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Biological control of rice sheath blight disease with formulation of indigenous *Trichoderma* strains under paddy field conditions

Shahram Naeimi¹, Vahid Khosravi², Mohammad-Zaman Nouri², Hassan Hoda¹, Csaba Vágvölgyi³, László Kredics^{3*}

¹Department of Biological Control Research, Iranian Research Institute of Plant Protection, Agricultural Research Education and Extension Organization, Tehran 19395, Iran

²Rice Research Institute of Iran, Mazandaran Branch, Agricultural Research Education and Extension Organization, Amol 46191-91951, Iran

³Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary

ABSTRACT The effectiveness of indigenous *Trichoderma* strains in preventing sheath blight disease was evaluated during two growing seasons under paddy field conditions. Broom sorghum seeds were used for mass production of *Trichoderma* strains. Colonized seeds were ground to powder and mixed with talc and carboxymethyl cellulose. Suspensions were made from the bioformulations and sprayed onto rice plants. Effects of *Trichoderma* strains on disease incidence and severity as well as yield and other growth parameters were determined and compared with a chemical fungicide and a commercial biofungicide. A combined analysis of variance across two years was performed and a statistically significant effect of year, treatment and their interaction was reported. Results indicate that environmental factors and different biological fungicides had a strong effect on disease development under natural conditions. According to the results, propiconazole and *T. harzianum* AS12-2 resulted in the least disease severity and incidence. Overall, the efficacy of *T. harzianum* AS12-2 in reducing sheath blight development was significantly better than other *Trichoderma* treatments and was comparable to the conventional fungicide.

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*Correspondent author

E-mail: kredics@bio.u-szeged.hu

Introduction

Rice (*Oryza sativa* L.) is the staple food crop of over half of the world's population living mainly in Asia (Qin and Zhang 2005). Sheath blight of rice caused by *Rhizoctonia solani* Kühn AG1-IA is a very destructive disease of high-yielding varieties under favorable conditions for the pathogen. This disease is widely spread in most of the rice growing areas of the world and causes considerable yield losses (Ou 1985). Chemical fungicides are primarily used for sheath blight control (Rabindran and Vidhyasekaran 1996), but management of the disease is difficult because *R. solani* survives in soil as sclerotia and in rice plant tissues as mycelia (Jayaprakashvel et al. 2010). However, the application of chemical fungicides has several disadvantages, such as the development of resistance in the pathogen, phytotoxicity, toxic effects on human health, pollution of the environment, high costs and loss of biodiversity (Groth et al. 1990; Sehajpal et al. 2009). Therefore, considering the negative impacts

due to indiscriminate use of synthetic pesticides, it has become necessary to adopt ecofriendly approaches to avoid environmental pollution and ensure better crop management. Biological control using microbial biopesticides provides an attractive and environmentally safe alternative to chemical fungicides in the management of rice sheath blight (Mew and Rosales 1986; Vasantha Devi et al. 1989). The application of beneficial microorganisms in agriculture has recently become more important and several commercial biopesticides and biofertilizers are already on the market (Kumar et al. 2014).

Fungi belonging to the genus *Trichoderma* are ubiquitous over wide geographic areas of the world with the broadest impact on mankind (Kredics et al. 2014). Among the fungal biocontrol agents, *Trichoderma* spp. are the most promising in controlling foliar and root diseases of several crops (Elad 2000; Mathivanan et al. 2004). At present, several commercial formulations of *Trichoderma* are available worldwide as biopesticides, biofertilizers, growth enhancers and stimulants of natural resistance. Due to changing of the public viewpoint on consumption

of chemical-free food and protecting the environment, use of *Trichoderma*-based products can be expected to increase in the future (Woo et al. 2014).

Several *Trichoderma* isolates showed good potential in inhibiting *R. solani* AG-IA *in vitro* and in suppressing the sheath blight under greenhouse conditions (Roy 1977; Gokulapalan and Nair 1984; Cumagun and Lapis 1993; Khan and Sinha 2007; Naeimi et al. 2010, 2011; Chen et al. 2015), but their application for the control of the disease under natural conditions is limited (Nagaraju et al. 2002; Mathivanan et al. 2005; De França et al. 2015).

Many biocontrol agents (BCAs) have been found effective in inhibiting the growth of different plant pathogens under *in vitro* and greenhouse conditions, but they often fail to control the disease in fields and orchards (Alabouvette et al. 2006; Gerbore et al. 2014). Ecological factors, plant growth stages, the time and rate of application, the inoculum levels of the pathogen and the applied BCA, the diversity of the natural population of the pathogen, the method of delivery and other factors may affect the biological control efficacy in natural environments.

