つる割病の発病を抑制した (ポット試験)。しかし, 培土をオートクレーブ (121°C, 40 分) 滅菌処理したものでは B-143 灌注による発病抑制効果が失われた。そこで培土中に存在する微生物が本抑制効果発現に関与していると考えられたため,

分離を試みたところ、*Penicillium simplicissimum* No. 8 が分離された。同菌を、滅菌処理した培土に戻し接種した後に B-143 を灌注したところ、発病抑制効果が再現された。*P. simplicissimum* No. 8 および B-143 による発病抑制効果はトマト (*Lycopersicon esculentum*) 萎ちょう病、キャベツ

(*Brassica oleracea*) 萎黄病、ストック (*Matthiola incana*) 萎ちょう病に対しても認められた。すなわち、B-143 と *P. simplicissimum* No. 8 は協力的に土壌伝染性フザリウム病に対して発病抑制効果を示すと考えられた。