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Biological control of potential antagonistic bacteria isolates to restrict *Magnaporthe grisea* infection on rice

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Rice blast caused by *Magnaporthe grisea*, frequently affects rice in the world. This research is intended to screen biological control agents for controlling *M. grisea*, referencing the study biological control agents testing approaches, since biological control is an environmentally friendly plant disease controlling approach. 710 bacterial isolates were discovered from rice tissues, of which hopeful biological control scores were discovered referencing their abilities in antagonism inhibition and secreting extracellular hydrolytic enzyme. Biological control discovery against *M. grisea* were experimented on 35 bacterial strains with hopeful biological control characteristic examining through amplified ribosomal DNA restriction analysis (ARDRA) and BOX analysis on isolates with high assessment scores. Five biological control agents (BCAs) with protection efficacy of more than 40% in greenhouse and field experiment were discovered. *Pantoea ananatis* HS-8 and *Bacillus cereus* DL-7 performed well in greenhouse experiment, and field test respectively. In general, correlation coefficient is 0.95 between assessment scores of 35 experimented BCAs and correlation coefficient between antagonism test and biological control efficacy show 0.72 against *M. grisea*. Biological control efficacies results in greenhouse and field experiments showed positive correlation with assessment scores, proposing that the BCAs evaluating and screening method set-up is reference for screening BCAs for controlling *M. grisea.*

Key words: Biological control agents (BCAs), biological control efficacy, extracellular metabolites, *Magnaporthy grisea.*

INTRODUCTION

Magnaporthy grisea is one of the most important diseases of the many diseases that attack rice. Failures of entire rice crops have resulted directly from rice blast epidemics. All of the plant disease management strategies and techniques that have been generated have been brought to bear against rice blast, but often with limited success. Rice blast has never been eliminated from a region in which rice is grown, and a single change

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in the way rice is grown or the way resistance genes are deployed can result in significant disease increase even after years of successful management. This disease is a model that demonstrates the seriousness, elusiveness, and longevity of some plant diseases. Rice blast has been widely studied throughout the world. Many investigators have considered it to be a model disease for the study of genetics, epidemiology, molecular pathology of host parasite interactions and biology (Xiao et al., 2015), which helped discover new biological control agents, for agriculture crop protection (Tokpah et al., 2016).

Biological control agents (BCAs) antagonistic to plant pathogens are a sustainable strategy for plant protection (Chen et al., 2013). Successful biological control based on plant associated antagonists not only requires a better knowledge of the complex regulation of disease suppression by antagonists in response to biotic and abiotic factors, but also requires a knowledge of the dynamics and composition of plant-associated bacterial communities and what triggers plant colonization (Cretoiu et al., 2013). However, research of screening efficient biological control agents could be used to limit the fungal pathogen (blast). The study intend to discover bacterial strains with potential biological control characteristic from different rice parts, through efficient and useful BCAs experimenting strategy based on activities of extracellular hydrolytic enzymes, antagonism inhibition ability and phylogenetic analysis and BOX fragment sequencing. The practical results of greenhouse and field experiments are conducted for controlling *M. grisea* and the correlation is analyze between antagonism and biological control efficacies, assessment score, and biological control efficacies. Finally, conidia germination and appressorium penetration on the rice leaf, which led to the control of *M. grisea* in actual production (Qi et al., 2012).

MATERIALS AND METHODS

Plants and bacteria culture

M. grisea strain *Guy11* was used in this study. Conidiation strain blocks were maintained on strew decoction and corn (SDC) media at 28°C for 7 days in the dark followed by 3 days of continuous illumination under fluorescent light (Shlipak et al., 2013). Biological control isolates were cultured on Luria-Bertani (LB) agar at 28°C for 1 to 3 days. Rice cultivar, particular Nanjing 47, used in this experiment is widely planted by framers. Plastic pots (30 cm bottom diameter and 35 cm height) were filled with soil rich in humus, which had been sterilized at 121°C for 1 h three times individually on three simultaneous days, were sown with rice seeds in greenhouse experiment. An insect-free greenhouse preserved at 20 to 35°C with relative humidity of 70% and a 12 h/12 h day/night photoperiod (600-µmol photons/m^2/s of light supplied during the daytime) was where plants were grown.

Separation of rice habitant isolates

Rice samples, collected from farmers' fields in Taicang, Jiangsu Province in China were separated from surface and interior of stems, endorhiza, rhizosphere, endosphere and phyllosphere individually for rice habitant isolates and bacterial isolates. Surface and interior of its stem, or its root system, three grams fresh weight (FW) of soil, roots, stems or leaves were placed into a sterilized Erlenmeyer flask and suspended in 27 mL of a sterile 0.85% NaCl solution while screening of bacteria from rice rhizosphere. The suspension was incubated at 25°C with shaking at 180 rpm for 30 min and then settled for 5 min; the resulting supernatant was serially diluted, plated on R_2A medium plate (for soil samples) or LB agar (for tissue samples), and incubated at 28°C for 72 h to obtain cultures (form, color and texture) containing 50 to 300 CFU. Colonies with different morphologies from each microenvironment were transferred to LB agar, purified, and then stored at -70°C in LB broth containing 40% glycerol referenced by Balsanelli et al. (2016). Three gram (FW) of sampled (leaf, stem and root), was first soaked in 1% sodium hypochlorite (NaClO) for 5 min and then in 70% ethanol for 2 min assumed to be surface-sterilized, rinsed three times with sterile water, and finally imprinted on R_2A agar plates to screen endophytic bacteria and check sterility. Sample of 3.0 g sterile, was placed in a sterilized mortar including 27 mL of sterile 0.85% NaCl solution and homogenized with a sterilized pestle. Later 10^{-1}, 10^{-2} and 10^{-3} aliquots were taken from 0.1 ml extracts of each sample and smeared on R_2A plates, and incubated at 28°C for 72 h referenced by (Tokpah et al., 2016).

Screen for antagonism towards *M. grisea*

Hundred microliters of the supematants were diluted to obtain aliquots of 10^{-4} to 10^{-6} ml, which were smeared on WA medium. A WA plate medium was inoculated with *M. grisea* hyphae block in the center, and with a candidate antagonistic strain, which one bacterial colony was picked with sterile toothpick, in 3 cm away from the block, and incubated at 28°C for 48 to 72 h. Activity of *in vitro* antagonistic was graded with 0, 1, 2 or 3 based on the diameter (in cm) of the semicircular hyaline zones after 48 to 72 h: Grade 0, no antagonism; grade 1, (1-5 cm); grade 2, (5.1-10 cm); grade 3, >10 cm described by Tokpah et al. (2016).