Before developing a biofungicide, production, formulation and application strategies need to be carefully studied (Burgess 1998). In addition, the adaptation of a promising BCA to the agroecosystem and specific habitat in which it will be applied should primarily be evaluated (Ojiambo and Scherm 2006). In the case of rice sheath blight, the soil borne *R. solani* causes foliar damage, therefore the ideal BCA should survive in the phyllosphere (Chen et al. 2015) and the biocontrol success depends on the application and survival of antagonist populations at the target site (Savazzini et al. 2009).

In our previous study, several native *Trichoderma* isolates obtained from the paddy fields of northern Iran were screened against *R. solani* *in vitro* and their efficiencies in controlling rice sheath blight were evaluated under greenhouse conditions, where significant disease reduction was achieved (Naeimi et al. 2010). The present study was conducted to investigate the biocontrol efficacy of the four most effective strains as a talc-based powder formulation in preventing sheath blight disease development in field plots artificially infested with the pathogen over two successive years.

Materials and Methods

Field design

All trials were conducted in a paddy field located at the experimental farm of the Rice Research Institute of Iran, Amol, Mazandaran (36° 28' 691" N | 52° 27' 744" E, 30 m altitude), over two growing seasons (April-September 2012 and 2013). The field was divided into 24 plots; each

with a size of 1 × 2 m, resulting in a total of 4 × 8 = 32 rice hills. Rice seedlings (Shiroodi, a susceptible, high-yielding cultivar) were raised in the seedling bed for 25 days and transplanted in the main field with a spacing of 25 cm between and within rows. Agronomic practices followed state recommendations for rice production and the recommended dose of fertilizers (100:30:80 kg of NPK/ha) was applied to the field. No chemical pesticides were applied to the experimental plots unless otherwise mentioned. The crop was harvested at maturity and threshed to calculate crop yield. The study was continued in the same field with the same layout during the next growing season.

Pathogen and biocontrol agents

The fungal strains applied in this study are listed in Table 1. A virulent strain of the sheath blight pathogen *Rhizoctonia solani* AG1-IA (RBL1) isolated from a naturally infected rice plant was used in all experiments. The *Trichoderma* strains used in this study (*T. harzianum* AS3-5, *T. harzianum* AS12-2, *T. virens* AD1-3 and *T. virens* AS16-22) had shown biocontrol activity against *R. solani* *in vitro* and suppressed the sheath blight incidence under glasshouse conditions in a previous study (Naeimi et al. 2010).

Table 1. Fungal strains applied during this study

| Fungal strain | Habitat | Location | GenBank accession numbers | |
|----------------------------|-----------------|---------------|---------------------------|--------------|
| | | | ITS | <i>tef1a</i> |
| <i>T. harzianum</i> AS12-2 | Paddy soil | Chaloos | EU821789 | FJ618586 |
| <i>T. harzianum</i> AS3-5 | Paddy soil | Amol | EU821780 | FJ618586 |
| <i>T. virens</i> AD1-3 | Rice debris | Freydoonkenar | EU821794 | FJ618575 |
| <i>T. virens</i> AS16-22 | Paddy soil | Sari | EU821795 | FJ618575 |
| <i>R. solani</i> RBL1 | Infected sheath | Amol | HM211085 | n/a |

Inoculum preparation of *R. solani*

Five mycelial discs (5 mm in diameter) cut from the margin of 3-day-old culture of the pathogen (*R. solani* strain RBL1) on potato dextrose agar medium (Merck, Germany) were inoculated into 2 L Erlenmeyer flasks containing 1 kg autoclaved rice husk and rice bran (1:1) and incubated at 28 ± 1 °C for 30 days.

