Biological Control of Plant Diseases With *Bacillus* Species

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ABSTRACT : Biocontrol is playing a more and more important role in plant disease management. Evidences show that there are optimum prospects for people to apply biocontrol approach to control plant disease or to study the mechanism of antagonism. "The study of Antagonistic Protein of *Bacillus* spp. to *Xanthomonas oryzae* pv. *oryzae*" has been worked in our laboratory since 1986. One hundred and thirty antagonistic bacteria were screened out, most of them belonged to *Bacillus* spp., and showed very strong inhibitive effect to various plant pathogens. Nine antagonistic proteins (peptides) were purified (P11-I, P11-II, B8, B826-I, B826-II, A30-I, A30-II, G35). Two antagonistic protein related DNA fragments (B826-I, A30-II) were cloned and sequenced. B826-I DNA fragment composed by 905 bp, and it contained two ORF encoding 95, and 54 amino acids, respectively. By using Rif^r and Kam^r as the selective markers, we found the bacteria could colonize on rice leaf for at least 40 days. In greenhouse the antagonistic bacteria showed certain degree of control efficiency.

A Brief Review on The Using of Antagonistic Bacteria

Plant diseases cause severe losses on agricultural products every year. Chemical pesticides have been widely applied to control plant diseases for a long period, but some unexpected results were also appeared associated with the unlimited using of these chemicals, such as the pollution of crop products and environment, the resistance of plant pathogen to the chemicals, and so on. On the other hand, there is an ecological balance between the pathogens and their antagonists in nature which have occurred for thousands and thousands of years. Biological control of plant diseases is to control plant diseases by direct introduction of the microbial antagonists to reduce the pathogen population or by manipulation of the environment and host to make a new ecological balance between the pathogens and their antagonists which is specially favor to the antagonists and also to the host .

Biological control can overcome the disadvantage of the using of chemical pesticides. People aware its potential prospect, more and more attentions are paid on this field. Until now, there are some reports on successful control of plant diseases by *Trichoderma* spp., *Pseudomonas* spp., *Streptomyces* spp., *Agrobacterium* spp., *Bacillus* spp. and other antagonists or their antagonistic substances. For example, Peng, Y. F. (17, Personal communication) applied a mutant strain of *Pseudomonas syringae* to control take-all of wheat in the field successfully. The mechanisms of the antagonistic activities are also gradually understood, and some antagonistic substances have been isolated and purified.

Bacteriocins, as an important type of antagonistic substances produced by bacteria, are

non-multiple proteins with self-immunity. It was first discovered by Gratia in 1925, and named as colicin in 1946. Jacob took the name of bacteriocin instead of colicin (21). As more and more works were reported, bacteriocin is no longer the substance which could inhibit only closely-related bacterial strains, it usually has wide antagonistic spectrum. Up to now, more than 200 isolates of bacteria belonging to more than 30 bacterial genus were known to be bacteriocin-producers, and some of them have been applied in control of plant diseases.

Kerr (9) first reported the successful application of *Agrobacterium* radiobacter K84 to control peach canker in Australia, following succeeded in the United States and Greece. A bacteriocin, Agricin K84 , was purified from strain K84. Later, Imler, J. K. also isolated a bacteriocin, Syringacin 4-A, from *Pseudomonas syringae* pv. *syringae* 4-A. Syringacin 4-A could protect soybean from the infection of *Pseudomonas phaseolicola* and increase the germination of soybean seeds (30). Smidt *et al.* (25,26) inoculated a bacteriocin-producing avirulent strain of *P. syringae* pv. syringae W-1 on the hypocotyl of bean seedling. After 7 days, a bacteriocin was isolated and purified from the inoculated seedling. The seedling showed resistance to the plant bacterial pathogens. Chen *et al.* (1) reported that a bacteriocin-producing avirulent strain of *P. solanacearum* could efficiently control tobacco bacterial wilt.