Activities of extracellular hydrolytic enzymes and siderophores evaluation

In vitro activities of their extracellular hydrolytic enzymes (cellulase, chitinase, glucanase, protease and siderophores), which were indicated by distinct semi-circular hyaline zones around bacterial colonies on specific agar media were examined for bacterial strains. Activity of cellulase was discovered as described by Marjamaa et al. (2013), chitinase activity was tested in minimal medium (Cretoiu et al., 2013), and glucanase activity was examined referencing to Tomkins et al. (2013). Skim milk agar (50 mL of sterilized skim milk mixed at 55°C with 50 mL of 1/5 WA medium containing 2% agar) was used for the detection of protease activity, which was indicated by casein degradation (Durante et al., 2013). Determined expression of siderophore's was done as previously referenced (Tokpah et al., 2016).

Evaluation of possible biological control agents for their biological control ability

A biological control evaluation method was discovered to assess each BCA with different importance referencing to their antagonistic activity and enzyme producing activity (Tokpah et al., 2016). The *in vitro* antagonistic reaction was graded with 0, 1, 2 and 3 based on the diameter (cm) of the transparent-circular zones: No antagonism (0 score); 1-5 cm (1 score); 5.1-10 cm (2 scores); > 10

cm (3 scores). The ability of strains to produce cellulose, chitinase and glucanase were reference the same way. For scoring protease and siderophore production, each value was halved, as protease might play better role in biological control of nematode and siderophores is better in biocontrol of bacterial pathogens, rather than controlling fungal pathogen (Siddiqui et al., 2005). In addition, grade test is the same as accounted in antagonism method and 12-referenced highest assessment score of each bacterial isolate.

Identification of bacterial isolates, by phylogenetic analysis and BOX fragment sequencing

For ARDRA analysis, 89 bacteria DNA was prepared using the Mini BEST Bacterial Genomic DNA Extraction kit Takara Bio Inc. The partial nucleotide sequence of the amplified 16S rDNA was determined using the following primers: L1494-1514 (reverse) 5'-CTA CG (or A) G TA CCT TGT TAC GAC-4' in an automated DNA sequencer (Durante et al., 2013). Amplification was performed with a Pettier Thermal Cycler PTC-200 (Bio-Rad, Watertown, MA, USA) using an initial denaturation step at 94°C for 5 min, and subsequently 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 2 min and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. The PCR products (10 µl) were digested for 2.5 h using the restriction enzymes AluI and MspI. The restriction fragments were separated on a mix gel (1.5% agarose + 2.25% Synergel) running in 1.0 × TBE buffer at 80 V for approximately 5 h, and then stained with ethidium bromide, and photographed under UV transillumination. The experiment was repeated three times to examine the reproducibility of the results.

35 bacterial DNA was prepared using the Mini BEST Bacterial Genomic DNA Extraction kit. BOX-PCR was carried out as described by Rademaker and De Bruijn (1997) using the BOX A1R primer 5'-CTA CGG CAA GGC GAC GCT GAC G-4'. Amplification was performed with a Peltier Thermal Cycler PTC-200 (Biozym Diagnostic, Hessisch Oldendorf, Germany) using an initial denaturation step at 95°C for 6 min, and subsequently 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min and extension at 65°C for 8 min, followed by a final extension at 65°C for 16 min. A 5 µl aliquot of amplified PCR products were separated by gel electrophoresis on mixed gel (0.5% agarose + 0.75% Synergel) in 1.0 × TBE buffer at 120 V for 6 h, stained with ethidium bromide, and photographed under UV transillumination (Bio-Rad). The reproducibility of the results was verified in three independent experiments.

Greenhouse experiment

Thirty-five bacterial strains were grown in LB individually at 28°C with 280 r/min for 24 h carefully shaking in greenhouse experiment. Then, bacterial cells were pelleted by centrifugation, washed and suspended in a sterile 0.85% NaCl solution, and well-adjusted to 5 × 10^5 CFU/mL with water for use. Thirty-five bacterial treatments and 2 control treatments were investigated for their biological control efficacies in greenhouse experiments with 24 rice plants per replicate.

Mycelia plugs were grow on PDA medium at 25°C for three days and then inoculated on SDC medium, incubated at 28°C for 3 days, and transferred to a dark chamber for 5 days. The fungus conidia were harvested using 15 ml sterile distilled water containing 0.5% gelatin and used to inoculate the plants. The suspension was filtered through four layers of sterile cheesecloth, and kept in a flask 4°C. Conidia concentration was measured using a hemocytometer and adjusted to 10^5 conidia/ml, before spraying. Inoculation was done in the evening by spraying the *M. grisea* spore suspension (at 15 ml/per replicate). After inoculation, the plants were well kept in the dark chamber and covered with black plastic sheets for 24 h, in order to stimulate infection. Thereafter, the plants were exposed simultaneously to 12 h of light and 12 h of dark for up to three to seven days. At 21 days after inoculation, recorded disease grade were reference and statistically assessed. Relative disease severity and biological control efficacy were calculated with the referencing formula. Rice plant were scored for the disease severity (DS) using a scale of (0-5) as described: 0 indicate no symptoms; 1 indicate typical blast lesions with elliptical shapes measuring 1 to 2 cm long and usually confined to the area of the two main veins and infecting < 2% of the total leaf area; 2 indicate typical blast lesions infecting 10 to 25% of the leaf area; 3 indicate typical blast lesions infecting 26 to 50% of the leaf area; 4 indicate typical blast lesions infecting 51 to 75% of the leaf area, and 5 indicate all leaves that are dead respectively (Harish et al., 2008).