Preparation of talc-based formulation of *Trichoderma* strains

For mass production of *Trichoderma* strains, broom sor-

ghum (*Sorghum vulgare* var. *technicum*) grains were soaked in water overnight. Then, 300 g of grains were filled in 1 L Erlenmeyer flasks and sterilized in an autoclave for 30 min in three successive days. After cooling the grains to room temperature, three mycelial discs (5 mm) cut from the edge of growing colonies of *Trichoderma* strains were aseptically transferred to the flasks, which were subsequently kept at ambient laboratory temperature (26-32 °C) for 30 days and periodically shaken in order to have uniform growth of BCAs. After this period, *Trichoderma* inocula were taken out from the flasks, air dried, ground to a powder with Mortar Grinder Pulverisette 2 (Fritsch, Germany) and mixed well with extra fine pure talc powder (Merck, Germany) and carboxymethyl cellulose (CMC; BDH, England) as sticker at a 1:2:0.005 ratio under aseptic conditions. The mixture of talc and CMC was autoclaved for 30 min for two consecutive days before adding the BCAs. All formulations were packed in plastic containers, sealed and stored at room temperature until use. In the first year of the experiment, the populations of *T. harzianum* AS3-5, *T. harzianum* AS12-2, *T. virens* AD1-3 and *T. virens* AS16-22 in the formulation were 6×10^9 , 2.5×10^{10} , 2.0×10^{10} and 8.0×10^9 cfu/g of formulation, respectively at the time of application. Similarly, in the second year the populations of these four strains were 6.1×10^9 , 2.5×10^{10} , 1.8×10^{10} and 7.9×10^9 cfu/g of formulation, respectively.

Pathogen inoculation and application of biocontrol agents

To ensure uniform disease incidence and severity during both growing seasons, 40 days after transplanting at the maximum tillering stage of rice (one week after the first application of biocontrol formulation), 5 g of the prepared *R. solani* inoculum was placed among tillers in the centre of twelve rice hills per replication near the waterline (Tang et al. 2007).

Formulations of each native *Trichoderma* strain were dissolved in water, thoroughly mixed, filtered through two layers of cheesecloth (after 30 min) and sprayed four times on rice foliage at 750 L/ha as I) 7 days prior to pathogen inoculation; II) on the following day after inoculation of *R. solani*; III) 7 days and IV) 14 days after the second application. Tween 20 (Merck, Germany) at 0.05% was added to the suspensions as a surfactant before spraying. Trium P (Koppert, the Netherlands), a commercial biofungicide which contains *Trichoderma harzianum* KRL-AG2 (T-22) (1×10^9 cfu/g) was also applied as above on rice foliage as a comparative treatment according to the manufacturer's instruction. All BCAs were sprayed on the phyllosphere of rice with a 2 L hand sprayer (EVA, Dimartino, Italy) after sunset. Propiconazole (TILT[®], 250 EC, Syngenta, Switzerland) was used as the chemical fungicide control and applied as a foliar spray (1 L/ha) 2 days after inocula-

tion. Plants inoculated only with *R. solani* were considered as inoculated control. Furthermore, for non-inoculated control, plants were sprayed with distilled water and no input (BCA and/or fungicide) was applied.

Disease assessment

Two weeks after artificial inoculation of the pathogen (AIP), 6 hills from each treatment per replication were randomly selected and tagged to observe the sheath blight incidence [in terms of the percentage of infected tillers (PIT)]. For assessing sheath blight severity, the relative lesion height (RLH) of 5 tillers from each 6 randomly selected hills was first calculated and then the disease severity was recorded according to the 0-9 scale of Standard Evaluation System developed by the International Rice Research Institute (IRRI 2002). The incidence and severity of sheath blight was also determined 35 days after AIP to see whether the formulations still had an effect on the prevention of disease development. Agronomic traits of rice including plant height (PH), number of tillers/hill, crop yield and 1000-grains weight (GW) were measured.

Meteorological data

All climate data including air temperature, relative humidity (RH), precipitation and sunlight hours were taken from Amol Weather Station located about 300 m from the experimental area.

Statistical analysis

All field experiments were established as a randomized complete block design with three replicates. A combined analysis of variance (ANOVA) was performed to determine the effect of year and treatments on disease development and agronomic traits, using the SAS software ver. 9.1.3 (SAS Institute, Cary, NC). All means of the treatments were compared by Duncan's multiple range tests at $P \leq 0.01$.

