

Ecological Characteristics and Biological Control of Mulberry Anthracnose

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Abstract

To develop effective control techniques against mulberry anthracnose caused by *Colletotrichum dematium*, ecological and biocontrol studies on the disease were carried out. It was confirmed that the causal fungus overwinters mainly in infected mulberry leaves on the ground, then first infects the leaves adjacent to the ground in the rainy season. The fungal infection secondarily reaches the middle and upper foliage of mulberry trees with the passage of time, being less associated with the leaf age. The diseased leaves subsequently fall in autumn, and the fungus overwinters in these leaves again. It was suggested that the removal of fallen leaves from the mulberry field in autumn may contribute to cutting off the above disease cycle efficiently. A potential antagonist, *Bacillus amyloliquefaciens* strain RC-2, was isolated from healthy mulberry leaves. The antagonism was due to the production of antifungal compounds by the strain. Seven kinds of antifungal compounds were purified, and one of these was identified as iturin A2. Based on antagonistic mechanisms of the strain, the inhibition ability of RC-2 could be efficiently used by the application of the strain onto the leaves adjacent to the ground when primary fungal transmission from soil occurs. Furthermore, the antibiotics inhibited several other phytopathogenic fungi and bacteria, besides *C. dematium*, *in vitro*, suggesting that the antibiotics produced by RC-2 might be useful as multiple control materials against various plant diseases.

Discipline: Plant disease

Additional key words: *Colletotrichum dematium*, *Bacillus amyloliquefaciens*

Introduction

Mulberry, a woody plant belonging to the genus *Morus*, family Moraceae, is widely distributed around the world. The leaves of the plant are used as feed for silkworms in the sericultural industry. Mulberry anthracnose, first reported in 1925⁷, is a common leaf disease of mulberry in Japan. Although the causal pathogen had long been considered to be *Colletotrichum morifolium* Hara⁷, our recent study¹² revealed that (1) anthracnose species isolated from diseased mulberry leaves were *C. dematium*, *C. acutatum* and *Glomerella cingulata*, (2) *C. morifolium* should be a synonym of *C. dematium* based

on morphological characteristics, and (3) *C. dematium* was the most aggressive pathogen. Mulberry leaves infected with *C. dematium*, the major fungus of the disease, display brown necrotic spots or streaks¹³, resulting in the decrease of the yield of leaves for silkworm feeding. The incidence of the fungal disease has tended to increase due to dense planting of the trees and mechanical harvesting of the shoots, which predispose plants to the disease. Control of the disease has been a major problem in mulberry cultivation. It is essential for developing effective control techniques to investigate the ecological characteristics of the disease. However, no substantial studies have been carried out on these aspects, although several brief reports have been published^{7,9}.

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In addition, integrated control techniques including chemical and biological control have recently been developed from an ecological and economic point of view for various plant diseases worldwide¹. Nevertheless, no biocontrol studies on mulberry anthracnose using antagonistic microorganisms had been conducted hitherto.

In this paper, we reviewed the ecological characteristics of *C. dematium* determined in our recent studies, and described a potential biocontrol agent against the fungal disease, *Bacillus amyloliquefaciens* RC-2, isolated from healthy mulberry leaves. Also, we discussed the beneficial effect of application methods of the antagonistic microorganism on the suppression of anthracnose, based on the ecological characteristics of the disease and antagonistic mechanisms of the strain.

Annual development of anthracnose in mulberry trees

To analyze the annual development of mulberry anthracnose caused by *C. dematium* in mulberry trees, changes in the occurrence of anthracnose were surveyed in individual mulberry trees at fixed points from August 1993 to November 1995 in a mulberry field located at the National Institute of Sericultural and Entomological Science. In the surveys conducted each year, the onset of

anthracnose symptoms was observed from late June to mid-July, which corresponds to the rainy season in the region, and the disease occurred only on leaves located near the ground. The disease became more frequent in August each year. Fig. 1 shows the number of diseased leaves at 3 different heights above the ground (lower, 0–50 cm; middle, 51–120 cm; upper, above 121 cm) per mulberry tree. The disease in August was mostly observed in the lower foliage each year. Infection reached the middle and upper foliage within one or two months. The total number of infected leaves increased, being maximum in November each year, although the incidence varied with the year of the survey. In addition, the severity of the infection was greater in the lower foliage than at other positions of the leaves in all the surveys.