Some mechanisms of bacteriocin activities have been studied. Konisky (11) found that bacteriocin combined with the receptors on the surface of sensitive bacteria and stimulated the specific transportation system on the membrane of protoplasm, as a result, it inhibited various physiological activity and the cell became unstable. Hardy (6) and Holland (7) reported that colicin increased the permeability of cell membrane, made the K^+ move outside of the cell, decreased the ATP concentrate, inhibited the movement of amino acids, saccharides and killed the cells. Obviously, different mechanisms are involved in the bacteriocin activities. Many bacteriocins, which produced by the genus of *Escherichia, Bacillus, Agrobacterium, Streptococcus, Clostridium, Rhizobium, Klebsiella, Erwinia* and etc., have been known as plasmid-encoded. Pugsley (18) identified the bacteriocin-related region in plasmid colN and cloned it into pBR322. Michael *et al.* (13) have transformed the genes of megacin A-216 and A-192134 into *Bacillus megaterium* VT-1660. San Millen *et al.* (22) reported that four regions on the plasmid pMCC B17 were related to the production of microcin 87.

Bacillus spp. are not important plant pathogens but potential biocontrol agents. With the development of study on biocontrol, more and more people become interested in the study on biocontrol by *Bacillus* spp. In many reports, *Bacillus* spp. were directly applied to control various diseases of different crops in different areas, especially to control the plant diseases caused by soil-born fungi. Some works involved in the study on antagonistic substances. Goodman found *B. cereus* could produce bacteriocin which inhibited plant pathogen. Guldner (5) purified the antagonistic substance "Iturins" from *B. subtilis* which could inhibited *Monilinia fructicola*, the pathogen of peach fruit brown rot. Pusley *et al.* (19,20) reported *B. subtilis* could control the above disease in post-harvest storage stage, and inhibit the root rot of soybean and *Rhizoctonia solani* as well.

Turner and Backman (28) studied the antagonistic mechanism of *Bacillus* spp. to fungi. They found that when the peanut seeds were treated with *B. subtilis*, the bacteria could colonize on the roots of peanut, and increased resistance of peanut to stress, improved the germination of seeds and the peanut's nutrition, and finally decrease the root rot caused by *R. solani*. Choe (2) reported that Phytophthora blight of green pepper was suppressed by *Bacillus* spp., and which could retained their activity at least 1 month after application. Ferreira *et al.* (4) reported that the extract of *Bacillus* spp. could inhibit the mycelial growth and ascospore germination of *Eutypa lata*. Tschen (27) reported *B. subtilis* F-29-3 could inhibit the growth of *R. solani*. The extract of F-29-3 culture contained bacilycia and fengycin, and could limit the expansion of local lesion, moreover it is stable in soil. Ni *et al.* (15) isolated *B. subtilis* PB-113 from papaya fruits. It inhibited *Colletotrichum gloeosporioides* in plate test. Strain PB113 produced a glycopeptides antibiotic at late growth phase, the maximum UV absorption was at 277 nm, and it was heat stable. Strain PB113 was directly used to control Citrus *Penicillium digitatum*. Mizubuti *et al.* (14) isolated five strains of *B. subtilis* inhibiting *Uromyces appendiculates* and 26 strains of *B. subtilis* inhibiting *Pyricularia oryzae*. Schmiedknecht *et al.* (24) applied *Bacillus* spp. to control stem canker and black scurf of potato (*R. solani*) in greenhouse and in field, which could decrease the disease incidence by 45~76%, 61~93%, respectively.