Results

Rice sheath blight severity and incidence were notably decreased in some BCA-treated plots under field conditions. The effect of year on RLH_{14} , PIT_{14} , 1000 GW, plant height ($P \leq 0.01$) and No. of tillers ($P \leq 0.05$) proved to be significant according to the results of the combined analysis of variance for both years (Table 2). Disease severity (in terms of RLH) and incidence (in terms of PIT) after 14 days as well as growth parameters were lower for the second year. The effect of treatment on all characteristics except for plant height and No. of tillers was significant at $P \leq 0.01$ (Table 2). In the case of traits with significant treatment \times year interactions, including RLH_{14} , PIT_{14} and RLH_{35} , ANOVA was performed for each

Table 2. F-values for the effects of year, treatment and their interaction according to combined ANOVA over two years (2012-2013)

| Source of variation | Degree of freedom | F-value | | | | | | | |
|---------------------|-------------------|-------------------|-------------------|-------------------|-------------------|--------|---------|---------|----------------|
| | | RLH ₁₄ | RLH ₃₅ | PIT ₁₄ | PIT ₃₅ | Yield | 1000 GW | PH | No. of tillers |
| Year | 1 | 40.21** | 1.14 | 28.6** | 0.61 | 5.31 | 308.9** | 99.74** | 16* |
| Treatment | 7 | 21.03** | 17.19** | 19.56** | 17.31** | 6.13** | 4.63** | 0.93 | 2.2 |
| Treatment × Year | 7 | 5.05** | 2.57* | 6.46** | 2.16 | 1.49 | 0.96 | 0.5 | 1.69 |

RLH= relative lesion height, PIT= percentage of infected tillers, GW=grain weight, PH=plant height. *, **: Significant at 5 and 1%, respectively.

growing season and means were compared using Duncan’s multiple range test (Table 3). The minimum sheath blight incidence was recorded for propiconazole (PIT₃₅ = 57.01%) and *T. harzianum* AS12-2 (PIT₃₅ = 60.67%) 35 days after AIP ($P \leq 0.01$) (Fig. 1). Non-treated plots showed the highest yield (6433 kg/ha) and 1000-GW (27.79 g). It is important to note that agronomical characteristics were not significantly different among *Trichoderma*-treated and fungicide-treated plots (data not shown).

According to the first year data, 14 days after AIP, *T. harzianum* AS12-2 and propiconazole provided the lowest disease scores (2.55 and 2.78, respectively) (data not shown). The minimum RLH₁₄ of 22.18 and 24.19 were recorded for *T. harzianum* AS12-2 and propiconazole, respectively, when compared to the inoculated control (Table 3). In addition, among the treatments, *T. harzianum* AS12-2 was found to be most effective in reducing disease incidence (PIT₁₄ = 36.35%), which was less than in the case of chemical fungicide-treated plots (PIT₁₄ = 72.27%). Other treatments were not significantly different in terms of disease incidence 14 days after AIP. Triarium P led to the lowest reduction of disease severity (RLH₁₄ = 36.36%) and incidence (PIT₁₄ = 98.21%) compared to the native biocontrol agents. Thirty five days after AIP, the lowest disease scale (3.44) and RLH₃₅ (27.87%) were

observed in propiconazole-treated plots. Formulation of native *Trichoderma* spp. together with Triarium P - although not significantly different from the chemical fungicide - resulted in relatively higher disease severity ranging from 44.22% to 51.73% (Table 3).

In the second year of the experiment, *T. harzianum* AS3-5, propiconazole and *T. harzianum* AS12-2 showed the lowest disease scores 14 days after AIP, as compared to the control (0.37, 0.48 and 0.55, respectively). The RLH₁₄ values of the aforementioned treatments were 4.03%, 4.85% and 6.4%, respectively, which were not significantly different (Table 3). These treatments also showed the minimum disease incidences (PIT₁₄ of 7.4%, 9.56% and 12.94%, respectively) and were not significantly different from each other and from the non-inoculated control (PIT₁₄ = 5.87%). Similar to the results of the first year, Triarium P was the least effective in suppressing sheath blight (RLH₁₄ = 25.49%, PIT₁₄ = 93.83%). The lowest disease scales and RLH values were recorded for propiconazole (0.97 and 8.93%, respectively), *T. harzianum* AS12-2 (3.54 and 13.5%, respectively) and *T. harzianum* AS3-5 (3.84 and 30.27%, respectively) 35 days after AIP.