When the naturally diseased leaves were observed on each shoot forming 3 different angles (ca. 0°, 45° and 90° to the ground) on the trees in October in both 1993 and 1994, most of the leaves of only the shoots parallel to the ground were severely infected regardless of the leaf age¹⁴. On shoots forming a 45° or 90° angle with the ground, the disease was sparsely observed from the middle to old leaves, whereas field inoculation tests¹⁴ showed that young leaves were more susceptible to *C. dematium* than older leaves attached at lower positions of the shoots.

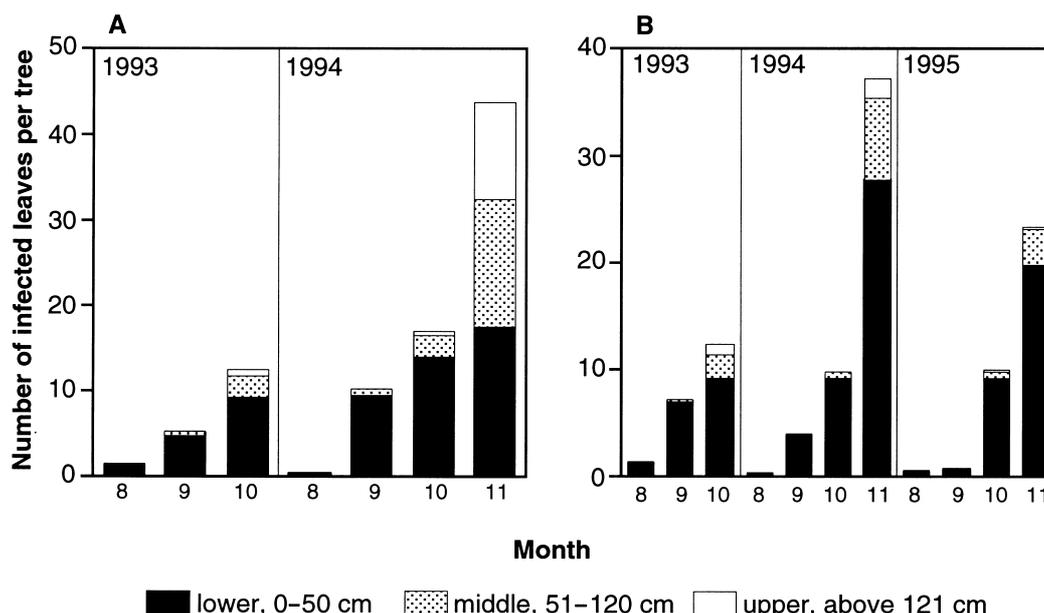


Fig. 1. Average number of leaves with symptoms of anthracnose per mulberry tree at fixed points of mulberry field

A: Spring-pruned trees (all the shoots were cut at the trunks in March),
 B: Summer-pruned trees (all the shoots were cut at the trunks in March and June).
 Diseased leaves were counted at 3 different heights of the leaves above the ground.
 The counting was performed once a month from August to November in each year.

The above surveys and results indicated that the development of anthracnose proceeded as follows: (1) the mulberry pathogen overwinters in or on the soil as the source of primary infection, and the infection starts from the leaves close to the ground in the following year; (2) the anthracnose symptoms first occur on lower foliage and subsequently reach the upper foliage on trees. Thus, the disease spreads from lower to upper leaves although younger leaves appear to be more susceptible.