Biological control efficacy and relative disease severity were calculated as follows:

Relative disease severity (%) = [∑ (The number of diseased plants in each grade × the number of grade) / (Total number of plants investigated × the highest disease grade)] × 100%

Biological control efficacy (%) = [(Relative disease severity of Control 1-Disease severity with bacterial treatment) / Disease severity of Control 1] × 100

Examination of Conidia germination, and appressorium penetration on the rice leaf

Bacteria strains were cultured on LB medium plates for spores germination while *Guy11* strains were maintained on straw decoction and corn (SDC) medium at 28°C for a week in the dark followed by 3 days of continuous illumination under fluorescent light. Conidial germination and appressorium formation were measured on a hydrophobic surface (Qi et al., 2012). Appressorium formation rate was counted according to preceding study (Zhang et al., 2011). More than 100 appressoria were observed for each replicate and the experiments were repeated three times. The conidia germination, and appressoria penetration on the rice leaf was observed using an epifluorescenc microscope (Shlipak et al., 2013).

Field test

The field tests were on private land located in Taicang, Jiangsu province, China, referencing GPS coordinate, as N 32.420364, E 121.398103 and the field studies did not involve protected or endangered species. Six biological control agent's treatments were set in the field trials (according to the assessment criteria) and water treatment as mock control, each treatment comprising of 3 replicates and 24 rice plants per replicate. Each treatment includes 1 m^2 normal growth rice field. BCAs suspensions were prepared as reference in greenhouse experiment. Rice ears were deal with by spraying 1 L 5 × 10^5 CFU/mL of BCAs suspension per treatment when rice field come to vegetation stage. After 5 days of BCAs treatment, 1 L 1 × 10^5 CFU/mL *Guy11* spore suspension was sprayed on each treatment and black plastic sheets were used to keep moisture around individual ear. The disease grade was statistically assessed and reference in one week after pathogen inoculation. Relative disease severity and biological control efficacy and relative disease severity were calculated as above according to Harish et al. (2008).

Statistical analysis

Clustering analysis was performed using the unweighted pair grouping method based on arithmetic averages (UPGMA) in order

to determine the population structure of the isolates. After deleting the isolates with same potential biological control characteristics and BOX fingerprint the selected bacterial strains were examine by sequencing their 16s rRNA gene, and the sequences were compared, using the basic local alignment search tool (BLAST), with the reference sequences in the Nucleotide Sequence Database of NCBI (National Center for Biotechnology Information).

Biological control efficacy and relative disease severity were subject to two ways analysis of variance (ANOVA) referencing the statistical software Data Processing System (DPS version 7.05) to determine the differences among the treatments. The means of treatments showing significant differences were separated at the 5% level of significance using the Fisher's least significant difference (LSD) test in order to determine the best treatment. Microsoft Excel 2010 (Microsoft Corporation) was used to calculate the conventional correlation coefficients of the biological control efficacy of isolates with their assessed biological control potential assessments basing on production of siderophores antagonism and extracellular hydrolytic enzymes *in vitro*, antagonism. In addition, Microsoft Excel 2010 Microsoft Corporation was used to calculate the coefficients of, conidia germination, and appressoria penetration on the rice leaf and was observed using an epifluorescenc microscope (Shlipak et al., 2013).

RESULTS

Examining isolates with potential biocontrol efficacy against *M. grisea* disease

Rice samples were gathered from farmers filed in Taicang of which bacterial population density was discovered and referenced in (Table 1). Seven hundred and ten bacterial strains were isolated from rice tissue, comprising root, surface and interior of stems, endorhiza, phyllosphere, soil, and rhizosphere. Generally, the amount of bacterial isolates from surface of plant tissues was higher than those from interior (Table 1). Activities of extracellular hydrolytic enzymes (cellulase, chitinase, glucanase, protease and siderophores) were measured, and the bacterial isolates and as well as antagonistic ability were evaluated with reference method (Table 1).

ARDRA and BOX analysis of potential biological control efficacy strains

Eighty-nine isolates with more than 2 in the evaluating score were chosen out of 710 bacterial isolates, and subjected to ARDRA/BOX fingerprint analysis to avoid redundancy in further analysis. The isolates were separated to 10 clusters on 65% similarity (Figure 1A), of the 10 clusters with 1 isolate accounted for cluster 1, 2, 7, 9, 10 and 2 clusters with at least 2 isolates cluster 6, 8, while, others clusters with 4 strains, 29 strains and 47 isolates accounted for 3, 4 and 5 (Figure 1A). Thirty-five bacterial strains with highest evaluating score were taken from each cluster; BOX-PCR experiment was taken to rearrange them into 6 clusters with very important variability (Figure 1B).16S rRNA gene fragments were amplified from genome of these 35 bacterial strains and identification of physiological and biochemical shows that HS-8 and DL-7 are *Pantoea ananatis* and *Bacillus cereus* strains, and other stains were identified using similar method (Table 2).

Biological control efficacy experiment against *M. grisea* under greenhouse state

Greenhouse test were carried out with 35 selected bacterial strains and 6 isolates with apparent biological control efficacy (from 50%, up Table 2) which approved HS-8, DL-7, DS-26, HL-22, HR-12 and DR-42. Two strains (HR-12, and DR-42) were isolated from rhizosphere, two (DL-7, and HL-22) from phyllosphere, and two (HS-8, and DS-26) from stem sample of rice plant.

Conidia germination and Appressorium penetration on rice leaf treated with (HS-8 and DL-7)

Examination of conidia germination and appressorium formation, found that there were 70% increased rate of conidia germination at 8 h and 20% decreased rate in appressorium formation at 24 h among isolates referencing significant difference at ($P<0.05$) in the rate of conidia germination and appressorium formation between isolates (HS-8 and DL-7) to control (Figure 2A and B).

Correlation assessment between assessment scores and biological control efficacy

Six isolates were received with significant biological control efficacy (more than 50%) in greenhouse experiment and two isolates with significant biological control efficacy in field experiment. Pearson correlation assessment was use to examine the association between their biological control efficacy and the assessment scores based on those statistics (Table 2). The correlation coefficient between antagonism test and greenhouse test results is 0.72 (Figure 3A), correlation coefficient between assessment scores and greenhouse test results is 0.95 (Figure 3B). However, *P. ananatis* HS-8 and *B. cereus* DL-7 showed significant results in greenhouse and field experiments. HS-8 and DL-7 evidently decrease *M. grisea* severity in greenhouse and field experiments (Tables 2 and 3).

DISCUSSION

With the alarming news of rice blast disease, and the aggravation of global warming on the rice crop, *M. grisea* has caused serious loss not only in China but also in other parts of the world (Graham et al., 2013).

