A novel soil-type biopesticide KNB422-soil against rice seedling diseases

Taiji MIYAKE,* Hideaki TATEISHI, Yoneko SAKUMA and Toshihide SAISHOJI
Agrochemical Research Laboratories, Kureha Corporation, 16 Ochiai, Nishiki-machi, Iwaki-shi, Fukushima 974–8686, Japan

(Received May 6, 2011; Accepted February 6, 2012)

KNB422 is a fungal isolate effective at controlling rice seedling diseases such as Bakanae disease, when applied to seeds. When a granulated soil for rice nurseries containing KNB422 (KNB422-soil) was used, several seed-borne diseases caused by fungal as well as bacterial pathogens, such as Gibberella fujikuroi and Burkholderia plantarii, were controlled efficiently during seedling production. Using KNB422-soil also controlled soil-borne diseases caused by Rhizopus oryzae, Pythium graminicola, Trichoderma viride, and Fusarium sp. as effectively as chemical pesticides. A combination of KNB422-soil and hot-water submersion of rice seeds completely controlled Bakanae disease without any chemicals. Since the use of KNB422-soil for rice seedling production was sufficiently controlled seed-borne and soil-borne diseases and hot-water submersion enhanced its activity, this new material would reduce chemical usage and labor.

© Pesticide Science Society of Japan

Keywords: Talaromyces, KNB422, KNB422-soil, biocontrol, soil-type biopesticide, rice seedling disease.

Electronic supplementary material The online version of this article contains supplementary material (Supplemental Figure S1), which is available at http://www.jstage.jst.go.jp/browse/jpestics/.

Introduction

Chemical pesticides are fundamental to crop protection. However, over use can make pathogens less sensitive to chemicals.1) Therefore, selection pressure on pests by chemicals must be reduced to avoid the emergence of chemical-resistant pests. Furthermore, concerns about the effects of chemical pesticides on human health and the environment have been growing for years. Various physical, biological, and chemical tools have been combined to protect crops from pests. Notably, biopesticides have been explored actively for several decades.2–9)

Rice is one of the world’s most important crops and a staple in several countries. In rice nursery cultivation, diseases caused by seed-borne or soil-borne pathogens occur if seeds or soils are not treated with pesticides. In most cases, the pathogens of seed-borne diseases such as Gibberella fujikuroi, which cause Bakanae disease, already contaminate the seeds when they are harvested and therefore are controlled by seed treatment with disinfectants. The pathogens of soil-borne diseases, such as Rhizopus oryzae and Pythium graminicola, usually exist in soils and, therefore, are controlled by seed treatment and/or soil treatment.10) Thus, the use of multiple chemicals is necessary to completely control rice seedling diseases.

Total chemical-application rates for the rice cultivation process are regulated by local authorities. Therefore, reducing chemical usage in the early stages allows for the use of chemicals for unanticipated diseases in later stages. Other approaches beside chemical protection have been explored recently,11–13) and biocontrol agents for controlling rice seedling diseases are considered useful. There are a number of reports about controlling seed-borne or soil-borne pathogens with biocontrol agents.14,15)

The fungal isolate KNB422, discovered by Kureha Corporation in 2002, can control a wide range of rice seedling diseases.16–19) From morphological observations of the sexual and asexual organs of KNB422, the fungus was determined to be a Talaromyces sp.16) In this paper, a unique and useful material for rice seedling production, a soil-type biopesticide containing KNB422, and its efficacy against rice seedling diseases are discussed.

Materials and Methods

1. Preparation of the spore suspension of KNB422
A mycelial sample of KNB422 (Stored at Kureha Corporation, Fukushima, Japan) was transplanted on a potato dextrose agar (PDA) (Difco Laboratories, Sparks, MD, USA) medium and incubated at 25°C for 10 days under light. The spores that developed in the medium were suspended in deionized water.

* To whom correspondence should be addressed.
E-mail: miyake@kureha.co.jp
Published online April 29, 2012
© Pesticide Science Society of Japan
and filtrated with a single layer of gauze to remove mycelia. The spore suspension was diluted with deionized water to prescribed concentrations.

