Chapter VII-4

Evaluation of microbial agents against rice pests

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1 Introduction

Rice is unique among the world's major food crops by virtue of the extent and variety of its uses and adaptability to a broad range of climatic, edaphic, and cultural conditions (Mikkelsen and Datta, 1991). It provides 20% of the per capita energy and 15% of the per capita protein for humans worldwide. Annual world rice production is approximately 460 million tons grown on more than 145 million ha. Over 90% of this area lies in Asia, while the remainder is divided among Latin America, Africa, Australia, Europe, and the USA (see Way and Bowling, 1991). Rice production should be increased to supply a rapidly expanding population; however, it has been hindered by a number of diseases and insect pests. Moreover, rapid changes in rice production technologies have created greater frequencies of pest epidemics (Reissig *et al.*, 1986).

More than 70 species of insect pests are known to feed on rice, and at least 20 of them can seriously affect rice production. They attack all parts of the rice plant at all growth stages and some serve as vectors of viruses that adversely affect the plant (Mikkelsen and Datta, 1991). Control of insect pests has primarily depended on the application of chemical insecticides, but because of the many disadvantages of chemicals, alternative biological control approaches are needed. In the rice agroecosystems, many types of entomopathogens may suppress insect pests. In this chapter, we detail techniques on the use and evaluation of entomopathogens for the potential control of a few key insect pests (*i.e.*, the brown planthopper, the white-backed planthopper and the rice water weevil) of rice.

2 Major insect pests of rice

A number of insect pests that attack rice plants account for yield losses of 24% worldwide. Some of the major ones are listed in Table 1. Below, we provide information on a few key pests.

A Homoptera (brown planthopper and white-backed planthopper)

The brown planthopper (BPH), *Nilaparvata lugens*, and white-backed planthopper (WBPH), *Sogatella furcifera*, are serious pests in many rice-growing regions of the Far East

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(Choo *et al.*, 1989). They occur in tropical to temperate areas and can cause extensive damage by feeding on rice plants or serving as vectors for grassy or rugged stunt virus of rice. Both sexes of BPH adults have short-winged (brachypterous) and long-winged (macropterous) types, whereas WBPH adult females have both short-winged and long-winged types, but all males are the long-winged type. The short-winged types cannot fly and remain in the field to feed and reproduce, whereas the long-winged types can disperse, mate, and reproduce (*i.e.*, BPH). A macropterous BPH female lays about 100 individual eggs and a brachypterous female lays about 300 eggs in the tillers using its saw-like ovipositor (Reissig *et al.*, 1986). A WBPH female lays from 300–500 eggs. Nymphs and adults suck sap from the base of plants, just above the waterline. In heavy infestations, these planthoppers can cause hopperburn resulting in browning and wilting of some or all tillers in a hill.

In the tropics, the planthoppers have multiple generations per year. In temperate regions, they have 2 or 3 generations per year. They cannot overwinter in the temperate regions, and reinfestion of rice occurs each summer when tropical storms assist their dispersal northward. Currently, chemical insecticides and host plant resistance are the major control tactics for BPH and WBPH.

B Lepidoptera (rice stem borer)

Although many lepidopterous species are associated with rice (Table 1), the rice stem borer, *Chilo suppressalis*, has been one of the most important pests, especially in Asia. However, the release of the short-stature, high-yielding rice varieties, planting of early maturing varieties, improved cultural practices including destruction of post-harvest stubble, and the use of synthetic insecticides have reduced the stem borers from a key to a secondary pest (Way and

Table 1. Some major insect pests of rice occurring in the USA and Asia

Insect species	Plant part attacked	Region	References
Homoptera			
*Nephotettix cincticeps**	Leaf and stem	Asia	Reissig *et al.* (1986)
*Nephotettix virescens**	Leaf and stem	Asia	Reissig *et al.* (1986)
*Laodelphax striatelius**	Stem	Asia	Reissig *et al.* (1986)
*Macrosteles fascifrons**	Stem	USA	Elliott *et al.* (1994)
*Nilaparvata lugens**	Stem	Asia	Reissig *et al.* (1986)
Sogatella furcifera	Stem	Asia	Reissig *et al.* (1986)
Coleoptera			
Oulema oryzae	Leaf	Asia	Reissig *et al.* (1986)
Lissorhoptrus oryzophilus	Root	Asia, USA	Reissig *et al.* (1986)
Diptera			
Chironomus spp.	Grain	USA	Elliott *et al.* (1994)
Cricotopus spp.	Grain	USA	Elliott *et al.* (1994)
Tanytarsus spp.	Grain	USA	Elliott *et al.* (1994)
Hydrellia griseola	Leaf	Asia	Reissig *et al.* (1986)
		USA	Elliott *et al.* (1994)
Pseudonapomyza asiatica	Leaf	Asia	Reissig *et al.* (1986)
Atherigona exigua	Tiller	Asia	Reissig *et al.* (1986)
Atherigona oryzae	Tiller	Asia	Reissig *et al.* (1986)
Lepidoptera			
Mythimna separata	Leaf and stem	Asia	Reissig *et al.* (1986)
Pseudaletia unipuncta	Leaf and stem	USA	Elliott *et al.* (1994)
Sesamia inferens	Stem	Asia	Reissig *et al.* (1986)
Spodoptera frugiperda	Stem	USA	Elliott *et al.* (1994)
Chilo suppressalis	Stem	Asia	Reissig *et al.* (1986)
Cnaphalocrocis medinalis	Leaf	Asia	Reissig *et al.* (1986)
Scirpophaga incertulas	Stem	Asia	Reissig *et al.* (1986)

* Virus vector

Bowling, 1991). Yet, it can still cause significant damage by reducing tiller numbers even on resistant varieties and, in spite of the successes with chemical insecticides, remain difficult to control because of its plant-boring habit (Reissig *et al.*, 1986).