Biological control agents were separated in different

Table 1. Antagonistic activities of metabolic enzymes on isolates screened from rice tissue.

Strain habitat	Crop growth stage[A]	Bacteria concentration (CFU/g)[B]	Number of bacterial strains	Isolates with antagonism activity and metabolite enzyme activity[C]					
				Antagonism test	Chitinases	Cellulases	Proteases	Glucanase	Siderophores
Surface stem	Tiller stage	6.4×10^{6}	59	25	5	8	4	4	4
	Booting stage	6.6×10^{6}	80	42	4	15	7	12	4
	Ripe stage	6.2×10^{5}	31	15	1	6	2	3	3
Interior stem	Tiller stage	6.3×10^{4}	22	9	2	5	1	1	0
	Booting stage	6.7×10^{5}	36	15	2	4	4	2	3
	Ripe stage	5.5×10^{6}	28	8	0	3	1	2	2
Endorhiza	Tiller stage	3.9×10^{3}	30	6	0	2	0	2	2
	Booting stage	3.8×10^{4}	43	13	1	5	3	2	2
	Ripe stage	6.9×10^{7}	21	4	1	1	0	2	0
Endosphere	Tiller stage	6.5×10^{3}	18	10	1	6	1	2	0
	Booting stage	3.3×10^{8}	12	7	0	3	1	1	2
	Ripe stage	4.3×10^{5}	8	4	1	0	1	0	2
Phyllosphere	Tiller stage	5.6×10^{6}	66	12	0	8	1	2	1
	Booting stage	7.5×10^{6}	80	21	6	10	0	2	3
	Ripe stage	6.4×10^{6}	28	6	0	2	0	1	3
Rhizophere	Tiller stage	4.9×10^{5}	39	16	3	8	0	3	2
	Booting stage	6.7×10^{5}	41	19	4	8	2	3	2
	Ripe stage	4.2×10^{6}	25	5	0	1	1	2	1
Soil	Tiller stage	3.2×10^{6}	11	5	0	3	0	1	1
	Booting stage	4.3×10^{5}	19	8	2	2	1	3	0
	Ripe stage	5.5×10^{6}	13	6	2	1	1	2	0

(A) BCAs screened from two-growth period of rice, tillering and heading periods. (B) Bacteria concentration represents the total bacteria concentration screened in tissue of rice samples. (C) *M. grisea* strain *Guy11* use for antagonistic experiment. Isolates which have a noticeable halo on WA medium confront cultured with fungal pathogen were called antagonist. The width of the clear semicircular halo surrounding the bacterial streak was measured after incubation.

positions (rhizosphere, surface and interior of stems, endorhiza, phyllosphere, soil, and root) from non-infected/infected rice in this experiment. Bacterial strains with antagonist activities were examined from rice *vivo* tissue (Table 1); demonstrating *vivo* plant habitats might include more bacteria cultural antagonism to plant pathogen as proposed (Villarroya et al., 2016). It might also be related with adaption in the microbial communities in infested fields (Bulgarelli et al., 2013).

To discover acceptable origin of potential BCAs is to obtain successful biological control and assess the vivo and *in vitro* of the crop. Previous, research has mainly focused on isolating nitrogen-fixing bacteria (Balsanelli et al., 2016) and plant growth-promoting bacteria (Corcione et al., 2013).