2. Preparation of KNB422-soil
To prepare soil containing KNB422 (KNB422-soil), the spore suspension was added to the granulated soil (Kureha) for rice seedlings and mixed well in a plastic bag. The volumetric ratio of the spore suspension to granulated soil was 1:100. The soil was maintained at 25°C for 15 days.

3. Measurement of the KNB422 population in KNB422-soil
To measure the population of KNB422 in KNB422-soil, an aliquot of the soil was mixed with a 9-fold volume of sterilized water in a plastic tube. The suspension was diluted several times with sterilized water to prepare various concentrations of KNB422, and the suspensions (0.1 mL) were transferred to a 90 mm-diameter PDA plate. After 2 days at 30°C, colony-forming units (cfu) were counted to calculate the fungal population in the soil with no replication.20 Because the granulated soil used contained very few microorganisms due to sterilization by heat treatment in the commercial production of the granulated soil, the formation of colonies of KNB422 in the PDA medium was not prevented by the growth of other microorganisms.

4. Evaluation of efficacy of KNB422-soil against Bakanae disease
4.1. Overview of raising rice seedlings
Rice seeds were soaked in water at 15°C for 1 day, soaked in fresh water for 4 days (water-soaking step), and incubated in fresh water at 30°C for 1 day (germination step). The volumetric ratio of the seeds to water was 1:1. The seeds were divided into three replicates and sown on the granulated soil in plastic seedling boxes (seed-sowing step). After incubation in a steam chamber at 30°C for 3 days (budding step), the plants were transferred to a greenhouse and raised (greening step).21 Raising of rice seedlings is shown in Supplemental Fig. S1. The plants were grown until the symptoms of the disease became visible in untreated plots, and the number of diseased plants was counted to obtain the disease incidence.

4.2. Preparation of infected seed of Bakanae disease
A mycelial sample of Gibberella fujikuroi G-1-1-8 (stored at Kureha) was transplanted to a PDA medium and incubated at 25°C for 10 days under light. The spores formed in the medium were suspended in deionized water and filtrated with a single layer of gauze to remove mycelia. To obtain infected seeds, rice plants (Oryza sativa, cv. Tanginbozu) in the greenhouse were artificially inoculated by spraying the spore suspension of G. fujikuroi (1 x 10^6 spores/mL) on the flowers in a field. The plants were raised normally, and the seeds infected with G. fujikuroi were harvested.

4.3. Measurement of the efficacy of KNB422-soil
KNB422-soil (1 x 10^6 cfu/g, 260 g) was placed into 100 x 150 mm plastic seedling boxes (45 mm depth). Rice seeds infested with G. fujikuroi were sowed in KNB422-soil and covered with 80 g of KNB422-soil. For untreated plots, granulated soil that did not contain KNB422 was used. After a 3-day incubation in a steam chamber at 30°C, the plants were grown until Bakanae symptoms, abnormally elongated seedlings and damping-off seedlings, occurred in the untreated plots. A protective index was calculated with the formula, 100 × (dc−dt)/dc, where dc is the disease incidence in untreated plots and dt is that in KNB422-soil-treated plots. Data are the mean of three replications.

5. Evaluation of efficacy of KNB422-soil against seed-borne pathogens
5.1. Preparation of seeds infested with seed-borne pathogens (blast, bacterial seedling blight, and bacterial seedling rot)
To obtain seeds with rice blast, rice seeds (cv. Koshihikari) naturally infected with Pyricularia oryzae were collected from a field where rice blast occurred. To obtain seeds with bacterial pathogens, rice seeds (cv. Nihonbare) were dipped in a suspension of Burkholderia plantariar YR8805 (1 x 10^7 cells/mL) or Burkholderia glumae NIAES1682 (1 x 10^7 cells/mL) under 200 mmHg in a vacuum container.