Survival of *C. dematium* in soil and infected mulberry leaves

As described in the above section, it appears that the causal pathogen, *C. dematium*, overwinters in or on soil. Several reports have indicated that pathogens can overwinter on or in soil in some forms, such as infested debris, and overwintered inocula play an important role as primary infection source in *Colletotrichum* spp.^{3,5,10}. Hence, we determined the possible source of primary inoculum of *C. dematium* under field conditions. Studies were focused on conidia in soil since the conidia of several anthracnose pathogens play a significant role as secondary inoculum^{2,4}. Tests were also conducted using infected leaves, because numerous mulberry leaves remained in the field until the following spring.

Conidia or infected mulberry leaves were mixed with soil and placed on the ground, indoors, or outdoors under a roof protected from rain and snow in early December. The viability of the conidia on the ground declined rapidly, and the fungus could not be detected

within 65 days¹⁶. This suggests that the conidia of *C. dematium* mostly fail to overwinter in soil under field conditions and are not likely to be a significant primary source of inoculum. Conversely, the fungus remained viable for at least 150 days in infected and latently infected leaves under field conditions¹⁶. In addition, laboratory experiments revealed that the fungus in infected leaves survived for at least 90 days at 25 and 35°C and for 600 days at 0°C¹⁶. Thus, *C. dematium* is considered to survive mainly in fallen mulberry leaves in winter, which become a source of primary inoculum in the following year.

Biological control of mulberry anthracnose

Mulberry anthracnose has usually been controlled with chemical fungicides, such as thiophanate-methyl. However, the application of chemicals should be reduced to preserve the agro-ecosystems. We attempted to develop a new control strategy using an antagonistic bacterium as a substitute for the fungicide.

Among the bacteria isolated from healthy mulberry leaves, a bacterial strain RC-2 was selected as a candidate for the antagonism, because it was most inhibitory on the growth of *C. dematium in vitro* and due to the development of anthracnose symptoms *in vivo* (Fig. 2). Using a bacterial identification kit (API 50 CHB test strip; Bio Merieux S. A., France), RC-2 was identified as *Bacillus amyloliquefaciens*. The inhibitory activity on the anthracnose symptoms on mulberry seedlings was due to the production of antifungal compounds by RC-2. When



Fig. 2. Inhibition of anthracnose disease on mulberry seedlings by *Bacillus amyloliquefaciens* RC-2
Right: Culture fluid of RC-2 was spread on the seedlings before spray-inoculation with a conidial suspension of *Colletotrichum dematium*. Left: Seedlings were inoculated with a conidial suspension, but not treated with RC-2. After 8 days, the seedlings wilted due to severe fungal infection.

aseptic culture filtrates of RC-2 were spread onto the leaves of mulberry trees just before inoculation with *C. dematium*, the development of anthracnose lesions on leaves was significantly inhibited, whereas treatment with washed bacterial cells did not inhibit the development of the symptoms (Fig. 3). The mechanisms of bio-control are generally classified into competition, parasitism or predation, and antibiosis⁶. Antibiosis was involved in the inhibition of mulberry anthracnose by the strain RC-2. When the culture filtrate was applied onto the leaves before or after fungal inoculation, symptom development on the leaves was inhibited only when the filtrate was applied before fungal inoculation (Fig. 4). These findings suggest that the filtrate containing antifungal compounds exhibits a preventive effect on the disease. In addition, this preventive effect was confirmed to be due to the inhibition of conidial germination¹⁷.

Antifungal compounds produced by RC-2 in the culture filtrate were also found to inhibit the growth of several other phytopathogenic fungi and bacteria *in vitro*. The aseptic culture filtrate strongly inhibited the growth of *Agrobacterium tumefaciens*, *Clavibacter michiganensis* subsp. *michiganensis*, *Erwinia carotovora* subsp.