On the contrary, the study focus is on biological

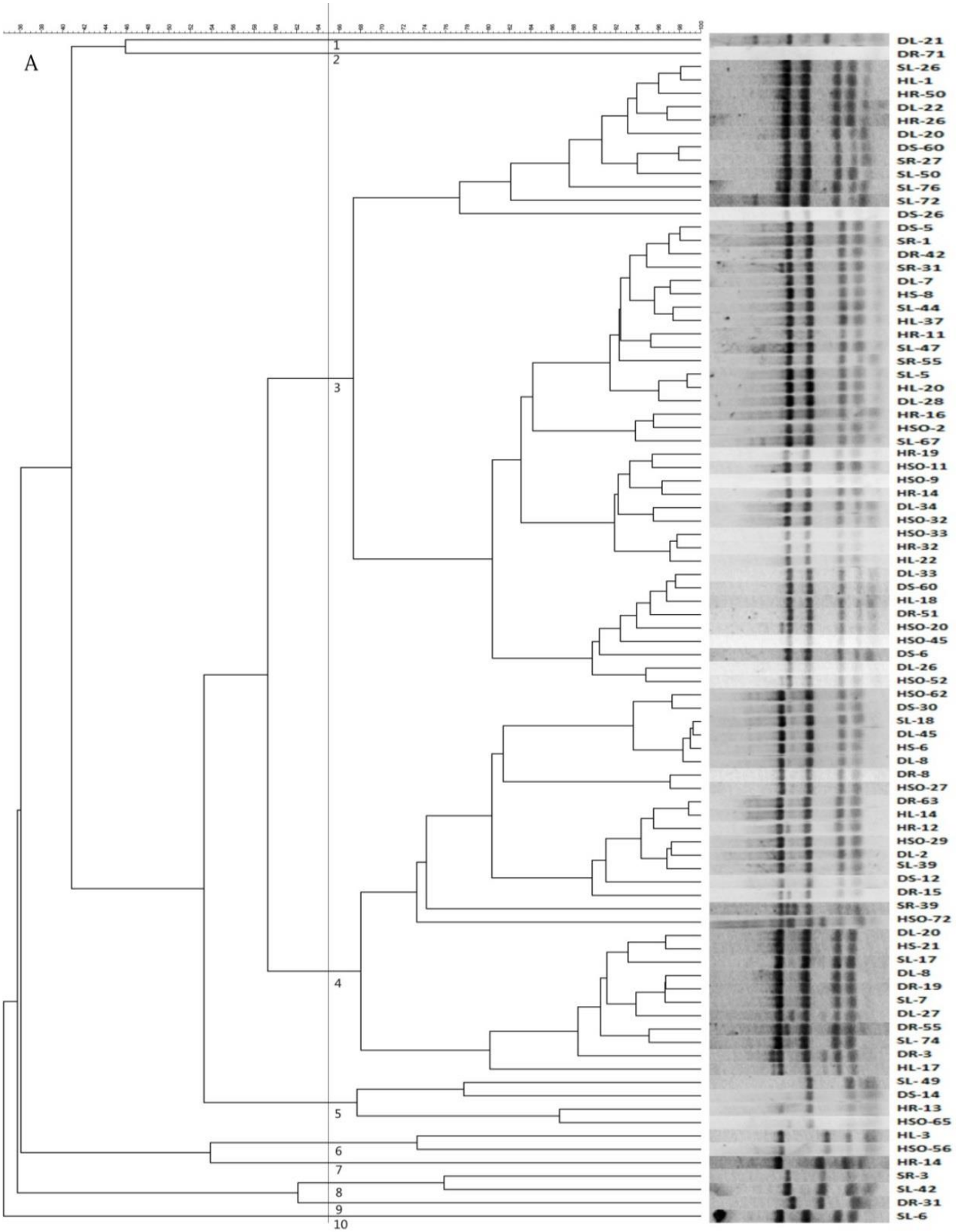

Figure 1. Analysis of the fingerprint (A) ARDRA and (B) BOX. The dendrogram was constructed using GelCompar®II version 4.5 (Applied MathsBVBA). The analysis was performed using Pearson correlation applied to the densitometric curves reference by Rademaker and De Bruijn (1997), followed by clustering analysis using the unweighted pair-rouping method based on arithmetic averages (UPGMA).

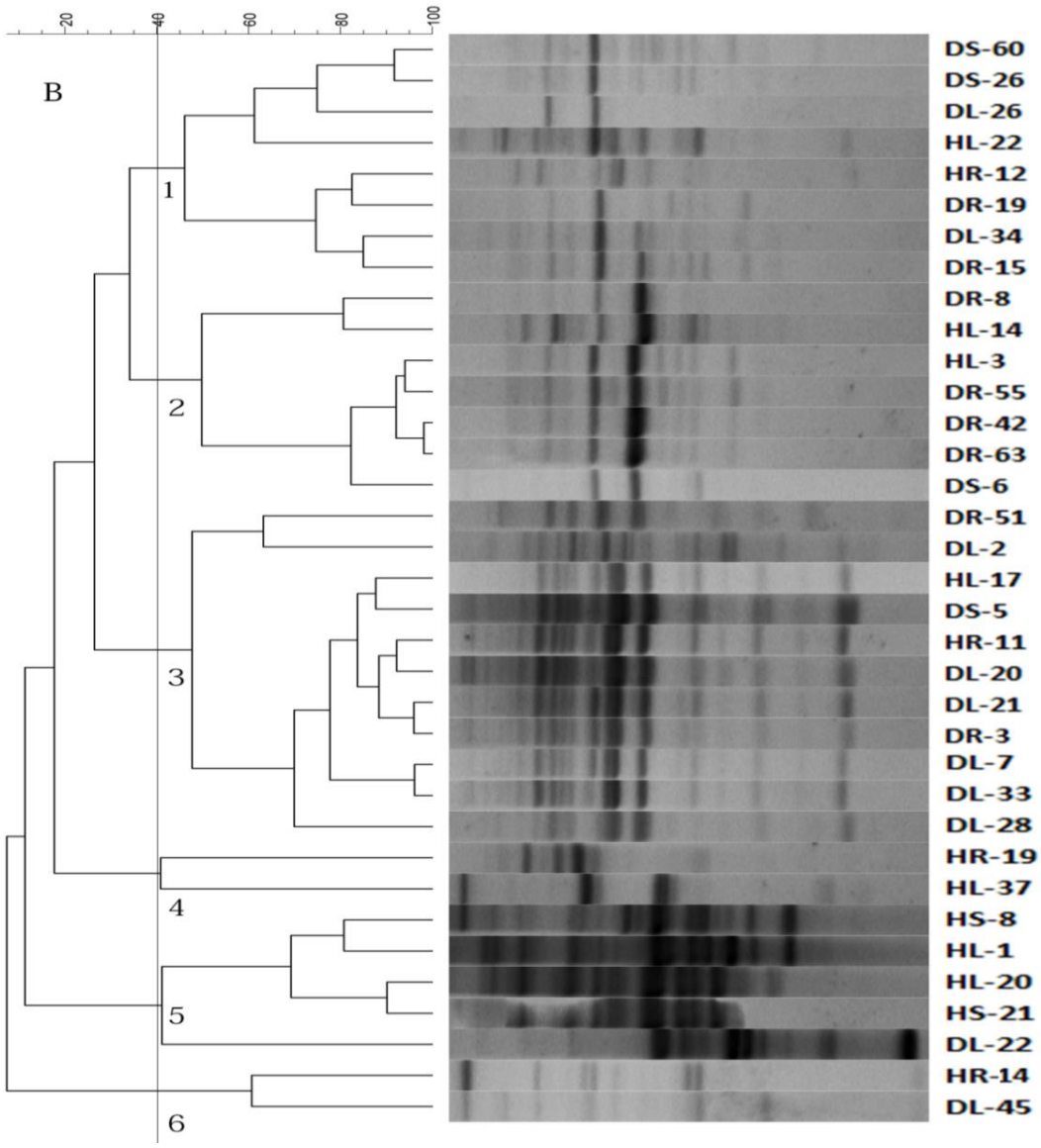

Figure 1. Contd.

control isolates to restrict *M. grisea* infection on rice (Figure 4A and B) which, suggest that antagonistic activity against *M. grisea* and activities of hydrolytic enzymes, were in essential to the biological control agents. Thus, recommending the study assessment strategy for testing biological control agents in similar works.

ARDRA and BOX classifies assessment strategy and BCAs helped avoid using biological control agents of same species in greenhouse and field test and receive different genetic background, which reduce workload in follow-up test. In other experiments, classifying assessment strategy based on genetic background was not included (Er et al., 2016), which resulted in duplication of same species, which is time-consuming.

Biological control success may depend on suitable formulations as well as survival of the microbial agents. Bacteria as biological control agents have advantages over fungal when applied as a preventive application to suppress the disease. In line with this study, several strains of bacteria isolated from rice plant were previously evaluated for their antagonistic ability against *M. grisea* (Tokpah et al., 2016). Six BCAs were acquired with more than 50% which displayed preferable biological control ability (Table 2).