5.2. Measurement of efficacy of KNB422-soil against seed-borne pathogens
For the bacterial seedling blight and bacterial seedling rot experiments, the incubation temperature in a steam chamber was 32°C. The other methods are described in Section 4.3.

6. Evaluation of efficacy of KNB422-soil against soil-borne pathogens
6.1. Origin of fungal isolates of soil-borne diseases
Four soil-borne pathogens of rice, each of which is a causal pathogen of seedling blight in rice, were used in this study. Rhizopus oryzae IFO 5440 and Trichoderma viride IFO 30498 were obtained from the NITE Biological Resource Center (Chiba, Japan). Fusarium sp. Osk-1 was obtained from the Agricultural, Food and Environmental Sciences Research Center of Osaka Prefecture (Osaka, Japan). Pythium graminicola OPU480 was obtained from Osaka Prefecture University (Osaka, Japan).

6.2.1. Preparation of KNB422-soil containing R. oryzae
Mycelia of R. oryzae were incubated in a PDA medium at 25°C for 7 days. A two-fold volume of deionized water was added to the medium, the mixture was homogenized, and the homogenate was mixed well with a 12-fold volume of KNB422-soil with 1 x 10^6 cfu/g KNB422.

6.2.2. Preparation of soil containing P. graminicola
Mycelia of P. graminicola were incubated in a PDA medium at 22°C for 7 days. A commercially available oatmeal powder and vermiculite were mixed at a volumetric ratio of 3:1, deionized water was added, and the mixture was shook vigorously by...
hand and sterilized by autoclave. A mycelial sample of *P. graminicola* was transplanted to the sterilized oatmeal-vermiculite mixture and incubated at 22°C for 14 days. Deionized water was then added, and the mixture was homogenized with a blender. The homogenate was mixed well with a 14-fold volume of KNB422-soil with $1 \times 10^6$ conidia/g KNB422.

6.2.3. Preparation of soil containing *T. viride*

Mycelia of *T. viride* were incubated in a PDA medium at 30°C for 7 days. The spores that formed were collected with deionized water, and the spore suspension was filtered with a single layer of gauze to remove mycelia. This suspension ($1 \times 10^6$ cells/mL) was added to KNB422-soil at about $1 \times 10^7$ conidia/g. The volumetric ratio of the spore suspension to KNB422-soil with $1 \times 10^6$ cfu/g KNB422 was 1:25.

6.2.4. Preparation of soil containing *Fusarium sp.*

*Fusarium sp.* was incubated in a PDA medium at 30°C for 10 days. A mycelia of the *Fusarium sp.* was transplanted to the sterilized oatmeal-vermiculite mixture and incubated at 30°C for 10 days. The other methods were as described in Section 6.2.2.

6.3. Measurement of efficacy of KNB422-soil against soil-borne diseases

KNB422-soil (520 g) infested with each pathogen was placed into $140 \times 200$ mm plastic seedling boxes (45 mm depth). Rice seeds not infested with the pathogens were sown into the soil and covered with 80 g of soil. The other methods were as described in Section 2.4; however, the incubation temperature in the steam chamber was 22°C for *P. graminicola*.

7. Efficacy of the combination of hot water submersion and KNB422-soil against Bakanae disease

Rice seeds infested with *G. fujikuroi* were dipped in 60°C water for 10 min and subjected to the water-soaking step. The seeds were sown on 260 g of KNB422-soil and covered with 80 g of soil. After a 3-day incubation in a steam chamber at 30°C, the plants were grown until symptoms of disease were observed in untreated plots.

**Results**

1. *Population of KNB422 in the nursery KNB422-soil*

KNB422-soil prepared with $1 \times 10^6$ spores/mL of KNB422 was maintained at 25°C in the dark to measure the growth of KNB422. On day 0, the population of KNB422 was calculated to be approximately $1 \times 10^4$ cfu/g. The number of KNB422 increased to about $1.5 \times 10^5$ cfu/g in 5 days, 200-fold more than the initial number (Fig. 1). This result indicates that Kureha-nursery soil is fit for KNB422 growth.