B. subtilis NCIMB could inhibit *Xanthomonas campestris* pv. *phaseoli*. An active substance was purified from the bacterial culture after precipitation using acid and butanol. The extract could also inhibit *Botrytis cinerea* (8). Most works on plant disease control by *Bacillus* spp. were taken in plate test or pot test, and some in field, all of which showed different degrees of control efficiency. Following plant pathogens have been tested to be inhibited by *Bacillus* spp. in various studies: *Fusarium oxysporium* f.sp. *ciceris* (cicer wilt), *F. oxysporium* f.sp. *lycopersici* (tomato wilt), *F. solani* (sunflower, soybean, Vinga, abelmoschus, carnation), *Rhizoctonia solani* (peanut, chrysanthemum, potato, bean, pea, rice, rape-seed), *R. betaticola* (sugar-beet), *Phytophthora capsici* (pepper), *Pyrenopora tritici* (wheat), *Macrophomina phaseolina* (soybean, Vinga radiota), *Colletotrichum gloeosporioides* (*Styloosanthes quianensis*, papaya), *C.corchrium* (jute), *Alternaria solani* (tomato), *Eutypa lata* (grapevine), *Sclerotium rolfsii, Claviceps fusiformis, Uromyces appendioulatus* (bean rust), *Pyricularia oryzae* (rice), *Botrytis cinerea* (apple), *Erwinia carotovora, Streptomyces scabies* (potato), *Xanthomonas campestris* pv. *phaseoli* (bean), *Pseudomonas parasitica* var. *nicotianae* (tobacco), *Dothiorella gregaria*, and even to nematodes-*Meloidogyne incognita*.

The Study of Antagonistic Proteins of Bacteria to
Xanthomonas oryzae pv. *oryzae*

Rice bacterial leaf blight is a severe disease in the rice planting area. The bacteria invade plants through rice leaf water pore or wound after flooding or heavy raining. There is still no effective method to control it. The studies on plant disease biocontrol by using antagonistic bacteria or antagonistic substances of bacteria have been great progressed since 1972.

As mentioned in part I, the development of molecular biology and the success in transgenic plant bring a new approach for crop improvement. The combination of traditional biocontrol with genetic engineering may provide a new way for diseases management.

The program of "the study of antagonistic proteins of bacteria to *X. oryzae* pv. *oryzae*" has been started in our lab (Biotechnology Institute, ZAU) since 1986. Attempts were focused as follows:

1. Screening antagonistic bacterial strains from rhizosphere, phyllosphere, atmosphere and etc., and studying their potential use in control of rice bacterial leaf blight (*X. oryzae* pv. *oryzae*) and other rice diseases, such as rice blast, rice sheath blight, and etc..

2. Purification and analysis of some antagonistic proteins: Isolating the antagonistic proteins produced by bacteria with chromosome coding or plasmid coding.

3. Characterization and cloning of antagonistic protein genes.

4. Transforming the antagonistic protein genes into rice plant to get disease-resistant one, or into the dominant bacteria colonizing on the rice leaf to obtain genetic engineered bacteria and use them in the practical control.

Screening the antagonistic bacterial strains. More than 800 bacterial isolates were collected from atmosphere, phyllosphere and rhizosphere of rice and other plants. Their antagonistic activity to *X. oryzae* pv. *oryzae* and other plant bacteria as well as fungal pathogens were tested.

One hundred and thirty bacterial strains were screened out, and most of them belonged to *Bacillus* spp. They could inhibit the growth of *X. oryzae* pv. *oryzae* and other pathogens (Fig. 1). Table 1 showed the antagonistic effects of strain B8, B826, P11, G35 on ten tested indicator strains of *X. oryzae* pv. *oryzae* in plate test .

Most of the isolated antagonistic bacteria could also inhibit the growth of several other pathogens, such as *X. campestris* pv. *campestris*, *X. oryzae* pv. *oryzicola*, *Pseudomonas solanacearum*, *Erwinia carotovora* subsp. *carotovora*, *Rhizoctonia solani*, *Pyricularia oryzae*, and etc..