The adults are nocturnal with a female capable of laying 200–300 eggs in masses near the base of rice leaves or leaf sheaths. The larvae penetrate tillers and feed on the inner surface of the stem walls, interrupting the movement of water and nutrients. The central leaves of damaged tillers of young plants turn brown (called dead hearts). If the damage occurs after spikelets form, panicles turn white (called whiteheads) and no grain filling occurs (Reissig *et al.*, 1986). The plant often dies and the larva moves to another stem. Therefore, one larva is capable of damaging several stems. There are 1 to 6 generations a year depending on the cultivation region. For example, *C. suppressalis* generally has two generations a year in temperate regions with the second generation causing more serious damage than the first generation.

C Coleoptera (rice water weevil)

The rice water weevil, *Lissorhoptrus oryzophilus*, is the most important and ubiquitous insect pest of rice in the USA (Bunyarat *et al.*, 1977; Bowling, 1980). Although Kuschel (1951) lists 6 species of *Lissorhoptrus* in the USA, *L. oryzophilus* is the major pest of rice in the southern states expanding to other rice growing regions of the world. It was found in California in 1959 (Lange and Grigarick, 1959) and was accidentally introduced to Japan in 1976 and has spread to other Asian rice-producing countries including Korea and China. The species is bisexual in the southern states but only parthenogenetic females occur in California and Asia.

The adults overwinter in sheltered areas in debris or leaf litter of forests, at the bases of perennial types of grasses, or on elevated areas such as irrigation levees. The overwintering adults may feed on aquatic grasses and sedges following emergence. They fly to the rice fields between April and June. The number of accumulated day degrees needed for muscle development in relation to peak departure flights from overwintering sites has been determined in Arkansas and Louisiana (Morgan *et al.*, 1984). Muscle degeneration occurs after landing in flooded fields of the southern states and California. Oviposition soon follows and may continue for several weeks. Adults feed on the rice leaves during this period, which produces characteristic linear slits on the dorsal surface. The earlier infestation in flooded rice paddies is generally more serious.

Most eggs are inserted individually in the submerged sheath tissue near to and above the crown (Grigarick and Beards, 1965). After hatching in 6–9 days, the legless white larvae mine the stem for a short time (about 1 day) and then move to and complete 4 instars of development in or on the roots. The larva obtains oxygen by tapping roots with its dorsal abdominal tracheal hooks (Way and Bowling, 1991). Pupation occurs in a waterproof, round cell that has an outside coating of mud and is attached to the root. Adults begin emerging from these cells in early to mid-July in California and continue to do so through the rice growing season depending on such factors as the oviposition period, temperature and possibly interrupted feeding on the rice roots. A small proportion of the emerging generation produce eggs in California (Grigarick and Beards, 1965) and Arkansas (Muda *et al.*, 1981), but the economic significance of this "partial" second generation (Tucker, 1912; Webb, 1914) is not known. Under optimal conditions, 4 generations of the rice water weevil can develop in southern Louisiana; however a 2nd, and perhaps a "partial" 3rd generation occurs more frequently. The "last" generation of emerging adults feed, build up fat reserves and fly to overwintering sites in late July and August (Knabke, 1970; Muda *et al.*, 1981).

Extensive adult feeding on the leaves can injure very small seedlings, but root pruning by the larvae causes the major problems. Plants with damaged roots may become stunted and produce less grain because of reduction of the number of tillers and panicles or cause grain maturity to be uneven at the time of harvest. The earlier the root pruning is to the plant growth stages, the greater is the level of injury for a given number of larvae. Distribution of adult activity and resultant injury in Arkansas and California are greater along the levees and field margins (Socksai and

Tugwell, 1978; Lange and Grigarick, 1959) and, in general, are greater in the sections of fields associated with open water. The use of chemical insecticides is the main control tactic for this insect.

3 Entomopathogens of major rice pests

The insect pests are attacked naturally by a number of microbial control agents such as nematodes, fungi, bacteria, and viruses (Table 2). These entomopathogens can reduce pest populations but often they do not occur at a high enough frequency to suppress the population. By understanding the biology and ecology of these entomopathogens, we may be able to use them effectively in the integrated pest management of rice through augmentation or inundative releases.

A Mermithid parasite of planthoppers

More than 200 natural enemies (parasitoids, predators, and entomopathogens) have been recorded from BPH (see Benrey and Lamp, 1994), and most of them have an overlapping host range with WBPH. Although BPH and WBPH have an impressive list of natural enemies, a mermithid nematode parasite, *Agamermis unka*, is the most important and common natural enemy in temperate regions. This unique parasite is widely distributed and commonly found in rice paddies at high densities in the southern part of Korean (Cho *et al.*, 2002). Parasitism is generally high. Over 50% of BPHs (Choo *et al.*, 1989; Choo and Kaya, 1990 1993, 1994; Choo *et al.*, 1995) and WBPH (Choo and Kaya, 1990) were parasitized by *A. unka* in Korea. Esaki and Hashimoto (1931) found that > 40% of BPH and > 70% of WBPH populations were parasitized by *Agamermis* in southern Japan. In Hunan Province, China, no control measures are needed when the population density of BPH is under 2000 insects on 100 rice plants, provided that natural infection rate is above 75% (Wang and Li, 1987). *Agamermis* parasitism castrates the reproductive organs of BPH and WBPH (Choo and Kaya 1990). Parasitism of the host usually occurs at the lower part of the rice stem where most planthoppers are found (Choo and Kaya, 1993).

The newly-hatched second stage mermithid is the infective stage (pre-parasite). Once the mermithid contacts the planthopper nymph, it uses its stylet to penetrate through the cuticle into the host hemocoel to initiate the parasitic phase. The 3rd and 4th stage juveniles occur in the hemocoel. Two to three weeks after parasitization, the 4th stage juvenile (postparasite) exits its adult host by boring through the thin intersegmental area of the abdominal segment, always causing death of the host. After emergence, the postparasite burrows into the soil, molts, and overwinters as an adult (Choo and Kaya, 1993).

Agamermis can be redistributed by artificial releases. When the pre-parasites of *A. unka* were released at a mermithid to BPH ratio of 10:1, parasitism of BPHs ranged from 33 to 63% (Choo *et al.*, 1995). Because egg production and hatchability of *Agamermis* are high, inoculative releases into areas where the mermithid population is low or nonexistent appear feasible.

