Efficacy of a simple liquid culture of *Bacillus megaterium* in suppressing grain discoloration disease of rice (*Oryza sativa*)

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Abstract Grain discoloration (GD) of rice (*Oryza sativa*) is a disease complex that occurs on rice panicles as a result of infection by various fungi. *Bacillus megaterium*, a bacterial antagonist that is effective against sheath blight of rice, was cultured using either nutrient broth, potato dextrose broth or a common household flavour enhancer. Preliminary data from a single experiment suggested that all three cultures supported growth of the bacterium. Spraying diluted bacterial nutrient-broth culture onto rice (var. RD 31) three times (41, 84 and 96 days after transplanting) reduced the mean severity of GD as a result of the field trials from three consecutive growing seasons. The spray did not affect the percentage germination of the rice seeds after they had been stored for three months in the laboratory at room temperature (26°C–32°C).

Keywords biological control, dirty panicles of rice.

INTRODUCTION

Rice (*Oryza sativa*) is considered to be one of the most important food crops in the world (Brar & Khush 2013). In Thailand, cultivating rice is an integral part of Thai culture as well as the main source of food and income for rural Thai people. In 2014, Thailand exported approximately 5.6 million tonnes of rice for a total value of around US\$3 billion (Thai Rice Exporters Association 2014). Growing rice, particularly in the dry season, is concentrated in both the central and northern regions where the farmers can access water for irrigation. However, continual rice cultivation can result in the occasional but widespread outbreak of both insect pests and rice plant diseases (Bureau of Rice Research and Development Thailand 2015).

Grain discoloration (GD), also known as dirty panicle disease of rice, is a disease complex that has been reported to be caused by fungal pathogens such as *Sarocladium* sp., *Bipolaris* sp., *Alternaria* sp., *Fusarium* sp., *Phoma* sp., *Curvularia* sp., and *Trichoconiella* sp. (IRRI 2002). This disease has also been reported in Brazil (Prabhu et al. 2012), Iran (Amini et al. 2016) and Pakistan (Arshad et al. 2009), indicating that it is a worldwide problem. In Thailand, GD is one of the emerging problems for rice cultivation, with *A. padwickii*, *C. lunata*, *F. semitectum* and *Helminthosporium oryzae* identified as causal agents (Prathuangwong et al. 2013). At present, no commercial rice varieties are resistant to GD resulting to the prevalence of this disease

throughout Thailand (Bureau of Rice Research and Development, Thailand 2015).

Fungicides, such as Mancozeb, have been recommended to control GD as a seed treatment. Also, Propiconazole has been recommended for use as a spray for rice plants to control brown spot disease, narrow brown spot disease and the causal agents of these diseases, which are also capable of causing GD (Bureau of Rice Research and Development, Thailand 2015).

Bacterial and fungal antagonists have been used to control diseases in rice (Gnanamanickam et al. 1992; Naeimi et al. 2010; Kumar et al. 2013; Mosquera-Espinosa et al. 2013; Prathuangwong et al. 2013). In Thailand, some bacterial products have been formulated and field tested to control sheath blight disease (Kanjnamaneesathian et al. 1998; Chumthong et al. 2008; Wiwattanapatapee et al. 2007) and GD in rice (Kanjnamaneesathian et al. 2009; Prathuangwong et al. 2013). These include the bacterium, *Bacillus megaterium*, isolated from soil in rice paddy fields.

The way that biological control agents are formulated is important to ensure not only their control efficacy, but also the ability to store, transport and commercialise them (Burgess 1998). However, only a few bacterial formulations have been commercialised for controlling diseases in rice and other crops in Thailand (Maketon et al. 2008). This lack of biological control products means that Thai rice farmers have only a limited number of tools for controlling rice diseases and this contributes to their reliance on the application of chemical fungicides.