2. *Efficacy of KNB422-soil against Bakanae disease*

KNB422-soils containing various concentrations of KNB422 were prepared, and their efficacy against Bakanae disease was investigated using Bakanae pathogen-infested rice seeds. The number of damping-off and abnormally elongated seedlings was counted to calculate the protective value. High efficacy was obtained with KNB422-soil containing more than $1 \times 10^6$ cfu/g of KNB422. Furthermore, KNB422-soil containing more than $1 \times 10^5$ cfu/g of KNB422 was as effective as Ipconazole-Cu flowable (active ingredient 5% and 4.6%, respectively; Kureha), one of the most useful chemicals against Bakanae disease (Table 1).

3. *Efficacy of KNB422-soil against seed-borne pathogens*

For the blast experiment, the number of seedlings with lesions on the first and the second leaf sheath was counted. For the bacterial seedling blight and bacterial seedling rot experiments, the number of damping-off and bleaching seedlings was counted. The protective value of KNB422-soil against blast disease was almost the same as that of Ipconazole-Cu flowable. Furthermore, the protective values against bacterial seedling blight and bacterial seedling rot were also almost identical to those of Ipconazole-Cu flowable (Table 2). These results indicate that KNB422-soil is effective against not only fungal but also bacterial seed-borne pathogens.

**Table 1. Efficacy of KNB422-soil against Bakanae disease**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of seedlings assessed</th>
<th>Incidence of diseased seedlings(^a,b) (%)</th>
<th>Protective value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNB422-soil(^c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^6$ cfu/g</td>
<td>500</td>
<td>1.8 a</td>
<td>96.6</td>
</tr>
<tr>
<td>$1 \times 10^5$ cfu/g</td>
<td>419</td>
<td>1.6 a</td>
<td>96.9</td>
</tr>
<tr>
<td>$1 \times 10^4$ cfu/g</td>
<td>479</td>
<td>6.2 a</td>
<td>88.4</td>
</tr>
<tr>
<td>$1 \times 10^3$ cfu/g</td>
<td>411</td>
<td>57.8 b</td>
<td>0</td>
</tr>
<tr>
<td>Ipconazole-Cu flowable(^d)</td>
<td>458</td>
<td>0.0 a</td>
<td>100</td>
</tr>
<tr>
<td>Untreated</td>
<td>456</td>
<td>53.7 b</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\)Contains conidia of *Talaromyces* sp. KNB422 and was used for both the nursery bed and cover soil.

\(^b\)Rice seeds were soaked in a 200-fold water suspension of Ipconazole-Cu flowable (a.i. 5% and 4.6%, respectively) for 24 hr before water soaking.

\(^c\)The number of damping-off and abnormally elongated seedlings was counted.

\(^d\)Numbers followed by the same letter are not significantly different at \(p<0.05\) according to Tukey’s multiple range test.
4. Efficacy of KNB422-soil against soil-borne pathogens

For the *R. oryzae* experiment, the number of seedlings with damping-off and clubbed roots was counted. For the *P. graminicola* and *Fusarium* sp. experiment, the number of seedlings with damping-off, browning roots, growth inhibition, and no germinated seeds was counted. For the *T. viride* experiment, the number of seedlings with damping-off and visible mycelial mat on the roots was counted. The protective value of KNB422-soil against *R. oryzae* was higher than that of TPN flowable (a.i. 40%), which is a chemical fungicide (Table 3). The protective values of KNB422-soil against *P. graminicola* and *Fusarium* sp. were higher than those of Hydroxyisoxazole (a.i. 30%) or Benomyl-TPN flowable (a.i. 20% and 50%, respectively), another chemical fungicide (Table 3). Furthermore, the protective value of KNB422-soil against *T. viride* was higher than that of Ipconazole-Cu flowable (Table 3). Thus, KNB422-soil is useful for protecting rice against several soil-borne pathogens in the seedling production process.