Moreover, those six BCAs with more than 50% showed significant correlation in greenhouse with strains' assessment score but some disparity of acting in field experiment against *M. grisea* (DR-42 and HR-12), showing that similar BCA isolates might give different

Table 2. Recognition and assessments of 35 bacterial isolates disease severity and biological control efficacy in controlling *M. grisea* caused by *Guy11.*

Strains	Identify results	Similarity (%)	Atangonim value	Proteae value	Cellulse value	Chitinse value	Glucanse value	Siderophores value	Scores	Disease severity (%)	Biological control efficacy (%)
HS-8	*Pantoea* sp.	80	3	1	3	1	2	2	12	16.67±5.56^{qr}	86.50
DL-7	*Bacillus cereus*	98	2	1	1	2	2	2	10	18.52±3.21^{qr}	84.00
DS-26	*B. cereus.*	99	2	1	3	0	1	1	7	33.21±4.97^{op}	77.00
HL-22	*Bacillus.* sp.	99	2	0	1	1	1	1	6	35.19±8.49^{nop}	71.50
HR-12	*Enterobacter* sp.	98	2	1	2	0	1	0	6	36.11±2.78^{mnop}	70.25
DR-19	*B. cereus*	99	1	0	1	1	0	0	3	45.19±7.14^{ghijklmn}	32.00
DL-34	*B. cereus*	99	2	0	1	0	0	1	3	49.81±3.16^{fghijkl}	32.75
DR-15	*B. cereus*	99	0	0	0	0	0	0	2	53.7±6.42^{defghij}	27.50
DR-8	*Bacillus subtilis*	99	1	0	1	0	0	0	2	54.32±13.4^{defghi}	26.67
HL-14	*B. cereus*	99	1	0	0	0	1	0	2	53.02±9.92^{efghijk}	28.42
HL-3	*B. subtilis*	99	2	0	0	0	0	0	2	54.07±6.42^{defghi}	27.00
DR-55	*B. subtilis*	99	2	0	0	0	0	0	2	46.11±10.2^{ghijklmn}	24.17
DR-42	*B. pumilus*	92	2	0	0	1	2	0	5	44.07±5.01^{hijklmno}	54.50
DR-63	*Enterobacter* sp.	99	1	0	0	0	0	0	2	47.04±0.32^{ghijklm}	28.50
DS-6	*B. subtilis*	99	2	0	0	0	1	1	3	50.99±7.86^{fghijkl}	31.17
DR-51	*B. subtilis*	99	1	0	1	0	1	0	3	41.67±7.35^{klmno}	33.75
DL-2	*B. pumilus*	85	1	0	1	1	0	0	3	43.09±4.69^{ijklmno}	31.83
HL-17	*B. cereus*	96	1	0	1	0	1	0	3	41.23±2.04^{lmnop}	31.33
DS-5	*B. cereus*	99	0	2	0	0	0	0	2	58.77±7.46^{cdef}	20.67
HR-11	*B. cereus*	99	1	1	0	1	0	0	3	50.68±3.26^{fghijkl}	31.58
DL-20	*B. subtilis*	99	1	0	0	1	0	0	2	54.94±2.47^{defgh}	25.83
DL-21	*B. subtilis*	99	1	0	0	1	0	0	2	52.96±9.71^{efghijk}	28.50
DR-3	*B. subtilis*	99	0	0	1	0	0	0	1	64.81±8.49^{cdef}	12.50
DL-26	*B. cereus*	99	1	0	0	0	0	1	2	56.67±4.01^{cdefg}	23.50
DL-33	*B. cereus*	99	1	0	0	0	1	0	2	54.32±13.4^{defghi}	26.67
DL-28	*Bacillus* sp.	99	1	0	0	0	1	1	3	51.23±4.66^{fghijkl}	30.83
HR-19	*B. cereus*	99	0	0	1	0	0	0	1	63.7±7.56b^{cde}	17.50
HL-37	*B. idriesis*	99	0	0	0	1	0	0	1	65.12±6.17b^{cd}	12.08
DS-60	*B. cereus*	99	0	0	1	0	1	0	2	56.51±2.2^{cdefg}	23.71
HL-1	*Pantoea* sp.	99	0	1	0	0	0	0	1	53.02±9.92^{efghijk}	19.25
HL-20	*Pantoea* sp.	97	0	0	1	1	1	0	3	41.13 ± 2.47^{mn}	30.46
HS-21	*B. cereus*	94	1	0	0	0	0	0	1	60.66 ± 1.43b	13.43
DL-22	*Acinetobacter* sp.	99	0	0	0	1	0	1	2	60.89 ± 3.49b^{c}	29.83

Table 2. Contd.

HR14	*B. cereus*	99	1	1	0	1	0	0	3	31.12 ± 3.35^{q}	35.51
DL-45	*B. cereus*	99	0	0	0	0	1	0	1	58.56 ± 3.52^{cde}	16.58

(A) Genbank library closest isolate, using nucleotide blasting of the 16S rDNA sequence in NCBI (National Center of Biotechnology Information. USA). (B) The percentage means the similarity between our BCAs known bacterial isolates with references in NCBI. (C) The values are Mean ± Std. Deviation; followed by the same letter within a column are not significantly different as discovered by the LSD test ($P < 0.05$).