B Entomopathogenic fungi from planthoppers

A number of entomopathogenic fungi have been isolated from BPH and WBPH (Table 2). In addition, other fungi such as *Beauveria brongniartii* and *Hirsutella saussurei* have been isolated from other planthopper and leafhopper species. Interestingly, several hoppers in the rice ecosystem are infected with *Nomuraea rileyi*, a common fungus of lepidopterous larvae (Li, 1985). Some of these pathogens have been evaluated for the control of planthoppers (Aguda and Litsinger, 1984; Aguda *et al.*, 1987). BPH and WBPH are susceptible to *B. bassiana*, but some isolates are more efficacious than others (Aguda and Litsinger, 1984). *B. bassiana* and *Metarhizium anisopliae* appear to be the most useful against planthoppers because of their ease of mass production, storage, virulence, and ease of application.

C Entomopathogens from lepidopterous species

Nematodes and entomopathogenic fungi, bacteria, and viruses have been isolated from lepidopterous pests (Table 2). *Bacillus thuringiensis* can be used against various lepidopterous pests. In addition, several transgenic rice plants have been developed that

Table 2. Some entomopathogens isolated from or tested against insect pests of rice

Insect species	Entomopathogens	Region	References
Homoptera			
Nephotettix cincticeps	Fungi		
	Entomophthora spp.	Asia	Reissig *et al.* (1986); Rombach *et al.* (1987b)
	Beauveria bassiana	Asia	Reissig *et al.* (1986)
	Nematode		
	Agamermis unka	China	Zhao *et al.* (1987)
Nephotettix virescens	Fungi		
	Entomophthora spp.	Asia	Reissig *et al.* (1986); Rombach *et al.* (1987b)
	B. bassiana	Taiwan	Chu and Hirashima (1981)
	Conidiobolus coronatus	Philippines	Rombach *et al.* (1987b)
	Metarhizium album	Philippines	Rombach *et al.* (1987a)
	Paecilomyces farinosus	Thailand	Rombach *et al.* (1987b)
	Nematode		
	Hexamermis sp.	Asia	Reissig *et al.* (1986)
Nilaparvata lugens	Fungi		
	Erynia delphacis	Japan	Rombach *et al.* (1987b)
	B. bassiana	Asia	Chu and Hirashima (1981); Rombach *et al.* (1987b); Li (1985); Rombach *et al.* (1987a)
	B. brongniartii	Asia	Reissig *et al.* (1986)
	Cephalosporium sp.	India	Rombach *et al.* (1987b)
	C. coronatus	Japan, Philippines	Rombach *et al.* (1987b)
	Entomophthora spp.	Asia	Rombach *et al.* (1987b)
	Metarhizium anisopliae	Asia	Reissig *et al.* (1986)
	M. flaroviride	Philippines	Rombach *et al.* (1986b)
	Paecilomyces farinosus	Japan	Rombach *et al.* (1987b)
	Nematode		
	A. unka	Japan	Kaburaki and Imamura (1932); Esaki and Hashimoto (1931)
		Korea	Choo *et al.* (1989, 1995); Choo (1991)
		China	Yan *et al.* (1986); Zhao *et al.* (1987)
	Hexamermis sp.	India	Manjunath (1978)
	Mermis sp.	India	Manjunath (1978)
Sogatella furcifera	Fungi		
	B. bassiana	China	Li (1985)
	B. brongniartii	Asia	Reissig *et al.* (1986)
	Entomophthora sp.	Fiji island	Rombach *et al.* (1987b)
	Hirsutella citriformis	Rep. Solomon Islands	Rombach *et al.* (1987b)
	Nematode		
	A. unka	Korea	Choo and Kaya (1990)
		Japan	Esaki and Hashimoto (1931); Kaburaki and Imamura (1932)
		China	Zhao *et al.* (1987)

(Continued)

Table 2. (Continued)

Insect species	Entomopathogens	Region	References
Coleoptera			
Lissorhoptrus oryzophilus	Fungi		
	B. bassiana	USA	Rice *et al.* (1994) Urtz and Rice (1997)
Lepidoptera			
Scirpophaga incertulas	Fungus		
	B. bassiana	Asia	Reissig *et al.* (1986)
	Nematode		
	Hexamermis cathetospicula	Malaysia	Poinar and Chang (1985)
Chilo suppressalis	Fungi		
	B. bassiana	Asia	Reissig *et al.* (1986)
	M. anisopliae	Philippines	Rombach *et al.* (1987b)
	Paecilomyces farinosus	Japan	Rombach *et al.* (1987b)
	Nematode		
	Amphimermis zuimushi	Japan	Kaburaki and Imamura (1932)
	Virus		
	Chilo iridovirus	Japan	Rombach *et al.* (1987b)
Cnaphalocrocis medinalis	Fungus		
	B. bassiana	India China	Rombach *et al.* (1987b) Li (1985)
	Virus		
	Granulovirus	India	Rombach *et al.* (1987b)

express *B. thuringiensis* toxin genes for insect control. These include *B. thuringiensis cryIA(b)* and *cryIA(c)* genes conferring resistance to the yellow stem borer (Nayak *et al.*, 1997, Cheng *et al.*, 1998).

D Entomopathogens of the rice water weevil

The role of natural entomopathogenic enemies in the suppression of the rice water weevil has not been determined (Reissig *et al.*, 1986). However, an unidentified mermithid nematode was isolated from the larvae (Bunyarat *et al.*, 1977). Weevils collected in mid-June had the highest parasitization rate (26%), with peak nematode emergence occurring during the last week in June. The mermithid decreased egg production of weevils. In a laboratory study, the entomopathogenic nematode, *Steinernema carpocapsae* Mexican strain, was applied against the weevil, but this nematode did not reduce weevil population on the roots even at 120,000 infective juveniles/liter/pot (Nagata, 1987).

Recently, the potential role that entomopathogenic bacteria and fungi may play in controlling the rice water weevil has been investigated. Both *B. thuringiensis* and *B. bassiana* isolates found in the rice ecosystem or obtained from other sources were pathogenic to this weevil.