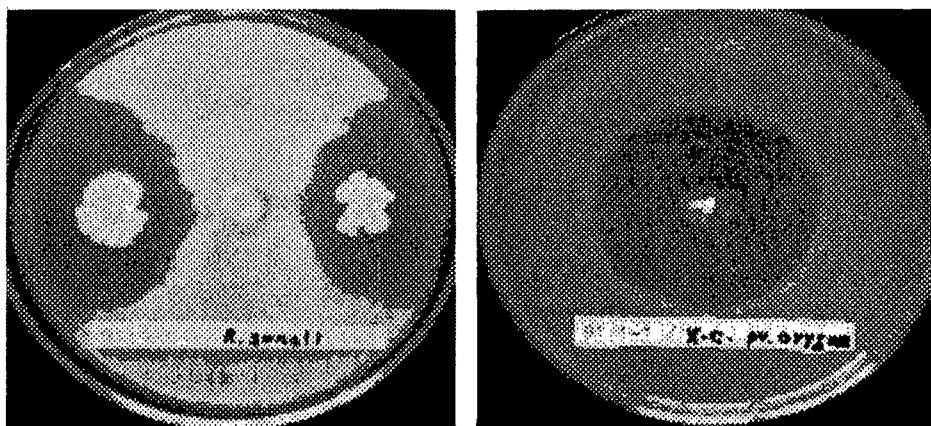Six of them, identified as *Bacillus subtilis* B826, P11, *B. cereus* G35, *Bacillus* sp. A30, B034

Fig. 1. The inhibiting zone of *Bacillus subtilis* to plant pathogens in plate test.

Table 1. The antagonistic effect of bacteria to different strains of *X. oryzae* pv. *oryzae**

	COX1	COX2	COX3	COX4	COX5	COX6	COX26	COX61	COX17	COX18
B8	3.4	4.0	3.0	4.0	3.2	3.7	3.5	3.4	3.5	3.4
B826	3.0	3.5	3.0	3.5	2.9	3.0	3.0	2.8	2.5	3.0
P11	2.8	2.5	3.0	3.0	2.8	2.8	3.0	2.7	2.4	2.7
G35	1.3	1.5	1.5	2.2	1.9	1.5	2.2	1.4	1.0	1.2

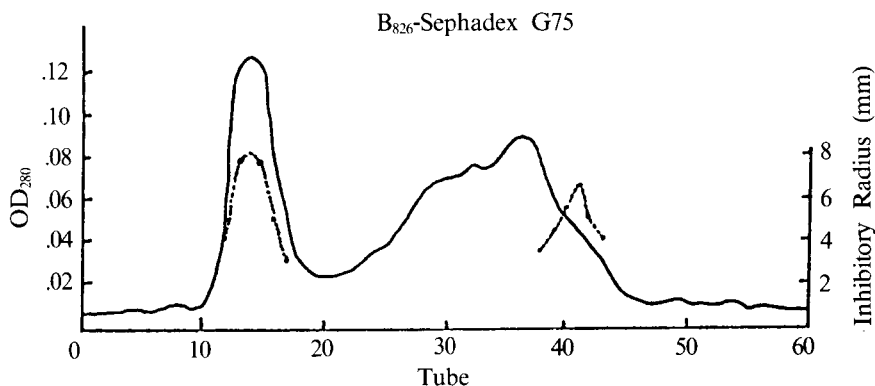*Numbers in the table were the radius zone (cm) of inhibition.

Fig. 2. Elution profile of B826 extraction on Sephadex G75. (—— OD_{280}, ----- Antagonistic activity peak to *X. oryzae* pv. *oryzae*).

and *Enterobacter cloacae* B8, were selected for the further study because of their strong inhibiting activities and wide antagonistic spectrum. For example, strain A30 could inhibit *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, *X. campestris* pv. *campestris*, *Pseudomonas solanacearum*, *Erwnia carotovora* pv. *cartovora*, *Rhizoctonia solani* (rice and cotton), *Fusarium oxysporum*, *F. vasinfectum*, *Pyricularia oryzae*, and etc..

Analysis of antagonistic proteins. It was found that there were different kinds of antagonistic substances in the antagonistic bacteria. Some bacterial strains could even produce different antagonistic substances which could inhibit the growth of *Xanthomonas* spp., *Pseudomonas solanacearum* (bacteria), *Pyricularia oryzae*, *Rhizoctonia solani* (fungi) and etc., respectively. For example, B826 could produce two kinds of antagonistic proteins, both of them inhibited *X. oryzae* pv. *oryzae* (Fig. 2~4). Moreover, A30, P11 and other strains could produce more than two kinds of proteins against *R. solani*, respectively (Table 2). Besides the proteins, other antagonistic compounds were also found in many strains. For example, strain H31 could produce four antagonistic substances, two of them were non-protein substances.