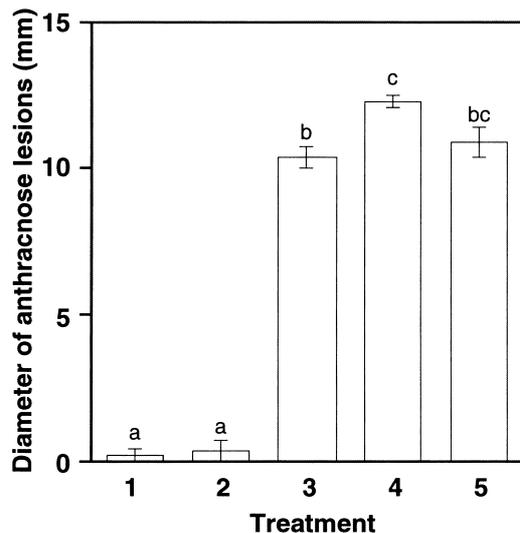


Fig. 3. Anthracnose disease on mulberry leaves treated with fractions of shake-cultured *Bacillus amyloliquefaciens* RC-2

Treatments: 1, Whole culture; 2, Culture filtrate; 3, Washed bacterial cells; 4, Uncultured 0.5% polypeptone-PD broth (control); 5, No treatment (control). Leaves were treated 2 h before inoculation with a conidial suspension of *Colletotrichum dematium*. Each value represents the mean of 8 replicates. Bars represent standard error of the mean. The same letters in each column indicate the absence of a significant difference ($P \leq 0.05$), according to Tukey's test.

carotovora and *Xanthomonas campestris* pv. *campestris* (Table 1), while the growth of 5 species of *Pseudomonas* was less inhibited. Growth inhibition by the filtrate was also observed with the phytopathogenic fungi, and the growth of *C. dematium* and *Glomerella cingulata* (mulberry anthracnose fungus), *Pyricularia oryzae* (rice blast fungus) and *Rosellinia necatrix* (mulberry white root rot fungus) was strongly inhibited. Thus, the antifungal substances showed a potential for the control of several fungal and bacterial diseases besides mulberry anthracnose.

The culture filtrate with a wide spectrum of antimicrobial activity was found to contain 7 compounds based on HPLC, FAB-MS and NMR analyses¹⁷. NMR spectra of compound 1, which gave the highest yield, showed that the chemical structure consisted of a cyclic peptide composed of 8 amino acids, as follows: β -amino acid with straight-chain \rightarrow Asn \rightarrow Tyr \rightarrow Asn \rightarrow Gln \rightarrow Pro \rightarrow Asn \rightarrow Ser \rightarrow . Also, based on the FAB-MS

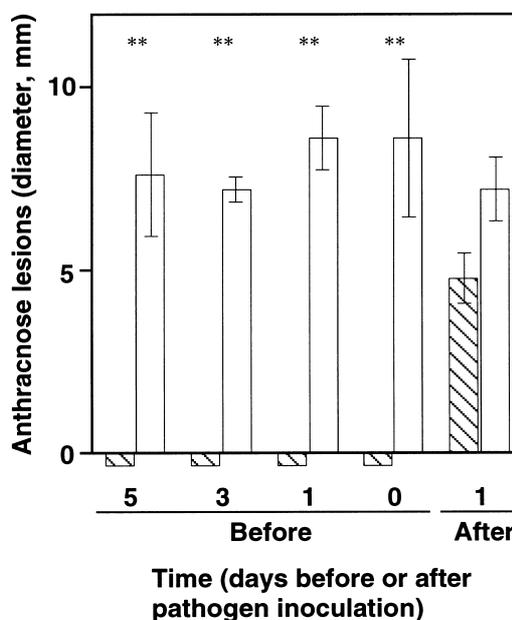


Fig. 4. Inhibition of anthracnose disease on mulberry leaves treated with a culture filtrate of *Bacillus amyloliquefaciens* RC-2 before or after inoculation with *Colletotrichum dematium*

Hatched columns indicate treatment with culture filtrate of RC-2. Hatched columns under the horizontal axis indicate that no anthracnose lesions appeared on the leaves treated with the filtrate before the fungal inoculation, while hatched column above the axis shows the presence of lesions. White columns: Treatment with uncultured 0.5% polypeptone-PD broth (as control). ** Significant difference at the 1% level between RC-2-treated and control leaves, according to Mann-Whitney's U test. Each value represents the average of 5 replicates. Bars represent standard error of the mean.