A systematic survey of suspected overwintering rice water weevil sites produced 17 potential *B. bassiana* isolates from diapausing and nondiapausing weevils (Rice *et al.*, 1994, Urtz and Rice, 1997). The methodologies developed during this survey are useful for isolating various fungal pathogens. This involves breaking the diapause of overwintering adult rice water weevil by submerging leaf litter in 36 °C water for a minimum of 4 h to a maximum of 18 h.

Protocol for isolation of fungi pathogenic for rice water weevil.

1. Collect 4 ($1\,m^2$) samples of leaf litter by raking until the soil is exposed.
2. Transfer the leaf litter and debris to individual 151 liter rubber garbage cans,
3. Add tap water at 36 °C until the litter is submerged. Place on the top of the leaf litter an aluminum trash can lid fitted with a wire screen (40 cm diameter with 1 cm space between wires) into a hole cut in the center of the lid to keep the litter submerged. Place an inverted funnel on the trash can lid over the screen so that the dormant rice water weevil adults float on the surface of the water.
4. Wait 4 h and then collect the viable adult rice water weevil that climb to the top of the funnel. Adult rice water weevil may be collected for up to 18 h.
5. Incubate adult weevils in glass jars (30–50/liter jar) at 25 °C containing paper tissue saturated with sterile H_2O, and periodically (2–3 day intervals) examine the jar for cadavers.
6. Remove the cadavers, suspend them individually in a test tube in 0.1% Tween 80, vortex vigorously, and plate 0.1 ml on an oatmeal-dodine-chlorotetracycline agar (ODTA), a medium selective for *Beauveria* species (Chase *et al.*, 1986).
7. Incubate ODTA plates at 25 °C until distinct colonies appear that are 2–5 mm in diameter.
8. Transfer subsamples of individual colonies to Sabouraud dextrose agar (SDA) plates containing 0.2% yeast extract.
9. Identify *B. bassiana* isolates based on colony appearance, the ability to grow on ODTA plates, and conidial morphology (Mugnai *et al.*, 1989). Koch's postulate needs to be conducted to verify the pathogenicity of the fungal isolates to the rice water weevil.

Other cadavers can be dissected and examined for other potential pathogens such as microsporidia or bacteria. An individual experienced with microsporidian spores can identify them with a compound microscope at 400 X. The spores can be partially purified by centrifugation and used to confirm their pathogenicity using Koch's postulates. The bacterial populations are more difficult to assess and will require isolation of individual colonies on bacteriological media selective for *B. thuringiensis*. Several types of media used in selective isolation strategies for *B. thuringiensis* are acetate (inhibiting the germination of *B. thuringiensis* spores) in conjunction with heat (Travers *et al.*, 1987), and antibiotic selection (Delucca *et al.*, 1981) in conjunction with media selective for *B. cereus* (Sekar *et al.*, 1997). Unless the individual is qualified, this isolation may be best done in conjunction with a bacteriologist.

In addition to isolating pathogens from rice water weevils, pathogens that are being developed for microbial control of other insects may be used for rice water weevil control. For example, a novel *B. thuringiensis* vegetative insecticidal protein, vip3A(a), with a wide spectrum of activities has been described (Estruch *et al.*, 1996). Vip3A(a) and its homologue vip3A(b) show no homology to the Cry class of insecticidal toxin but appear to have a similar mode of action as observed on the midgut of lepidopterous insects.

B. thuringiensis spore crystal-toxin mixture(s) of selected strains containing potentially coleopteran active Cry toxins was produced using Schaeffer sporulation media (Schaeffer *et al.*, 1965) and evaluated in greenhouse and field trials. Results of a greenhouse trial involving spore crystal-toxin mixtures produced from various *B. thuringiensis* strains are shown in Table 3. This study compared several *B. thuringiensis* strains that contained different cry toxin genes (based on PCR analysis). The experimental protocol was conducted as described (see greenhouse assay) with rice water weevil feeding scars (no. of feeding scars on newest leaf) and larval densities being recorded to measure effectiveness of treatments. With respect to feeding scars, a significant reduction was observed with strains 4AC1 and 4H2 on the 1st sample date, while on the 2nd date (5 and 11 days following infestation with two adult rice water weevil per plant) a reduction was observed with 4H2 and HD201. However, rice water weevil feeding activity is not always correlated with rice water weevil larval densities. Application of strain 4G1 resulted in the greatest reduction of rice water weevil larvae on both sample dates (21 and 28 days post-permanent flood). These results suggest that there may be differential activity of *B. thuringiensis* toxins against adult versus larval rice water weevil.

Table 3. The effect of *Bacillus thuringiensis* (*Bt*) strains after treatment on rice plants on leaf scaring by adult rice water weevil (RWW) and number of larvae/plant on two different sample dates

Bt strains	Leaf scars		RWW larvae/plant	
	1st date	2nd date	1st date	2nd date
4AC1	9.1	5.4	22.5	35.5
4G1	15.7	4.3	0	0
4H2	10.1	0.5	8.5	11.5
HD201	18.6	2.1	4	10.5
HD537	20.6	4.5	9.5	5
Control	19.4	8.2	7	13

4 Protocols for studying, applying and evaluating selected entomopathogens

A Agamermis unka

1 Naturally occurring Agamermis *in BPH and WBPH populations*

BPH and WBPH generally feed on the lower part of the rice stem close to the water surface. Thus, sweeping using a general-purpose net is not efficient for these insects. Instead, beating with hand or sucking with an aspirator or D-Vac machine is recommended for BPH and WBPH sampling.

To evaluate parasitism of *A. unka*, one approach is to transplant or seed BPH and WBPH susceptible rice cultivars at intervals of 20 × 20 cm between hills in a 662 m field. The paddy is divided into three main sampling plots of the same size. The plot is bordered by 5 rows of rice hills between plots. Three to five subsampling sites within each plot are randomly chosen to collect insects. The two or three rows of rice plants along the paddy banks should be excluded from the study to avoid edge effects. The size of the subsampling sites is 2 × 2 m with 3 rows of rice hill border.