It is aimed to introduce a simple liquid culture system to produce an effective and cheap tool for Thai rice farmers to use for controlling rice diseases. This research aims to evaluate the efficacy of a simple liquid culture of *Bacillus megaterium* in suppressing GD in rice. By providing simple guidelines on how to apply the diluted bacterial culture during the rice growing seasons, Thai rice farmers should be able to use biological control to control GD.

MATERIALS AND METHODS

Strain of *Bacillus megaterium*

Bacillus megaterium was isolated from a water-soluble granule formulation (Chumthong et al. 2008) that had been produced in February 2014 at the laboratory of the Faculty of Agricultural Technology, Songkhla Rajabhat University, Songkhla, Thailand. This bacterium was originally isolated from bulk soil in a paddy rice field, Satun province, Thailand (Kanjnamaneesathian et al. 1998) and was used as an active ingredient in various formulations for controlling sheath blight disease (Pengnoo et al. 2000; Wiwattanapatapee et al. 2004; Wiwattanapatapee et al. 2007; Chumthong et al. 2008; Wiwattanapatapee et al. 2013; Chumthong et al. 2016). One gram of water-soluble granule was suspended in sterile water (9 mL) in a test tube and the mixture was vigorously agitated with a vortex. One loopful of the bacterial suspension was streaked onto potato dextrose agar (PDA). The pure culture of *B. megaterium* was maintained on PDA and also on nutrient agar (NA) in the refrigerator (4°C) until further use.

Culturing of *B. megaterium*

Bacillus megaterium was cultured in three translucent (20 litre) plastic drinking water tanks. Ten grams of either nutrient broth [commercial dehydrated NB (HIMEDIA®, India) containing beef extract and peptone], potato dextrose broth [commercial dehydrated PDB (HIMEDIA®, India) containing potato starch and dextrose] or a common household flavour enhancer [CHFE (Knorr brand, Unilever, Thailand)] were added to each tank containing 10 litres of clean drinking water. The CHFE is composed by weight of 40% iodised salt, 31.9% monosodium glutamate, 13% palm oil, 8% sugar, 3.8% dehydrated pork meat, 1.5% soy sauce, 1.4% spice and 0.4% concentrated pork broth. Ten grams of *B. megaterium*, as water-soluble granule formulation (1×10^9 CFU/g), were added to each tank. The culture was aerated using an aquarium air pump and the air tube entered the tank through a cotton wool plug to prevent contamination. The cultures were incubated at room temperature (25–32°C) for 3 days.

Determination of bacterial growth

The turbidity of the cultures was assessed every 6 hours from the time *B. megaterium* was added, using a spectrophotometer at 610 nm to determine which of the three culture media (NB, PDB or CHFE) produced the most bacteria. Turbidity was measured in triplicate and the values were averaged and recorded as an optical density (OD).

To determine the number of bacteria in the NB culture when preparing it for spraying onto rice in the field, the turbidity of the bacterial suspension was compared with McFarland turbidity standards, a reference to adjust the turbidity of bacterial suspensions to determine the number of bacteria (Sutton 2011), after 24 h of growth.

In addition, 5 mL of the bacterial culture from each medium was collected and one loop was streaked onto NA and PDA to detect possible contaminants. The air supply was terminated after 5 days of culturing the bacterium. The physical characteristics of the liquid fermentation product, such as colour, turbidity, odour and foam formation, were recorded.

Application of the bacterial culture in NB to a rice field

After 24 hours, 10 litres of the bacterial suspension from NB was added into 100 litres of tap water and the mixture was sprayed onto one rice plot (var. RD 31), over 0.4 ha (2.5 rai; a Thai area unit). Applications were made 41 days after transplanting (DAT), 84 DAT and 96 DAT. Another rice plot with the same size, which was opposite the treatment plot and was separated by the 4-metres width walkway, did not receive any bacterial spray. This plot was used as the nil control. Experiments were conducted in the same plots for three consecutive seasons; when the first trial was carried out between April-July 2015, the second trial was conducted between October 2015-January 2016, and the third trial was done between April-July 2016.