Table 1. Antimicrobial activity of the culture filtrate of *Bacillus amyloliquefaciens* RC-2

Microorganisms ^{a)}	Dilution of culture filtrate (-fold) ^{b)}			
	1	2	4	Control ^{c)}
Bacteria				
<i>Agrobacterium tumefaciens</i>	26.7 ± 1.0 ^{d)}	22.7 ± 1.0	19.7 ± 0.7	NI
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i>	12.0 ± 0.5	7.3 ± 1.0	5.7 ± 0.3	NI
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>	16.0 ± 0.5	13.3 ± 0.3	9.7 ± 0.3	NI
<i>Pseudomonas chichorii</i>	7.7 ± 0.7	2.7 ± 1.2	NI	NI
<i>Pseudomonas marginalis</i> pv. <i>marginalis</i>	9.0 ± 0.5	5.3 ± 0.3	NI	NI
<i>Pseudomonas syringae</i> pv. <i>mori</i>	13.0 ± 0.9	6.7 ± 0.7	NI	NI
<i>Pseudomonas tolaasii</i>	7.7 ± 1.1	4.3 ± 1.8	NI	NI
<i>Pseudomonas viridiflava</i>	10.7 ± 0.3	6.3 ± 0.5	NI	NI
<i>Xanthomonas campestris</i> pv. <i>campestris</i>	25.0	21.0 ± 0.5	16.3 ± 0.7	NI
Fungi				
<i>Bipolaris leersiae</i>	NG ^{e)}	NG	3.0 ± 1.3	34.3 ± 1.8
<i>Colletotrichum acutatum</i>	NG	NG	5.0 ± 2.6	30.3 ± 0.3
<i>Colletotrichum dematium</i>	NG	NG	NG	20.3 ± 1.0
<i>Diaporthe nomurai</i>	NG	NG	1.7 ± 1.4	29.7 ± 1.5
<i>Fusarium lateritium</i> f.sp. <i>mori</i>	2.0 ± 0.9	7.0 ± 1.4	15.0 ± 0.5	23.7 ± 0.5
<i>Glomerella cingulata</i>	NG	NG	NG	40.3 ± 0.3
<i>Myrothecium roridum</i>	NG	1.0 ± 0.8	10.7 ± 0.7	15.0
<i>Pyricularia oryzae</i>	NG	NG	NG	18.3 ± 0.3
<i>Rosellinia necatrix</i>	NG	NG	NG	23.7 ± 1.5
<i>Sclerotinia sclerotiorum</i>	NG	NG	13.7 ± 5.6	>90.0

a): All the isolates were preserved in our laboratory.

b): Culture filtrate obtained from an incubation mixture of 0.5% polypeptone-PD broth was filter-sterilized. Undiluted filtrate taken as 1-fold.

c): Sterilized distilled water (20 µL) was placed as control.

d): Each value is the mean of 3 replicates of the diameter (mm) of the inhibition zone in the bacterial layer. Variance is given as the standard error of the mean. Activity was determined 1 day after treatment with the filtrate. Twenty µL of each dilution of the culture filtrate was placed on each bacterial layer of potato semi-synthetic agar. NI: No inhibition.

e): Each value is the mean of 3 replicates of the diameter (mm) of mycelial growth. Variance is given as the standard error of the mean. Activity was determined 3 days after placement of the filtrate. Twenty µL of each dilution of the culture filtrate was placed on each mycelial block (0.5–1.0 mm³) on a PSA plate. NG: No growth.

spectrum of compound 1, the molecular weight was found to be 1042. These chemical properties enabled to identify compound 1 as iturin A2 (Fig. 5), an antimicrobial cyclic peptide first isolated from *B. subtilis*⁸. Other compounds may also have a similar chemical structure to that of iturin A2, based on the NMR and FAB-MS spectra.