1. Collect insects randomly from within a plot with a mouth aspirator for 20 min and place them into a plastic container (*e.g.*, 10 cm diameter × 6 cm height) containing two or three rice seedlings. The top of the container should be covered with a gauze or screen and provisions made to place the insects into the container through an aperture in the gauze or screen. The aperture can be plugged with cotton.
2. Label each container and transport to the laboratory in a cooler.
3. Segregate the insects by planthopper species, sex and developmental stage in the laboratory and dissect them individually in Ringer's solution to determine *Agamermis* parasitism.

A second approach is to collect BPH and WBPH with a mouth aspirator from 50 rice plants from a farmer's field. The sampling site can be adjusted according to shape or size of the paddy field. A third method is to use an insect-collecting plate (*e.g.*, 23 cm × 30 cm plastic or wooden plate) that is painted black or white to obtain BPH and WBPH. Place the plate at a right angle to the bottom of the rice plant and shake the plant three to four times. Insects that drop onto the plate are knocked into a container (*e.g.*, 20 × 15 × 10 cm) that includes rice seedlings. Thirty to 50 rice plants are shaken to obtain a representative sample of insects. This method will collect all stages except the macropterous adults that may disperse before they can be placed into the cage. A fourth method described by Pea and Shepard (1986) sampled BPH using a D-Vac machine to determine parasitoid parasitism in farmers' rice fields. They obtained planthoppers and leafhoppers by sampling for 1 min at each of five to seven sites at each location.

2 Evaluation of Agamermis *against planthoppers in the laboratory*

1. Rear BPH or WBPH continuously on susceptible rice seedlings (*e.g.*, cv. Hwaseong or Chucheong or other suitable rice cultivars) in screened cages (*e.g.*, 30 × 25 × 30 cm) in an enclosed room or other suitable sites at a 15 h light: 9 h dark cycle. Add new rice seedlings into the cages as needed. Augment laboratory colony with field-collected adults every year.
2. Select 100 BPH or WBPH in the second and third instars from the colony and release them into a plastic box (40 × 30 × 20 cm) containing 45-day-old rice seedlings susceptible to the planthoppers. BPH-susceptible rice cultivars are recommended because they reduce insect mortality. Maintain the water level in the plastic box at about 3 cm in depth.

3. Place screen over the plastic box by inserting a 30 cm high wooden stake at each corner and the center of the box. Secure the bottom of the screen to the box with string or wire.
4. Add *A. unka* to the water at a ratio of 10 pre-parasites:1 BPH or WBPH.
5. Segregate the leafhoppers 10 to 13 days later to males and females and macropterous and brachypterous adults and dissect to determine the presence of the mermithid. Record the number of mermithids per host as more than one may occur in a host. Three or four replicates are sufficient to evaluate mermithid parasitism (Choo *et al.*, 1995).

3 *Evaluation of* Agamermis *against planthoppers in the field*

There are two approaches to assess *Agamermis* in the field. One approach is to select rice paddies that are free of this mermithid and add pre-parasites into the plots. The second method is to use plots that have an established population of the mermithid. The protocol below is to assess the impact of the mermithid in areas where it does not occur.

1. Transplant 30-day-old rice seedlings, susceptible to planthoppers, in the rice paddy free of *Agamermis* (240 m^2). Susceptible rice varieties should be used because they are better for the reproduction of BPH and WBPH.
2. Divide the paddy into three plots and place 1.2 × 1.2 × 1.2 m screened cages in each plot with three or four replicates. Prepare a temporary levee with paddy soil or a plastic panel. This will keep the applied mermithids (see next step) in the plots.
3. Apply a ratio of 100 to 200 *Agamermis* pre-parasite: 1 BPH or WBPH. (This rate is recommended over the 10:1 laboratory ratio because a higher ratio is needed for field parasitism.)
4. Place 30 pairs of BPH or WBPH adults into each cage for 45 days.
5. Sample the planthopper progeny 25 days later using an aspirator. Segregate the planthoppers into males and females and brachypterous and macropterous adults and categorize the nymphs into their respective developmental stage.
6. Dissect the planthoppers to determine mermithid parasitism (Choo, 1991)

4 *Sampling for adult* Agamermis *in soil*

The presence and the population density of *Agamermis* adults can be determined when the rice fields are fallow or standing water is absent. If the impact of a natural population of this mermithid is to be determined, the above protocol (*i.e., Evaluation of* Agamermis *against planthoppers in the field*) can be followed with slight modifications. The area selected will have the mermithid present and the temporary levees are not needed. The following protocol can be used to determine the density of the mermithid.

1. Dig a soil sample, 20 × 20 × 20 cm, with a shovel to confirm the presence of the mermithid (Choo *et al.*, 1995).
2. Break the soil sample with your hands into smaller pieces and pick up *Agamermis* adults with your hands or an applicator stick because they are large (28.3 × 18.8 mm in female and 16.3 × 9.1 mm in male) and can be seen with the naked eye.
2. Count the number of adults per sample
3. Take several (15 to 20) soil samples from various locations in the rice paddy to obtain the number of nematodes per cm^3.
4. Return mermithids to the soil or collect them for laboratory studies.

Collected nematodes are placed in a plastic container (*e.g.*, 10 cm diameter × 6 cm height or other suitable containers) with field soil to avoid desiccation and transported in an ice chest to the laboratory. In the laboratory, they may be placed individually or in a group in a Petri dish containing distilled water (Choo *et al.*, 1995). They may be stored at 4 °C until they are needed. Nematodes stored for 4 months will produce viable eggs (Choo, unpublished data); when stored for 12 months, they are alive and vigorous but egg production cannot be guaranteed. Eggs can be collected daily or every other day depending upon the purpose of the study.

5 *Sampling for pre-parasite* Agamermis *in the field*

a. Sampling from rice stems

1. Transplant a planthopper-susceptible rice cultivar in the field as above and delineate

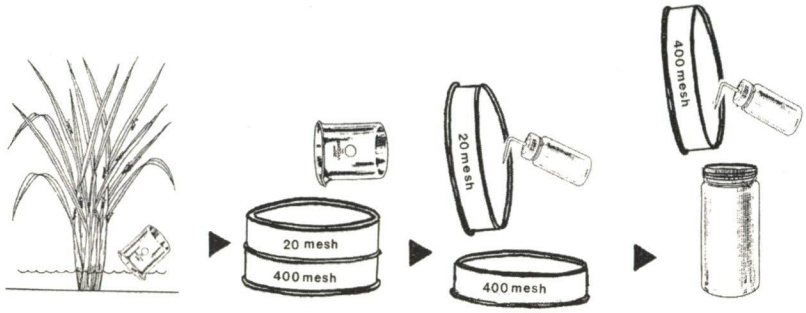

Figure 1. Method for sampling *Agamermis unka* pre-parasites from a flooded rice field.