Sampling rice with GD symptom for disease assessment

The severity of GD was assessed at 110 DAT based on the standard evaluation system for rice (SES) (IRRI 2002). The scale of GD severity was classified based on the grains with severely discoloured glumes in the panicle. The scale 0 represented no incidence, scale 1 represented less than 1%, scale 3 represented 1-5%, scale 5 represented 6-25%, scale 7 represented 26-50%, and scale 9 represented 51-100%.

In the growing season 1 (April-July 2015), rice panicles with GD symptoms were collected diagonally from ten locations across the rice plots which had been either sprayed or not sprayed with bacterial culture. Within this diagonal path, five rice panicles with GD symptoms were collected from each location at approximately equal length from the start until the end of this path. Of the 50 panicles collected, 40 with GD symptoms collected from the rice plot which had been sprayed with the bacterial culture were assessed by five assessors who were not involved in this research. These assessors were also asked to assess the severity of another 40 rice panicles with GD symptoms that had been collected from the nil control rice plot. After 10 minutes of inspecting both groups of rice panicles, assessors were asked to differentiate the severity of GD symptoms and determine which group of the samples had the lower GD severity, after which their responses were recorded. The other remaining ten rice panicles with GD symptoms from each treatment were used to assess the severity of GD based on SES (IRRI 2002).

In the growing season 2 (October 2015-January 2016), rice panicles with GD symptoms were collected from seven locations along the width of the rice plots that had been either sprayed or not sprayed with the bacterial culture. The sample collection path was started two metres from the inner walkway that was used to separate the two treatments. Within this path, ten rice panicles with GD symptom were collected from each location at approximately equal length from the start until the end. Seventy rice panicles with GD symptoms from each treatment were used to assess the severity of GD based on SES (IRRI 2002).

In the growing season 3 (April-July 2016), the rice panicles with GD symptoms were collected in the same manner as described in the growing season 2, except that ten rice panicles with GD symptom were collected along the width of the rice plot from ten locations across the rice plots which had been either sprayed or not sprayed with the bacterial culture. One hundred rice panicles with GD symptom from each treatment were used to assess the severity of GD based on SES (IRRI 2002).

Seed germination test after storage for 3 months

Rice panicles from the field sprayed with bacterial culture and that of the nil control which had been used for GD assessment as described above, were put separately in paper bags and stored in a plastic box for three months, under the laboratory room temperature (26°C–32°C). After three months, both samples of the rice seeds were threshed and healthy seeds without GD symptoms were collected. The germination rate of these seeds was determined using the rolled towel (RT) technique (Canadian Food Inspection Agency 2012). Four hundred seeds (with 100 seeds/replication) from each treatment were used to determine the percentage of seed germination.

Statistical analysis

Data collected were subjected to statistical analysis using the R program (R-language and environment for statistical computing and graphics). Both mean values ($\pm$ SE) of the severity of GD and the percentage of seed germination

of the three consecutive growing seasons (as replicates) were compared using the Duncan's Multiple Range Test.

RESULTS

Growth of the bacterium

The growth of the bacterial culture using NB peaked six hours after adding the water-soluble granules to the solution of the medium with the OD at 0.67 and no further bacterial growth in NB was detected after 12 hours. In contrast, growth of the bacterial cultures using either PDB or CHFE peaked simultaneously at 30 hours with the OD at 0.58 and 0.18 respectively. At 36 hours, further bacterial growth in both PDB and CHFE cultures was not detectable.

After 24 hours, the turbidity of the bacterium in NB culture was at 6×10^8 (CFU/mL) based on the McFarland turbidity standards (McFarland scale 2).

The efficacy of bacterial culture in NB in suppressing GD and its effect to rice seed germination

When asked to select the samples with comparatively low GD severity, all five assessors choose the samples that had received the bacterial sprays. Bacterial spray obtained from NB was effective in reducing the severity of GD based on SES (IRRI 2002) when the rice field sprayed with the bacterium was compared with that of the nil control with statistical significant difference (Table 1). Bacterial spray did not affect the percentage of seed germination of healthy rice seeds (Table 1).