Table 3. Effect of bioformulation of native *Trichoderma* strains on sheath blight incidence (as percentage of infected tillers = PIT) and severity (as relative lesion height = RLH) 14 and 35 days after artificial inoculation with *Rhizoctonia solani* in two successive years

| Treatment | RLH ₁₄ | | RLH ₃₅ | | PIT ₁₄ | |
|----------------------------|-------------------|------------------|-------------------|----------------|-------------------|-----------------|
| | 2012 | 2013 | 2012 | 2013 | 2012 | 2013 |
| <i>T. harzianum</i> AS12-2 | 22.18 ± 5.5 b | 6.4 ± 2.2 bcd | 50.18 ± 14.3 ab | 29.73 ± 7.5 ab | 36.35 ± 16.7 b | 12.94 ± 6.8 bc |
| <i>T. harzianum</i> AS3-5 | 31.28 ± 2.2 ab | 4.03 ± 2.0 cd | 51.73 ± 10.2 ab | 30.27 ± 3.8 ab | 94.79 ± 6.5 a | 7.4 ± 4.6 bc |
| <i>T. virens</i> AD1-3 | 30.10 ± 7.1 ab | 10.19 ± 4.3 abcd | 46.67 ± 2.0 ab | 58.1 ± 5.3 a | 91.67 ± 7.2 a | 25.73 ± 14.3 bc |
| <i>T. virens</i> AS16-22 | 31.10 ± 1.6 ab | 22.57 ± 7.7 abc | 44.22 ± 17.1 ab | 61.63 ± 0.8 a | 79.36 ± 18 a | 58.85 ± 20.9 ab |
| Triarium P | 36.36 ± 4.4 a | 25.49 ± 0.45 ab | 51.24 ± 4.1 ab | 44.94 ± 3.3 a | 98.21 ± 1.5 a | 93.83 ± 5.4 a |
| Propiconazole | 24.19 ± 4.9 b | 4.85 ± 2.4 cd | 27.87 ± 16.6 b | 8.93 ± 4.2 b | 72.27 ± 14.4 a | 9.56 ± 6.5 bc |
| Inoculated control | 35.97 ± 2.5 a | 30.06 ± 1.5 a | 55.50 ± 3.3 a | 48.07 ± 4.8 a | 98.1 ± 2.5 a | 99.57 ± 0.7 a |
| Non-inoculated control | 0.20 ± 0.07 c | 2.86 ± 1.4 d | 0.72 ± 0.34 c | 13.5 ± 7.4 b | 1.52 ± 1.1 c | 5.87 ± 3.4 c |

Values (means ± SE) within a column followed by the same letter(s) are not significantly different according to the Duncan’s multiple range test ($P = 0.01$)

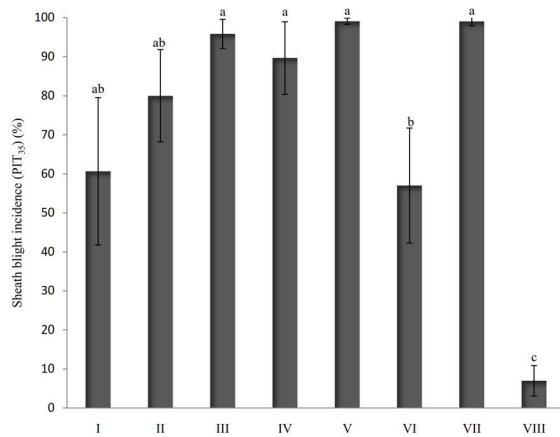


Figure 1. Effect of different treatments on sheath blight incidence (as percentage of infected tillers = PIT), 35 days after artificial inoculation with *Rhizoctonia solani* in two successive years.

I: *Trichoderma harzianum* AS12-2; II: *T. harzianum* AS3-5; III: *T. virens* AD1-3; IV: *T. virens* AS16-22; V: Biofungicide (Triatum P); VI: Chemical fungicide (propiconazole); VII: Inoculated control; VIII: Non-inoculated control. Values represented by the same letter(s) above the bars are not significantly different according to Duncan's multiple range test ($P = 0.01$). Each value represents the mean of three replicates.

Discussion

In order to commercialize a promising BCA as a microbial biopesticide, it has to be evaluated under the natural environmental conditions with a suitable formulation (Fravel and Larkin 1996; Alabouvette et al. 2006). In this study, four native *Trichoderma* strains were examined for prevention of the rice sheath blight disease and promotion of plant growth and grain yields under paddy field conditions. Application of talc-based formulations of some autochthonous *Trichoderma* strains significantly reduced the sheath blight severity and incidence caused by *R. solani*, which was comparable with the chemical fungicide propiconazole.

Although Shiroodi is a rice cultivar susceptible to sheath blight, the natural infection by the pathogen was very low in both years. On the other hand, the disease score recorded for inoculated control was not very high. This suggests that mild severity of sheath blight is probably related to environmental conditions. Optimum conditions for sheath blight infection and development are high temperature (30–32 °C), high RH (>95%) and shading (Ou 1985). Meteorological data indicate that temperature, RH and sunlight hours during AIP to the second disease evaluation were below the optimum conditions of infection and development of pathogen in the field.