2.5 × 2.5 m plots with a 0.5 m border between plots to determine pre-parasite activity and behavior in the rice paddy.

2. Follow normal rice cultural techniques except that no insecticides or nematicides are used.
3. Examine the rice stems for presence of the pre-parasites by cutting 10 stems just above the water level (> 1 cm above), and rinse in 60 ml of water in an 80 ml (or similar size) container for 1 min. This can be done at various time during the rice growing season.
4. Place the containers in an ice chest for transport to the laboratory.
5. In the laboratory, pour 10 ml of water into a Petri dish (100 × 15 mm) and count the number of pre-parasite under the microscope. This procedure is repeated until the entire water sample is examined for pre-parasites.

b. Sampling from the water

1. Obtain the density of pre-parasites in a flooded rice field by scooping rice field water from a 2.5 × 2.5 m plot into a 300 ml beaker and pouring the water sample through a 20 mesh (850 μm) and a 400 mesh (38 μm) sieve.
2. Rinse the 400 mesh sieve with distilled water several times; then rinse the sieve material into a 100 ml bottle (Figure 1).
3. Take 10 samples from each 2.5 × 2.5 m plot.
4. Place the 100 ml bottles into an ice chest and transport to the laboratory.
5. Pour the water into a Petri dish and count the number of pre-parasites.

In an unflooded field, a 30 × 25 cm pool is made between the rice plants, and water is collected and scooped into a beaker. In addition, 300 ml beaker is inserted at the level of soil surface until sufficient water is collected. The beaker is removed and the water is processed as above from Section IVA5b, step 1.

6 *Sampling time for* Agamermis

The sampling time for each developmental stage of *Agamermis* is different. In temperate regions, adults are sampled after rice harvest in September or October until planting in May the following year. Pre-parasite sampling is done during the rice growing season from July to August. In tropical regions, adult samples should be taken during periods when no water is present in the paddy and pre-parasite samples taken when water is present as well as planthopper hosts.

B Beauveria *and* Metarhizium *spp.*

Conidial suspensions of both entomopathogenic fungi have been used against a limited number of rice insect pests. The target pest species and its distribution in the field will affect how the plot size is determined. Other considerations for determining the size of the plots include fungal species, the number of replicates, paddy size, and availability of labor. Plots 50 m^2 in size is recommended for evaluation of low mobility pests, but plots 200 or more m^2 is required for highly mobile insects such as BPH or leafhoppers. In addition, the spray equipment and application time are dependent upon the target pest species. The fungal rate required to effect a desired mortality is a function of the susceptibility of the host and the virulence of the microorganisms (Reichelderfer, 1985).

1 Protocol and evaluation of entomopathogenic fungi against planthoppers

a. Pot assay in small field cages

1. Plant three rice seedlings susceptible to planthoppers in a suitable pot and maintain in the greenhouse in water. (The number of rice seedlings may vary according to the size of the pot.)
2. Cover each pot with a screen cage.
3. Release 100 third instar BPH nymphs on the plants and allow them to acclimatize for 1 day.
4. Place the pots into the rice paddy plots with a 0.3 m border between pots with plants of similar growth age. The pots can be placed into a random design or a random block design depending upon the experiment and size of the rice paddy.
5. Apply control and fungal treatments with a hand sprayer until runoff.
6. Take live insect counts every 3 to 4 days after treatment up to 21 days; if possible, collect dead insects and examine for fungal infection.
7. For multiple treatments, apply second or third treatment at 7 to 10 day intervals and take counts as in step 6 above.

b. Field treatment

A randomized block design with three or four replicates are usually employed for the field evaluation of entomopathogenic fungi for BPH or WBPH control.

1. Establish a plot size of 2×2 m (18–20 hills) and plant susceptible rice cultivars with a 20×20 cm spacing between rice hills. There should be a minimum of four replicates for each treatment.
2. One day before treatment, enclose each plot with a screen cage to prevent fungus-infected planthoppers from moving to neighboring plots (Aguda *et al.*, 1987).
3. Apply the conidial suspension at a rate of $4.0 \sim 5.0 \times 10^{12}$ conidia/ha (Rombach *et al.*, 1986b) to 7.5×10^{12} conidia/ha (Aguda *et al.*, 1987). The concentrations can be varied depending on the fungus or field situations. Dry mycelia of *B. bassiana* can be also applied at rates equivalent to 200g/ha in a formulation with 5% Liqua Gel®.
4. Three hundred ml of suspension is applied per plot by ultra low volume applicator. The nozzle of the applicator is adapted for the mycelium size and application is made at panicle stage of the rice.
5. Live BPHs from 12 randomly selected hills in each cage are counted because infected insects fall into the water and are difficult to find (Aguda *et al.*, 1987).

2 Protocol and evaluation of entomopathogenic fungi against rice water weevil

Adult rice water weevil can be bioassayed using one of two methods; (1) a test tube or submersion bioassay and (2) a droplet bioassay. Although this is a laboratory procedure, the rice water weevil is difficult to assess in the field, and more controlled tests against laboratory and/or greenhouse procedure (see protocol outlined for greenhouse assay below in Section 4C2) may be needed before field tests are done.

a. Submersion bioassay

1. Collect either over-wintering adults or adults from rice fields.
2. Place 10 weevils in a 15 ml polypropylene test tube containing a 1 ml suspension of conidia (10^4 to 10^8 conidia/ml).
3. Gently vortex for 5 sec and incubate for 30 to 60 sec.
4. Transfer weevils to a 100 mm Petri dish containing a moist filter paper (sterile H_2O) and rice leaves for 1–20 days. Replace with fresh rice leaves every 2–3 days.
5. Transfer Petri dishes containing inoculated weevils to a partially closed plastic container containing H_2O saturated paper towels and incubate at 28 °C. Re-wet filters as necessary to maintain a humid environment. Alternatively, transfer individual adult weevils to a test tube (1.8×10.5 cm) that has a rice seedling and assess mortality for the next 20 days. Replace with fresh rice seedling as needed.
6. Record adult mortality at 3, 5, 7, 10, 14 and 20 days following treatment.