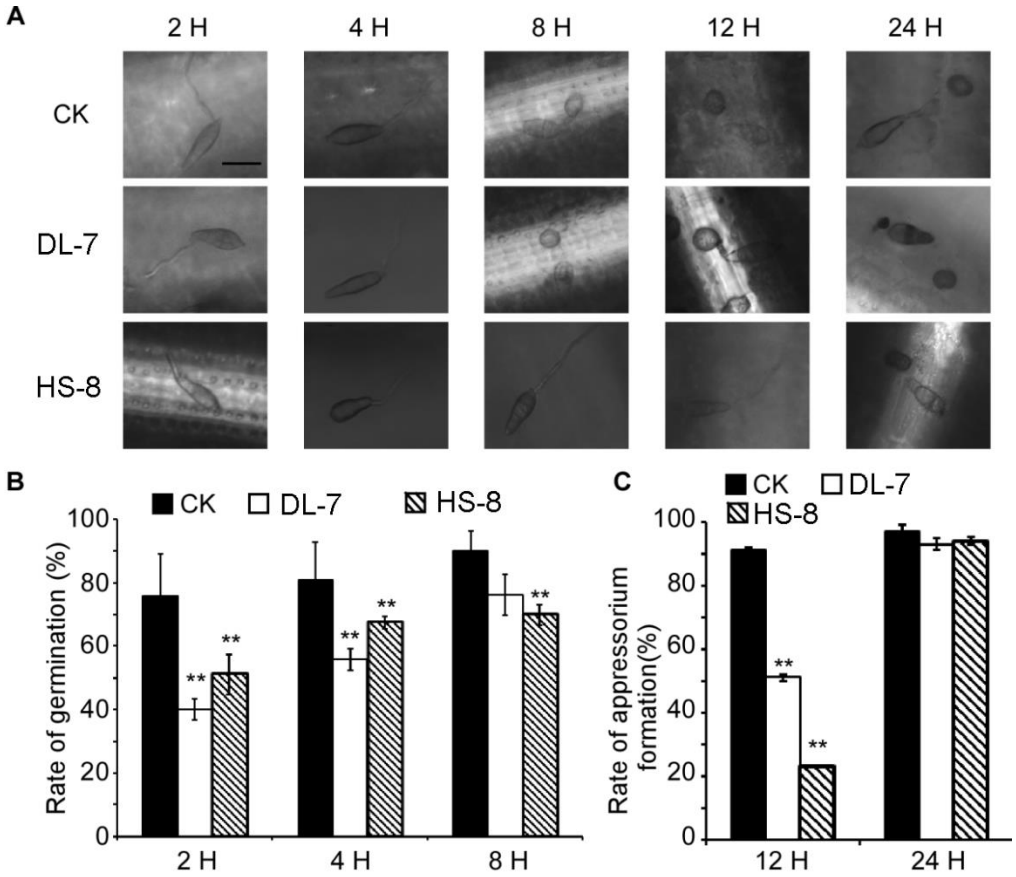

Figure 2. Infectious conidial germination and appressoria growth on rice leaves of strains (HS-8 and DL-7) and control. Analysis for each type of infectious hyphal shape and 100 infecting hyphae were coun1ted per replicate and the experiment repeated three times. (A) Appressoria transformant examined under an epifluorescence microscope and infectious growth was observed at different time points, post-inoculation (hpi). (B) Rice leaves from 14-day-old rice seedling inoculated with conidial suspension (1 ×10^5 spores/ml). (c) Appressorium penetration on the rice leaf.

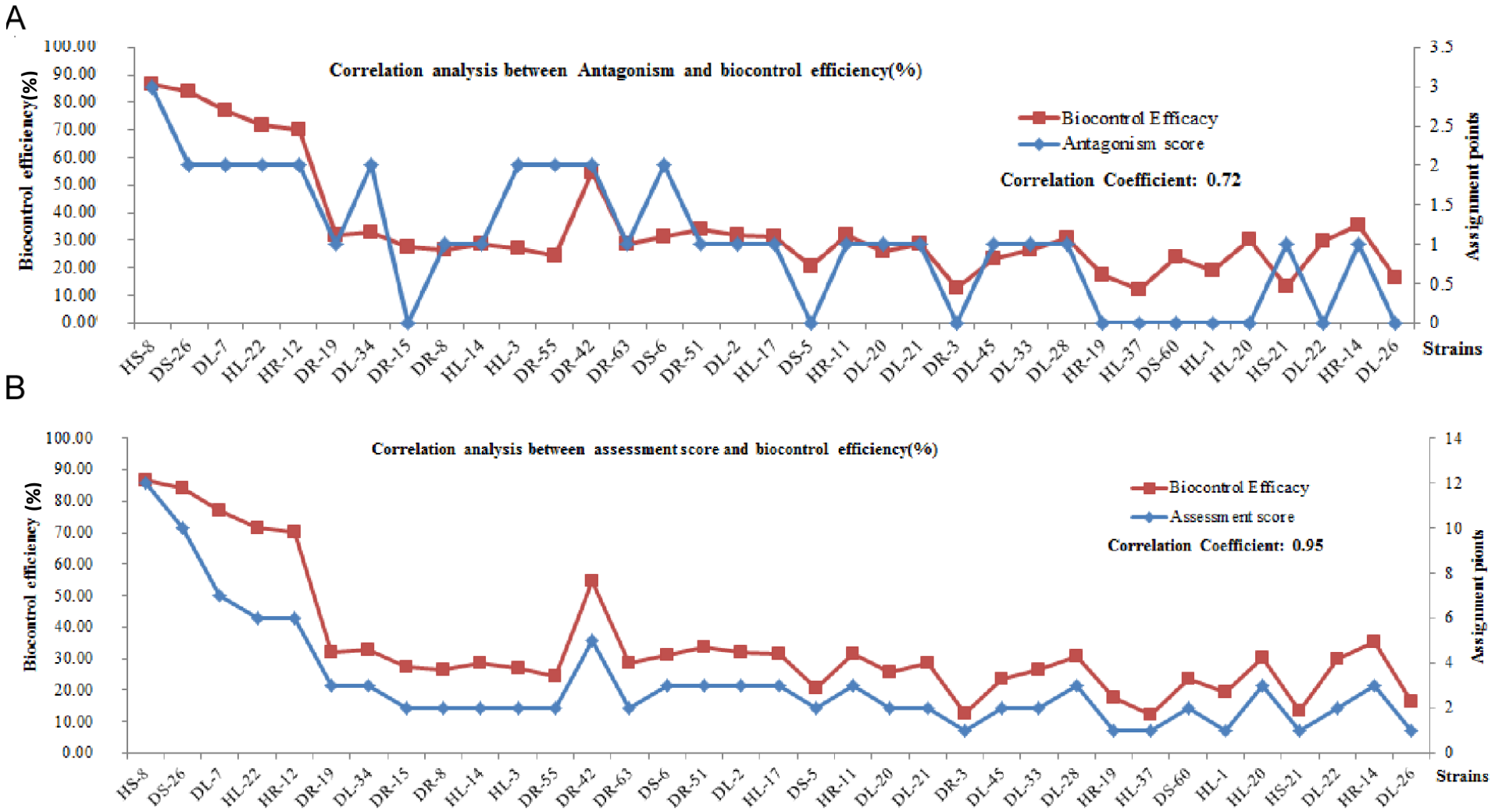

Figure 3. (A) Correlation analysis between antagonism and biological control efficacy to *M. grisea* disease is coefficient 0.72. (B) Correlation analysis between assessment and biological control efficacy to *M. grisea* disease in greenhouse test; Correlation analysis and the coefficient is 0.95. (C) The dark red square rhombus lines representing bacterial biological control efficacy uses the ordinate on the left, while the blue diamond rhombus lines representing the assessment of antagonism and hydrolytic enzymes activities on the right ordinate.