Several antagonistic proteins (peptides) were purified with ion-exchange column chromatography, gel filtration chromatography and HPLC. The purified antagonistic proteins showed very strong activities, 1~2 µg purified proteins could inhibited *X. oryzae* pv. *oryzae* very strongly in plate test. All these purified proteins showed a single band on SDS-PAGE, which suggested that each protein was composed of one homogeneous subunit (Table 2).
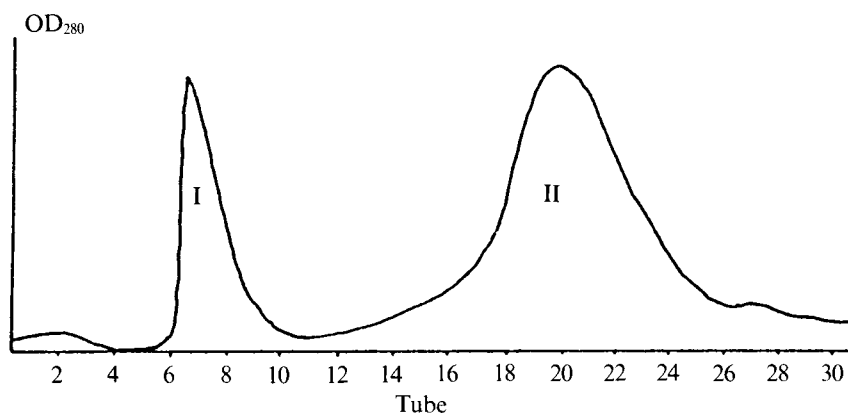
Fig. 3. Profile of the crude antagonitic proteins extract (after precipiated with ammonium sulfate) produced by B826 strain on Sephadex G75. (Mobile phase : 0.02 M phasphate buffer, pH 7.2, Flow rate : 10 ml/30 min. tube, Detector : 280 nm).
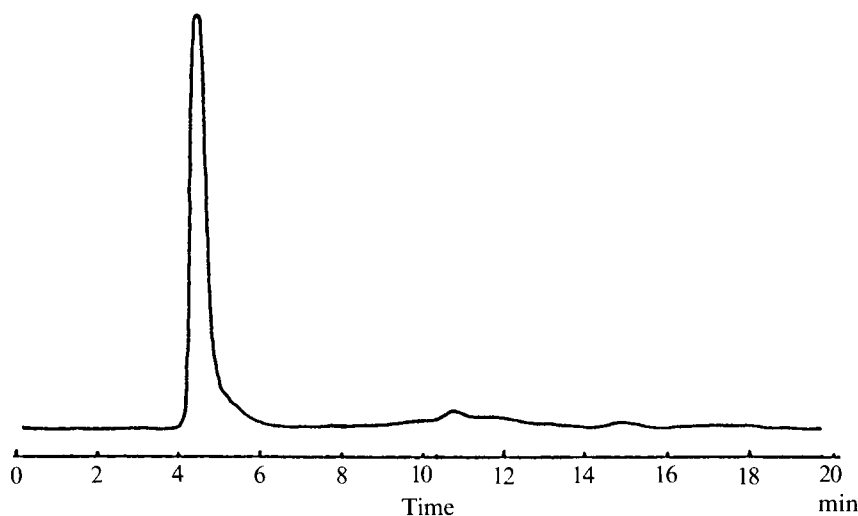

Fig. 4. Purified B826-I by HPLC (Pak-300sw).

Table 3 shows these protein properties of the antagonistic substances. It was proved that these antagonistic proteins didn't have the properties of proteinase and amylase themselves. The amio acid compositions and partial amino acid sequences of some antagonistic proteins (peptides) were shown in Table 4 and 5.