Table 1 Mean ($\pm$ SE) of the scale of grain discoloration (GD) severity (scale 0-9) and germination (%) of the rice seeds (*Oryza sativa* L.) (var. RD 31) of the three consecutive growing seasons. Means in each column followed by the same letter are not significantly different at $P=0.05$.

Treatment	GD severity	seed germination (%)
Bacterial spray	4.36 $\pm$ 0.34 b	93.33 $\pm$ 0.64 a
Nil control	6.97 $\pm$ 0.30 a	97.17 $\pm$ 0.56 a

DISCUSSION

Various formulations of *B. megaterium* have been produced previously and their efficacy have been tested in a greenhouse and a small rice plot to control rice sheath blight disease (Kanjana-manee-sathian et al. 1998; Wiwattanapatapee et al. 2004; Wiwattanapatapee et al. 2007; Chumthong et al. 2008; Wiwattanapatapee et al. 2013; Chumthong et al. 2016). Water-soluble granules of *B. megaterium* (Chumthong et al. 2008) were also effective at controlling GD when sprayed in rice field trials (Kanjana-manee-sathian et al. 2009). However, effective GD control with the spray of water-soluble granule containing *B. megaterium* could be achieved only when a large amount of this formulation (at 200 g/20 litres of water for 0.16 ha/one spray) had been applied three times to the rice field (Kanjana-manee-sathian et al. 2009). This requirement may be too demanding for rice farmers to implement and they may choose to use available chemical fungicides instead due to their assured control efficacy after spraying only once. For this reason, the water-soluble granule containing *B. megaterium* has been investigated for use as an inoculum to produce active cells of *B. megaterium* in the simple liquid culture, using NB, PDB or CHFE as possible sources of nutrients for supporting the growth of the bacterium. A single culture of *B. megaterium* using NB produced more cells in a shorter time than the other cultures tested so it was chosen for subsequent field trials. Previous results for another beneficial bacterium for rice production *Klebsiella variicola* C1DO (Wimalasena et al. 2017) also showed that NB is much more suitable than CHFE for use both to support growth of *K. variicola* C1DO and to induce the synthesis of indole acetic acid (IAA). However, CHFE is much more accessible by the farmers because it is much cheaper than NB and it is readily available in all convenient stores. The bacterium in CHFE as nutrient could be used to grow cultures in the amounts required to encourage farmers to use beneficial microorganisms, like *B. megaterium*. The impact of CHFE as nutrient to the improved efficacy of *B. megaterium* in growth promotion and disease suppression in rice should be investigated further.

Bacterial spray was effective in reducing the severity of GD in the field trials (Table 1) but still requires three bacterial sprays which may limit implementation by farmers. However, the bacterial sprays may suit the need of those farmers who produce rice organically. Possible replacement of NB with CHFE as a source of nutrient for supporting bacterial growth should also boost the possibility of transferring this simple liquid culture to organic rice farmers for use to control GD.

Although the efficacy of the bacterial sprays in reducing GD severity in this preliminary study has been demonstrated in the same rice plot for three consecutive growing seasons (Table 1), these field trials had been conducted without spatial replications. Future field trials with proper spatial replications (either in the same rice field or in the field in the different locations) should be carried out to validate the efficacy of bacterial sprays in controlling GD.

In general, the bacterial sprays did not affect seed germination (Table 1) and there are no previous reports of *B. megaterium* reducing seed germination in rice. In fact, *B. megaterium* (isolate A12ag) has been reported to be effective in enhancing rice germination (Sapsirisopa et al. 2009).

Further research should also be carried out to produce the inactive, but viable, endospores of *B. megaterium* in other forms (such as in a sterile filter-paper matrix) so that these can be used as inoculum starters in simple liquid cultures instead of water-soluble granules to increase the shelf-life of the spores.

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