biological control characteristic against *M. grisea* in the same environment. Biological control efficacy was higher in greenhouse and lower in field test (Tables 2 and 3), perhaps resulting to different environmental sites, and temperature (Zaida et al., 2014), with further consequences

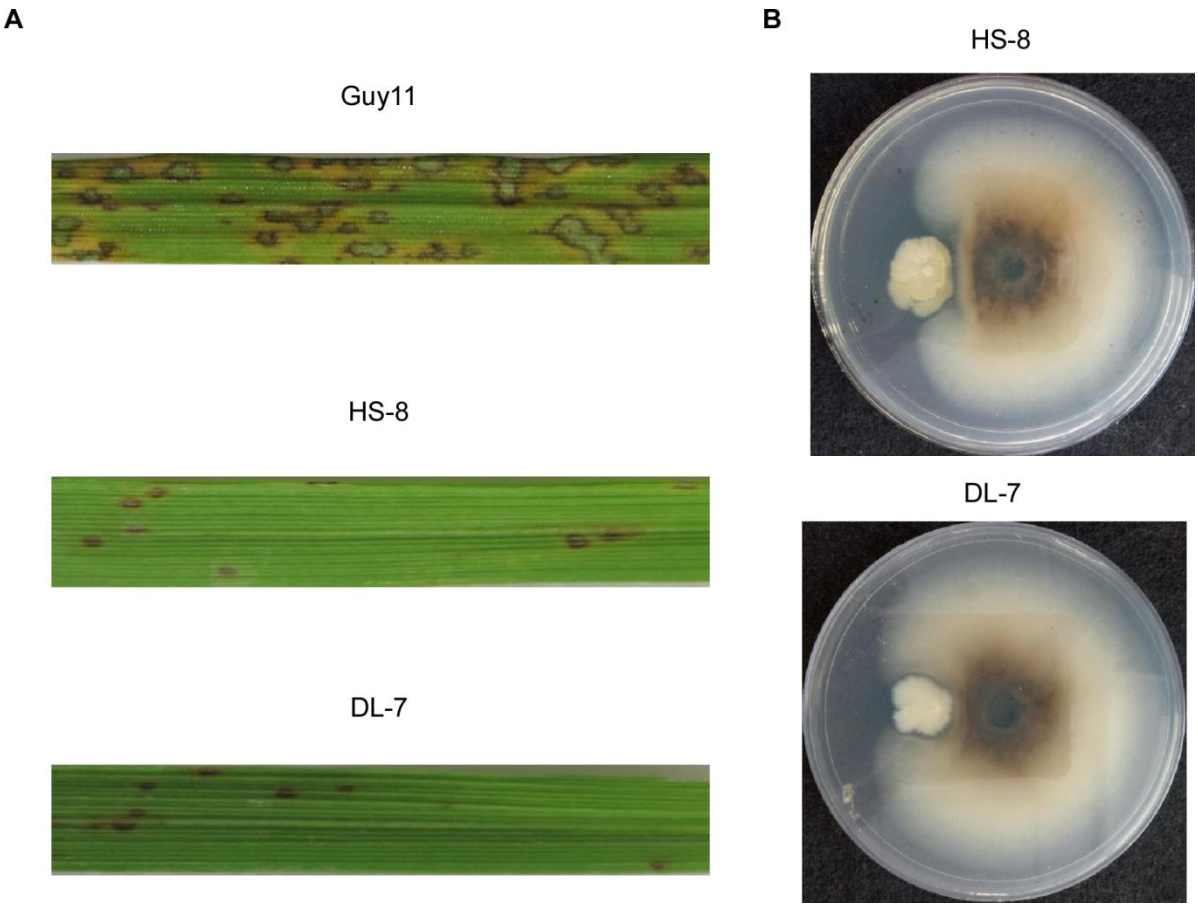

Figure 4. (A) Pathogenicity test on rice leaf plants and conidia suspension (5×10^5 spores/ml) sprayed on rice leaves. Diseased leaf was photographed at 5 day after inoculation. (B) Biological control strains inoculated on WA medium and cultured at 28°C for 7 days.

in different of colonizing abilities. On the other hand, few other characters of bacterial isolates, such as motility and nutrient competing ability, are possibly altered by colonizing condition as well (LeRoux et al., 2015). For separating successful biological control bacteria in different environment, climates and soil types were also considered. Like most other pathogens, conidia of *M. grisea* play a central role in the disease cycle. The infection process is initiated with attachment and conidia germination on the plant surface and appressoria formation from the end of the germ tubes (Figure 2A and B) (Turrà et al., 2015). In this study, more experiment that will focus on the mechanisms of the biological control agents was discovered.

With reference to the authors knowledge, this research is the newest to discover collections of an effective bacteria's, namely *P. ananatis* spp. strain (HS-8) and *B. cereus spp*. strain (DL-7) as BCAs against *M. grisea* based on the study assessment strategy (Tokpah et al., 2016), which showed good feasibility resulting in high correlation coefficient (Figure 3A and B). *P. ananatis* HS-8 and *B. cereus* DL-7 show obvious biological control capacity against *M. grisea* in greenhouse and field experiments while, other isolates with good biological control efficacy in greenhouse (DR-42 and HR-12) did not do well in field experiment (Tables 2 and 3). It can be hypothesize that strains HS-8 and DL-7 may ascribe to better adaptability in various environment as in different colonizing sites even one rice plant might face complete disparate environmental condition. Focusing will be on the mechanism of strains HS-8 and DL-7 referencing BCAs against rice diseases.

Conclusions

In this work, greenhouse experiment results recorded significant correlation with isolates assessment score (0.95), and antagonism and biological control efficacy of greenhouse experiment record 0.72 referencing the study assessment method for choosing BCAs, is also acceptable for *M. grisea* disease. By the study assessment method, five potential BCAs was discovered, especially *P. ananatis* HS-8 and *B. cereus* DL-7 with

good biological control ability against *M. grisea* disease on rice.

CONFLICT OF INTERESTS

Authors declare no conflict of interests.

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