Cloning of the antagonistic genes.

1. DNA Library Construction: Using lambda ZAPII/pBS cloning system, we have constructed four DNA libraries: B826, A30, P11, and B8.

2. Antisera Preparation: Rabbit antisera were prepared against the antagonistic proteins of P11 and A30. The monoclonal antibody (McAb) to B826-I protein had been prepared.

3. Immunological Screening of Specific Recombinant Clones: Using these antisera and McAb, we screened out the positive clones from the DNA library by IPTG inducing.

Table 2. List of the purified proteins (peptides)

Protein	Strain	MW	
P11-I	*Bacillus subtilis* P11	14.0 KD	Cloned
P11-II	*Bacillus subtilis* P11	10.0 KD	
B8	*Enterobacter cloacae* B8	5.0 KD	
B826-I	*Bacillus subtilis* B826	37.0 KD	Cloned
B826-II	*Bacillus subtilis* B826	8.0 KD	
A30-I	*Bacillus* sp. A30	3.0 KD	
A30-II	*Bacillus* sp. A30	1.3 KD	Sequenced, cloned
G35	*Bacillus cereus* G35	3.5 KD	Sequenced

Table 3. Stability of antagonistic protein to the different treatments

	Proteinase K	Pronase E	Trypsin	60℃ 30 min	80℃ 30 min	100℃ 15 min	Phenol
P11-I	+	+	+	−	−	−	+
P11-II	+	+	+	−	⊤	−	+
B826-II	+	+	+	−	−	−	+
B034	+	+	+	+++	+++	+*	+

+ : Sensitive, − : Stable, +++ : Partial sensitive, * : 20 min.

Table 4. Amino acid composition of P11-I and P11-II

	Asp	Thr	Ser	Glu	Pro	Gly	Val	Ile
P11-I	11.9	4.76	7.14	9.54	4.76	11.9	7.14	4.76
P11-II	8.82	2.94	4.14	4.41	8.82	22.05	2.94	7.35

	Leu	Trp	Arg	Tyr	Phe	Ala	Cye	Lys
P11-I	2.38	9.52	7.14	11.9	7.14	−	−	−
P11-II	8.82	4.41	1.74	−	−	16.18	2.94	4.41

Table 5. Partial amino acid sequences of peptide A30-I and G35

A30　: Met-Tyr-Met-Ile-Lys-trp-met-arg-thr
G35* : Fragment 1. Tyr-Trp-Ala-Asn-Lys
　　　　Fragment 2. Ile-Leu-Gly-Trp-Ile-Ser-Tyr-Ser-Asn-Lys
　　　　Fragment 3. Ser-Ile-Val-His-Pro-Arg

*Cooperated with Dr. Z. L. Chen, Beijing Univ.

We have gotten: Twelve positive clones expressing B826-I protein: Among 12 positive recombinant from B826 strain, clone 9 which contained a 2.1 kb insertion fragment showed higher antagonistic activity against *X. oryzae* pv. *oryzae*, and the antagonistic protein expressed by clone 9 was identified by western blot.

Three positive clones expressing A30 protein: All 3 positive recombinants from strain A30 had antagonistic activity when induced by IPTG. But two positive clones of P11 showed no antagonistic activity to *X. oryzae*.

4. Analysis of the Clones Inserts: The insert DNA of B826 was about 2.1 kb. The insert DNA of A30 was about 0.5 kb.

5. Sequencing of the Genes: By using subcloning strategy, an about 1 kb inserted subclone from 2.1 kb positive clone, which also showed antagonistic activity, was obtained and sequenced. The inserted fragment is 905 bp, in which there were two open reading frames which could code two proteins with 95 and 54 amino acids, respectively. Further work is to be carried out on the functions of the proteins and genes.