Initially, some biocontrol agents demonstrated effective biocontrol properties, but for a longer period of time

they were not able to satisfactorily suppress the disease. For instance, although *T. harzianum* AS12-2 promisingly controlled sheath blight, even better than the chemical fungicide at first, disease severity increased at the harvesting stage in the plots treated with this strain. This is consistent with findings of Perelló et al. (2009), who reported that although *Trichoderma* spp. effectively reduced the severity of *Septoria* blotch at the tillering stage of wheat, they failed to control the disease at the heading stage. It is speculated that despite four times of application of *Trichoderma* strains in the field, probably due to inappropriate environmental conditions, high microbial competitions and presumably other factors, they could not maintain or increase their populations and adopt to the rice phylloplane, therefore they failed to inhibit the disease development until the end of the season. Effective antagonists must become established in crop ecosystems and remain active against the target pathogens during the periods favorable for infection (Lo et al. 1998). *Trichoderma* strains are not common phyllosphere inhabitants (Elad and Kirshner 1993; Latorre et al. 1997), and if they are applied to above-ground plant parts, their populations may decrease by a factor of 10–100 within a period of 2 weeks (Freeman et al. 2004). According to the climate data for the time span between the first application of BCAs and the second disease assessment (40 days), the average air temperature was 28 °C in both years which favored the fungal BCAs. However, the limiting factor was very likely the RH and wetness time of the rice phyllosphere. The average RH values during the mentioned time in 2012 and 2013 were 55% and 61%, respectively. Moreover, the average precipitation was less than 1 mm and 1.6 mm, respectively. Many fungi, including *Trichoderma* strains need moisture for germination, growth and colonization, therefore the low moisture and dryness of the rice phylloplane may ultimately have negative effects on the antagonistic potential of the applied BCAs in the field. The average sunlight hours in the first and second year were 7 h and 8 h, respectively. Direct exposure to solar radiation reduces conidial production, survival, germination, and can kill the conidia of most fungal species, which limits the size of the fungal population and reduces the efficacy of fungal BCAs (Braga et al. 2015). The deleterious effects of ultraviolet (UV) radiation on conidia have been demonstrated in several fungi including *Trichoderma* spp. (Moody et al. 1999; Stevenson and Weimer 2002). Solar UV may therefore have negatively affected the survival and biocontrol potential of native BCAs in the current study after field application. Under the aforementioned conditions, *R. solani* developed to panicles as well as neighboring tillers during the time between the two disease evaluations. This suggests that although native *Trichoderma* strains effectively suppressed the pathogen

during the initial stage of disease development, but for an extended period of time, when environmental conditions are conducive to *R. solani* or the BCAs can not adapt to the rice phylloplane, sheath blight may eventually develop, although still at a slower and less severe rate. The high inoculum load of *R. solani* applied in this study might also have contributed to the failure of the BCAs to provide striking disease control.

The BCAs applied during this study did not promote plant growth and grain yield. This is in disagreement with other works which were stating that talc formulations of *Trichoderma* not only significantly control the sheath blight disease, but also increase the growth and grain yields in the paddy field (Mathivanan et al. 2005; Khan and Sinha, 2007; Soe and De Costa, 2012). Lorito and Woo (2015) stated that although *Trichoderma* spp. are regarded as plant growth promoting microbes, this property is not present in every strain. It is important to note that in this study, the talc powder inside the formulation formed a white layer on rice leaves, which did not disappear for a long time due to low precipitation. This may negatively affect photosynthesis, respiration and ultimately the plant growth and yield. Consequently, changing the carrier/bulking agent or reducing the amount of talc powder in the current formulation may result in growth promotion along with an acceptable level of disease control.

In conclusion, findings of the present field study indicate the feasibility of *Trichoderma* strains for the biocontrol of rice sheath blight disease in the field as an alternative disease control strategy and formulations of *T. harzianum* AS12-2 can be recommended as one of the crop protection strategies for the management of sheath blight. Future research should be directed toward the optimization of the existing formulation, in order that the applied strain can be more capable of becoming established and surviving in the rice phylloplane. This will facilitate the commercialization of the biocontrol *Trichoderma* strain and the introduction of the resulting biofungicide to the market. Moreover, further experiments are needed to find out the appropriate amount, time and frequency of application.

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