Special precautions are required to record mortality accurately. Mortality is determined by positioning adult weevils on their backs. After a 10 min interval, viable adult weevils will right themselves. At this time an accurate viability determination can be made. Observation of fungus sporulating on or dissection of cadavers can confirm mycosis.

b. Droplet bioassay

1. Transfer 10 adult rice water weevils/sterile 100 mm Petri dish containing a pre-wetted filter paper (1 ml sterile H_2O).
2. Dilute conidia serially to the appropriate suspension in 0.1% Tween 80 to prevent aggregation.
3. Place a 10 µl drop of the desired dose to the dorsal side of the adult rice water weevil using a pipetman.
4. Follow steps 4–6 described above in the "Submersion bioassay."

C Bacillus thuringiensis *assay against rice water weevil*

Two types of assays (leaf dip and greenhouse) can be used to evaluate *Bacillus thuringiensis* suspensions against rice water weevil adults.

1 Leaf dip bioassay

1. Streak a fresh nutrient agar plate from an appropriate culture of *B. thuringiensis* strain and incubate at 30 °C overnight.
2. Transfer a single colony to 1 ml of LB Medium (Difco) and incubate at 30 °C overnight.
3. Inoculate 20 ml of Schaeffer sporulation media in a 250 ml Erlenmeyer flask with 0.02 ml of an overnight *B. thuringiensis* culture and incubate at 30 °C for 5 days.
4. Harvest the spore crystal-toxin suspension by centrifugation at 5,000 rpm for 10 min.
5. Resuspend the spore crystal-toxin pellet in 10 ml of TNT (20mM Tris, 10mM NaCl, 0.05% Triton X-100); wash by centrifugation 2 times with TNT and resuspend in 5 ml of TNT.
6. Determine the spore crystal-toxin concentration using a hemocytometer as described by Klein (1997). Spore counts may also be made using the dilution and plating method described by Thiery and Frachon (1997).
7. Dip the rice leaves from young plants (at the 4–6 leaf stage of growth) into *B. thuringiensis* spore crystal-toxin suspension containing 0.04% Sil-Wet 77 or other compatible wetting agent (aids in dispersing and attaching the spore crystal-toxin suspension on the rice leaves).
8. Air-dry the rice leaves and transfer to bioassay dishes as previously described except that the filter paper is not saturated with water.
9. Remove old treated leaves and replace with freshly-treated leaves every 3–4 days.
10. Record mortality at 3, 5, 7, 10 and 14 days after initial treatment.

2 Greenhouse assay

This protocol is used for *B. thuringiensis* but may also be used for testing conidial suspensions of fungal pathogens such as *B. bassiana* or *M. anisopliae*.

1. Seed pots (10 cm × 15cm height) with 6–10 rice seeds per pot that contain an equal mixture of potting soil and topsoil. Rice is grown in small pots in greenhouse conditions at 14 h light (30 °C):10 h dark (22 °C)
2. At the 2–3 leaf stage, thin to 3 plants/pot to achieve uniform plant size and maturity.
3. Transfer 10 pots each into aquariums (45 cm wide × 60 cm long × 45 cm height).
4. At this time, apply a *B. thuringiensis* spore crystal-toxin mixture to the rice foliage and soil surface using a CO_2 powered sprayer fitted with a single 8015VS nozzle at a rate of 5×10^{12} spore crystal-toxin particles/ha.
5. Add rice water weevil to the aquariums at a density of 50 adult weevils/aquarium. Adult weevil may be collected from either overwintering sites or from field plots
6. Immediately cover the aquarium with a cage (made with a wood frame and gauze mesh) of equal size to that of the aquarium to prevent the adult weevils from escaping.
7. Make a second treatment application 3–4 days following the initial application.
8. Establish a permanent flood in the aquariums 1–2 days following the 2nd pre-flood application and follow with 1–2 more post-flood treatment(s) of the biological agent.
9. Check for rice water weevil mortality at 14 days after the second treatment.

5 Field experimentation for rice water weevil

A randomized complete block plot design is generally used for field trials evaluating the effectiveness of fungi (*B. bassiana* or *M. anisopliae*) or bacteria (*B. thuringiensis*) for their potential

for rice water weevil control. Generally, there are 4–6 replications per treatment with 3 to 5 subsamples obtained per treatment. Treatments (1–2) are made prior to and after the establishment of the permanent flood. Sampling may involve the counting of adults (number of rice water weevil adults within a 0.093 m^2 ring), adult weevil leaf feeding scars (on newly-emerged leaf), and rice water weevil egg and/or larval samples. Rice plant responses to treatments are evaluated by measuring root mass (volume and dry weight) and root length, plant height, plant maturity, and grain yield.

A Preparation of soil bed – water seeded or drill seeded rice

1. Till soil using conventional tillage practices to prepare a fine seed bed. Rice plant health is assured by a pre-plant incorporation of nitrogen-phosphorus-potash at a rate of 112-67-67 kg/ha.
2. Water seed the rice crop for experiments to be planted early in the growing season and drill seed the rice crop for planting later in the growing season. Draw the levees around the bays (paddies containing the experimental plots) to allow for flooding of the rice plots.
3. Soak the rice seed for 24 h in 2–3 volumes of water (non-chlorinated), drained, and allowed to set for another 24 h at ca 30 °C. Examine the rice seed for the presence of pips (sprouting).
4. Broadcast by hand the pre-germinated seed into the previously flooded designated rice plots. The rice seed is allowed to develop for 3–5 days (dependent on temperature) under flood or until radicle and coleoptile have developed sufficiently. Drain the water and allow the rice plant to peg (for the root to firmly attach to the soil) for 2–3 days after which a shallow permanent flood is applied.
5. Prior to application of the permanent flood, apply a top dress of N (34–67 kg/ha).
6. Follow the protocol outlined below (*i.e.*, section 5B) for treating rice water weevil adults.