From the partial amino acid sequence of the antagonistic protein of A30-II, we synthesized a 25-mer degenerate primer, and got a DNA fragment about 0.5 Kb by polymerase chain reaction of A30. The fragment was sequenced and showed that it coded only 16 amino acids. The sequence is as following:

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···TGCAGGAATTCGAT ATG TAT ATG ATT GAT TGG TGG ATG AGG GAT ACG CAG
                  Met Tyr Met Ile Asp Trp Trp Met Arg Asp Thr Gln
   A30 aa seq.     Met Tyr Met Ile Asp Trp Trp Met Arg Asp···
AGC CTT GCA GGA TAAGATGATCCTTGTACACTAA AACGTGATGTGATTTTTTTCG
Ser Leu Ala Gly
TTTTTTAAAATTAAGATGTAACATAGAGGA···
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The 16AA protein from the sequence is conformed to the M.W. of the purified active protein (A30-II).

6. Study on the Plasmid Coding Antagonistic Protein: We also studied the bacteria with antagonistic protein coded by plasmid, and found that 18 from 86 antagonistic bacterial strains harbored plasmids. Among them, *Bacillus* sp. B034 had a 8 kb plasmid and had very strong antagonistic activity *X. oryzae* pv. *oryzae*. After cured its plasmid with the treatment of acridine orange, it lost antagonistic activity. It is possible that there is a relationship between the plasmid and the antagonistic activity.

The restriction map of the B034 plasmid showed it had 4 *Eco*RI sites. All four *Eco*RI fragments were cloned in the shuttle vector pMK4 respectively. The recombinant DNAs were expressed in *E. coli* XL1-Blue and *B. subtilis* DB104. One recombinant harboring fragment-II (−3 kb) had some antagonistic activity to *X. oryzae* pv. *oryzae*.

Study on the colonization of antagonistic bacteria and their effect on rice bacterial blight in greenhouse. In order to study whether the antagonistic bacteria could be used directly in field, we studied the colonization of strain B8 on rice. First, we obtained a drug resistant mutant of strain B8 (Rif^r and Km^r) by stress selection and Tn5 mutagenesis, which could grow normally in rifampicin and kanamycin and still had strong antagonistic activity. Then the bacterial suspension of the mutant of B8 (about 10^8 cells/ml) was sprayed on rice leaves, the drug resistant bacteria were recovered and count from the rice leaves at different time. It was shown that B8 could colonize on rice leaves and its population becomes stable even 40 days after spraying (Fig. 5). But it was affected strongly by the weather.

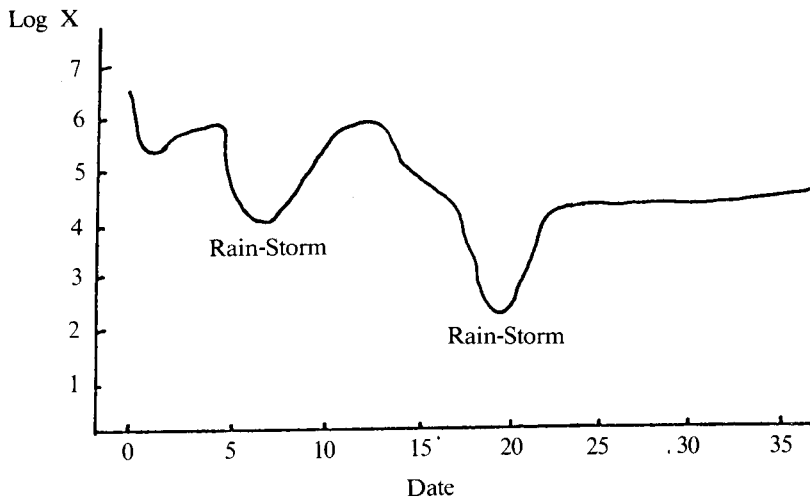The colonization of other antagonistic bacteria on rice leaves were also being studied

Fig. 5. Change of the population of strain B8 on rice leaves. (X: cfu/cm^2 leaf).