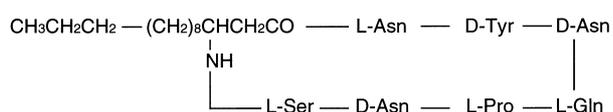


Fig. 5. Chemical structure of iturin A2 produced by *Bacillus amyloliquefaciens* RC-2

Iturin A2 is a cyclic peptide composed of 8 amino acids.

Conclusions and future perspectives

Colletotrichum dematium, the causal fungus of mulberry anthracnose, overwinters mainly in infected and latently infected mulberry leaves which remain in mulberry fields, and these leaves presumably become a source of primary inoculum. The primary inoculum first infects the lower leaves of mulberry trees in the rainy season. The infection appears to occur through fungus-infested soil; the propagules in soil may be disseminated to healthy plants within water droplets or by plowing¹⁵. Once anthracnose symptoms occur on the leaves near the ground, the disease reaches the middle and upper leaves of mulberry trees with time. The disease progression is opposite to the susceptibility of leaves on shoots. Diseased (and latently infected) leaves subsequently fall in

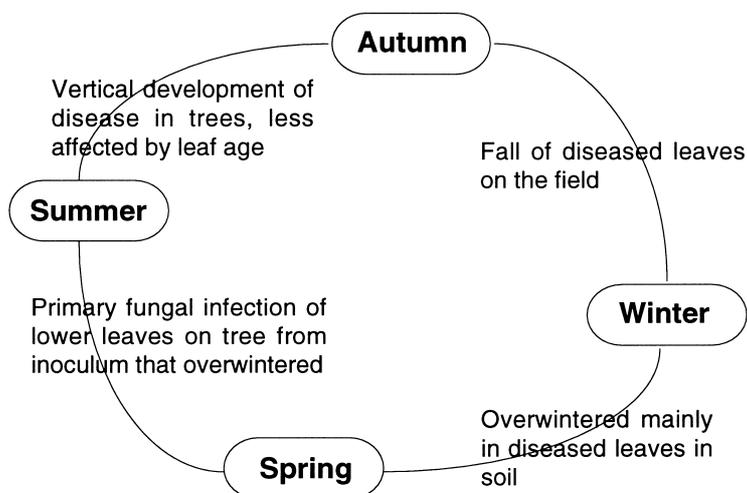


Fig. 6. Putative life cycle of *Colletotrichum dematium*

autumn, and the fungus overwinters in these leaves again. The putative life cycle of the fungus is depicted in Fig. 6. Based on the life cycle, the removal of fallen leaves from the mulberry field in autumn should interrupt the disease cycle efficiently, resulting in the prevention of the disease in the following year.

Using the potential antagonist (*B. amyoliquefaciens* strain RC-2) may also provide a new method of control of mulberry anthracnose. The strong prevention activity of the strain against the disease was due to the production of antimicrobial compounds, including iturin A2. Obviously, a single application of the bacterial materials could not completely suppress the disease since they do not suppress the causal fungus present in leaf tissues. Application of strain RC-2 onto mulberry leaves near the ground may suppress the primary infection from the soil. Though the form of the primary inoculum has not been identified in mulberry anthracnose, secondary conidia are likely to occur from the overwintering residues, as was the case with other *Colletotrichum* species¹¹. The antibiotics of RC-2 strongly inhibit conidial germination of the fungus, which leads to the interruption of the disease cycle (Fig. 6). The antibiotics produced by strain RC-2 appear to be useful as a substitute for chemical fungicides to prevent the disease, by minimizing the total amount of fungicide application. Field experiments should be conducted to reveal the source of primary infection and to determine the effectiveness of strain RC-2 as a biocontrol agent against mulberry anthracnose. Furthermore, the antibiotics derived from RC-2 inhibited the mycelial growth of several taxonomically diverse phytopathogenic fungi and bacteria, in addition to *C. dematium*, indicating that the antifungal compounds in the filtrate exhibit a wide spectrum of antimicrobial activity. Thus, the anti-

biotics produced by RC-2 might be used as multiple control materials against various plant diseases.

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