For drill-seeded rice, use conventional drills to deliver the seed to a depth of 1–2 cm. If the soil contains enough moisture the seed will germinate. Alternatively a flush (temporary flood) can be applied to cause uniform plant germination. The desired outcome in either case is to achieve uniformity in emergence of the rice plant.

B Transplanted rice cultivation

1. Transplant rice with a 20 × 20 cm spacing between rice hills. The plot size is 2 × 2 m (18 ~ 20 hills) and is blocked to avoid contamination of the entomopathogenic fungus to control and/or other plots.
2. Enclose the plot with screen or gauze to prevent the overwintering adults from moving to neighboring plots. The transplanting time of rice, adult activity, oviposition, adult longevity, persistence of pathogens, and fungus species determine application time.
3. Apply the fungus once during the peak time of oviposition at a given concentration of *B. bassiana* or *M. anisopliae* evenly during the evening on a windless day.
4. Collect weevil adults 7 and 14 days after treatment and examine for fungus infection. A randomized block design with three replicates can be employed for the field evaluation of entomopathogenic fungi for rice water weevil. For the first generation adult of rice water weevil, entomopathogenic fungi mixed with white carbon are applied with a duster at the beginning of adult occurrence. The adults are collected with a sweep net for 3 weeks at 2- to 5-day intervals and fungus infection determined.

C Pre- and post-treatment sampling

Before treatment, the rice fields should be sampled for rice water weevil adults directly or indirectly. In the indirect method, the presence of adult weevils can be assumed by the presence of feeding scars on the leaves. For the direct method, sampling rings (0.093 m^2) can be placed in the rice plots after the establishment of the permanent flood. A treatment threshold has been reached if there are 1 or more adult rice water weevils per sample ring (2–3 subsamples) per plot. Weevil densities should be checked 2 days before treatment and from 7 days after treatment and at 3- or 4-day intervals after that. Dead insects can be collected with forceps along the rows by zigzagging and making 10 rounds. Thus, insect density and pathogen infection can be determined.

D Inoculum

Inoculum may consist of fungal conidial powders or formulated *B. thuringiensis*. Dry, powdered conidia from various fungal isolates containing approximately 4×10^{10} conidia/g have been formulated in either water or oil-based suspensions by Mycotech Corporation (Butte, MT) using a proprietary novel semi-dry fermentation system. Selected strains of *B. thuringiensis* containing potentially active coleopteran Cry toxins (based on PCR analysis, *e.g.*, cry3Aa1, cry3Aa2, cry1Ia1, etc.) can be produced using Schaeffer sporulation media (Schaeffer *et al.*, 1965) as previously described. A wetting agent such as Sil-Wet 77 is needed to suspend either the conidia or the bacteria for wettable suspensions. Alternatively a 5% crop oil-water suspension can be applied for the conidia or *B. thuringiensis*.

E Application methodology

Application of various conidial or *B. thuringiensis* suspensions can be made using CO_2 powered backpack sprayers at a rate of 140 liters/ha using VS110015 nozzles (R & D Sprayers, Opelousas, LA) or similar spray equipment. Conidia or *B. thuringiensis* can be applied at a rate of 6.2×10^{12} particles/ha (particles are either the number of conidia or number of spore crystal-toxin particles). At the time applications are made, the leaf area index of the rice crop needs to be determined. The leaf area index will rapidly increase in value at this stage of rice plant growth and thus indicates the relative amount of rice leaf foliage to which applications are being made as opposed to soil or water if the field is flooded. Generally, 1–2 pre-flood and 1–2 postflood applications of the biological control agent should be made due to the life cycle of the rice water weevil.

F Treatment assessment – efficacy

Several methods (direct and indirect) can be used to assess the effectiveness of conidial formulations in controlling adult rice water weevil populations. Immediately following application of treatments, adult rice water weevils are collected from treated and control plots and transferred to Petri dishes with food. They are held for 14 days, checked for mortality every other day, and food added as needed. In this way a determination of initial contact of the pathogen and insect can be made. At various times following application (generally at 12 h intervals), adult rice water weevils are collected and bioassayed as described above. To assess the potential of leaf surface contact for infecting rice water weevil; leaves from control and treated plots can be collected at random from experimental plots (5 subsamples per plot) and placed in Petri dishes. Adult rice water weevils are collected from field plots several hundred meters away from the experimental plots and transferred to the bioassay plates. Rice water weevil mortality is determined as previously described. Rice water weevil egg densities are obtained in a time period of 5–9 days following establishment of the permanent flood, while rice water weevil larvae are sampled over a 1-month period starting approximately 3 weeks following establishment of the permanent flood

G Treatment assessment – persistence

Leaf samples can be collected at random in experimental field plots and transferred to the lab at the time of collection. Subsequently, the center of the leaves can be cut into $^1/_2$ cm length and placed into 5 ml of an extraction buffer (phosphate buffered saline pH 6.7) in 20 ml screw-capped vials. The sample vials are incubated at 27 °C for 2 h on an orbital shaker rotating at 250 RPM. An aliquot is removed from each vial, diluted appropriately and plated on ODTA plates for fungal conidia or Schaeffer's medium for *B. thuringiensis*. After incubation for 7 to 9 days for conidia and 2 to 3 days *for B. thuringiensis* at 27 °C, the colony forming counts (CFU) are recorded. The total numbers of CFU/ml are determined and from the leaf surface area of the samples, a determination of the number of CFU/cm^2 of leaf surface can be made.

H Environmental data

1. Obtain environmental information (abiotic factors) to aid in assessing the effectiveness of entomopathogens in field trials against rice insect

pests. The relevant environmental data that are obtained are temperature, relative humidity, solar radiation, and rainfall.

2. Obtain the information from a weather station located from a nearby weather station or make provisions for weather data to be collected on site. From this information, it is possible to determine the influence of abiotic factors on the persistence and stability of entomopathogens applied to rice plants.

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