in greenhouse. We inoculated rice leaves at the end of tillering stage by clipping methods with spraying suspension of *X. oryzae* pv. *oryzae* ($10^5 \sim 10^6$ cells/ml). The antagonistic bacteria suspension (about 10^8 cells/ml) of strains B8, P11, B826, and A30 or their culture filtrates were sprayed on rice leaves before, while, and after inoculation of *X. oryzae* pv. *oryzae*. Then the lesion length was recorded at different time. All tested strains reduced the lesion by 9~39% (the highest was 50%) of rice bacterial leaf blight compared to the control. The effect of antagonistic bacteria sprayed at the same time with *X. oryzae* pv. *oryzae* was the best and that sprayed before *X. oryzae* pv. *oryzae* inoculation was better than that sprayed after *X. oryzae* pv. *oryzae* inoculation.

Discussion

From the works mentioned above, more than 130 strains of antagonistic bacteria were screened out from rhizosphere, phyllosphere and other sources. Among them, *Bacillus* spp. are always easy to isolate and have strong inhibiting effects with wide antagonistic spectrum to various plant pathogens. It is also indicated that *Bacillus* spp. are very important in the biocontrol. Although most were plate tests, some showed very promising results on pot test in green-house. Further study is going to be undertaken. The potential use of *Bacillus* spp. as a biocontrol agent have a good prospect.

We isolated *Bacillus* spp., which have strong inhibiting effect to various plant pathogens from rice leaf surface, such as B8, A30, B034, etc. The study of bacterial colonization on rice leaves provides the evidence for the direct use of those antagonistic *Bacillus* spp.. After spraying the bacteria, it could colonize on rice leaves, even become the dominat strain showing stronger antagonistic function-decrease the density of pathogen inoculum, and prevent the disease before the invasion of pathogen.

Eight antagonistic proteins (peptides) were purified and analyzed from the antagonistic *Bacillus* spp.. As mentioned above, more than one kinds of antagonistic components with different antagonistic activity to different pathogen are occurred. Evidences showed the complexity of antagonistic mechanism. Anyway, the study on the antagonistic proteins and their relation will be very important to understand the antagonistic mechanism.

After the study of antagonistic genes, two DNA fragments which involved in the encoding of antagonistic proteins were sequenced. Works are being undertaken to transform these two genes into plants or bacteria which could colonize on plants to prevent the infection of related plant pathogens with strong antagonistic activity. More genes should be studied in detail, especially the relationship and the interaction between the antagonistic genes.

Based on the results mentioned above, followings are the research objects necessary to be studied recently.

1. The direct utilization of antagonistic bacterial strains or genetically engineered bacteria in rice field to control rice bacterial leaf blight.
    1) Studies of the colonization of the antagonistic bacteria continuously.
    2) Plant tests of antagonistic action in greenhouses and in the field.
    3) Bacterial ecology of rhizosphere and phyllosphere of rice.
2. Protein analysis and a comprehensive study of the relationship between antagonistic genes and their products. In our research, we studied only a few genes. It is necessary to study the complexity of antagonistic bacteria.
    1) Purification and measurement of antagonistic proteins.
    2) Amino acid sequences of antagonistic proteins.
    3) Mutagenesis and a comprehensive study of antagonistic proteins and the relationship of them.
3. Antagonistic protein gene cloning
    1) Genomic library construction and DNA cloning.
    2) Subtractive library and transposon tagging.
    3) DNA sequencing
    4) Isolation of plasmids coding antagonistic proteins.
4. Mechanism of antagonism
    1) Signal transport of antagonistic proteins.
    2) Interaction between the antagonistic bacteria and the pathogens.
5. Construction of transgenic plants or bacteria to control the plant diseases. The construction of expressing vectors for the introduction of these genes into rice plant and bacteria
    1) Transgenic rice plants.
    2) Transgenic bacteria which can colonize on the surface of rice with higher antagonistic activity.
6. Screening more antagonistic bacterial strains especially with wide inhibiting spectrum and plasmid-coding antagonistic proteins. There are plenty of different antagonistic bac-

teria in nature which can be isolated for construction of transgenic plants and bacteria and for using as biocontrol agents directly in the field.

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