5. Efficacy of the combination of hot water submersion and KNB422-soil against Bakanae disease

Treatment with hot water (60°C) submersion of seeds infested with *G. fujikuroi* for 10 min alone did not completely control the disease. However, when KNB422-soil was used for the nursery bed and cover soil in addition to submersion in hot water, the disease was almost completely controlled (Table 4). Thus, using KNB422-soil in the rice seedling process after hot-water submersion of the seeds results in full control of Bakanae disease.

Discussion

In the growing process of rice seedlings, the humidity and temperature are maintained relatively high and these conditions might be suitable for pathogens to propagate in the plants or soil. Therefore, protecting rice plants from diseases is necessary. Biocontrol agents would also grow easily under such conditions. In that case, their biocontrol activities will be maximized and become high compared with those under paddye field conditions. Although several biocontrol agents for rice seedling diseases have been developed, the diseases which can be controlled by these biocontrol agents are rather limited. KNB422 has a broad spectrum and can control Bakanae disease, seedling blights, and bacterial diseases. KNB422 grows actively in Kureha-nursery soil and KNB422 is highly effective against seed-borne and soil-borne pathogens when mixed with nursery soil and used for sowing. This observation indicates that KNB422 can be a dominant microorganism in Kureha-nursery soil, even though other pathogenic microorganisms contaminate the soil through rice seeds or circumstances in the rice seedling process. Thus, KNB422-soil, which is Kureha-nursery soil containing KNB422, is a potent material to control rice seedling diseases.

*Talaromyces flavus* SAY-Y-01, already available commercially as Tough block® (Idemitsu, Tokyo, Japan) in Japan, controls several rice seedling diseases to the same extent as KNB422 when used as a seed-treatment agent and has been registered for the seed treatment of rice but not as a soil-type biofungicide. Therefore, KNB422-soil is a novel and unique crop protection material to control pathogens during rice seedling production.

Hot-water submersion of rice seeds is now applied in wide areas of Japan as a way to reduce the use of chemicals in rice seedling production. However, some studies have reported that this treatment is insufficient for the control of Bakanae disease. When KNB422-soil is used in rice seedling production with seeds treated with hot-water submersion, KNB422 may grow more easily in the seeds because other microorganisms are reduced in number. Consequently, the efficacy of KNB422 to control diseases in the seedling production process will be promoted. Thus, the combination of KNB422-soil and hot-water submersion achieves good control of diseases in rice seedling production and reduces the number of pesticide applications in the early stages of rice production.

There are usually 5,000 to 7,000 rice seedlings in a small nursery box. Therefore, even though the percentage of seeds originally infected with the pathogens may be low, the number
of infected seedlings is sufficient to cause substantial damage in paddy fields. In the case of *G. fujikuroi*, which grows inside plant tissues, the disease occurs in the paddy field, and spores are distributed from the damaged plants. The spores spread and infest rice ears at the flowering stage, and these infested seeds then transmit the pathogen to the next generation. Once Bakanae disease occurs in a paddy field, it is very difficult to control because the fungus has already grown enough to spread. Therefore, the control of Bakanae disease in nursery boxes is very important.
In this paper, a new soil-type biopesticide for rice seedlings, KNB422-soil, is presented. Seed-borne diseases are usually prevented by seed disinfectants and soil-borne diseases by seed disinfectants and/or soil treatments. Since KNB422-soil achieves good control of both seed-borne and soil-borne diseases, it can reduce labor for seed disinfection, soil treatments with chemicals, and the disposal of waste liquid. As a result, it will reduce the environmental impact of chemicals and human labor in the rice production process. Nowadays, Integrated Pest Management (IPM) has been promoted in agricultural practice. This new soil-type biocontrol agent might contribute to the IPM of pathogens in rice production.

Acknowledgements
We express our sincere gratitude to Dr. Shin’ichi Kusakari (Research Institute of Environment, Agriculture and Fisheries, Osaka Prefectural Government) and Dr. Motoaki Tojo (Osaka Prefecture University) for their valuable advice and for kindly supplying the isolates.

References