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An improved strategy for stable biocontrol agents selecting to control rice sheath blight caused by *Rhizoctonia solani*

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ABSTRACT

Rice sheath blight caused by *Rhizoctonia solani* Kühnis increasingly threatening rice production in China. DNA fingerprints of 220 *R. solani* strains isolated in 11 provinces of China were established by random amplified polymorphic DNA (RAPD)-PCR. Cluster analysis of strains isolated from the same region showed high similarity, indicating that the genetic diversity of *R. solani* strains is significantly related to geographical origin. We assessed potential bio-control abilities of bio-control agents (BCAs) by values according to inhibition zones against *R. solani*, extracellular hydrolytic enzymes activity and siderophores production in vitro. Fourteen strains with diverse expected bio-control potential were tested for their bio-control efficacy against rice sheath blight caused by 11 pathogenic exemplars and for growth promoting ability, separately. Bio-control efficacy of single bacterium against various *R. solani* strains differed significantly (-36.23% ~ 88.24%), while *Pseudomonas fluorescens* 4aYN11 achieved a relatively stable control efficacy of 32.26%–78.79% and growth promotion of 18.43%. Pearson correlation coefficient between bio-control efficacy of each BCAs and their assessment is 0.717. In the present study, we established an improved strategy for screening stable bio-control agents based on an assessment system, their growth promotion potential and phylogenetic diversity of pathogen *R. solani*, and the result provides us not only one promising bio-control strain 4aYN11 with an average bio-control efficacy of 56.50%, but also a practical way for future screen of novel BCAs.

1. Introduction

Rice sheath blight caused by *Rhizoctonia solani* Kühn is one of the most wide spread rice diseases, causing quality issues and severe loss in rice production worldwide (Lee and Rush, 1983; Rush and Lindberg, 1996). Due to extensive use of nitrogen fertilizers and promotion of high-yield rice varieties (Singh et al., 2010), this disease has gradually become one of the three most severe ones in Chinese rice production.

Since no high resistant rice variety against sheath blight is available, validamycin is still widely used as the main cure for rice sheath blight in China (Xie et al., 1992; McClung et al., 1997; Peng et al., 2014). However, pesticide resistance and environmental issues that may arise with long-term use urges people to search for safer and more effective

means against rice sheath blight (Willocquet et al., 2000). Biological control is widely recognized as both safe and reliable solution for these worries. BCAs are natural origin pesticides and their complex control mechanisms minimize the risk of possible resistance under selection pressure. Recently, a large number of potential BCAs, such as *Harzianum*, *Pseudomonas*, *Bacillus*, *Ceratobasidium* and *Burkholderia* have been reported (Singh et al., 2010; Mosquera-Espinosa et al., 2013; Cuong et al., 2011). The enhanced efficiency by applying the mixture of BCAs and the antibiotic validamycin in controlling rice sheath blight indicating, even larger potential by application study (Peng et al., 2014). Moreover, BCAs have long been used to promote the growth of plants. This phenomenon is widely reported over the last few decades as a great amount of bacterial strains, including species of *Pseudomonas*,

Azospirillum, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus*, *Rhizobium* and *Serratia*, are recorded with growth promotion potential (Saharan and Nehra, 2011). Except for increasing nutrition available for plants, for instance, by solubilizing phosphate or facilitating absorption of iron, BCAs also affect root development and morphology resulting in larger root surface for better growth (Vessey, 2003). BCAs has been applied on model plants including rice to study the growth promotion and proved to be effective in both stress and healthy conditions (Bal et al., 2013; de Souza et al., 2013).

Biological control has notable advantages in consideration of environmental safety, but it is less practical compared to traditional chemical pesticide or chemical fertilizer. Unstable bio-control efficacy is one of the problems encountered in practical applications. Complex environmental condition and lack of compatibility with other pesticides are regarded as the main reason for the instability (Deacon and Berry, 1993). However, in our previous field application, BCAs with quite stable effect against rice sheath blight in one region might partially or even completely lose their control effect in some other region. Thus, we deduced that one of the most important causes of unstable bio-control efficiency is that we only screened BCAs according to their inhibition towards a single strain of a pathogen, regardless of possible different bio-control effect caused by pathogen diversity. This hypothesis was tested in bio-control of *Ralstonia solanacearum* recently (Xue et al., 2013), leading us to believe that more practical BCAs could be obtained after taking pathogen intraspecies genetic variation into consideration in the procedure of bacteria screening.

R. solani, the causal agent of rice sheath blight, has great genetic diversity according to RAPD study on field isolations (Duncan et al., 1993; Ceresini et al., 2002). Here, we collected 220 *R. solani* strains from 11 main rice planting provinces, divided them into 11 clusters according to RAPD analysis, and developed a new strategy for selection of more stable potential BCAs based on assessment including in vitro antagonistic, extracellular enzymes activity and siderophores production and greenhouse assay testing their effect on rice sheath blight caused by pathogens with diverse RAPD characteristics.

2. Materials and methods

2.1. Isolation and identification of R. solani from rice fields

Total 220 rice sheath blight samples were collected from 40 counties in 11 provinces (Anhui, Fujian, Guangxi, Guizhou, Hebei, Henan, Hubei, Hunan, Jiangsu, Sichuan and Zhejiang) (Table S1). Pathogens were isolated from collected samples with water agar method (Zhou and Yang, 1998). The morphology of isolated strains was first validated (Fig. S1). Cultures were grown on PDA media. The pathogenicity was tested using Koch's postulates. Basically, the purified putative pathogens hyphae disks were tied on rice sheath with plastic film. Thirty days later, the plant appears the typical rice sheath blight disease phenotype. The tissues from the junction of disease and health were rinsed with sterile water, cut into small pieces and cultured on PDA medium. The hyphae of acquired fungi were then confirmed to be identical to the inoculum under microscope.

2.2. Anastomosis group identification

Purified *R. solani* hypha was inoculated into 100 mL PDB medium contained in 500 mL flask, shaken at 25 °C for 5d. Mycelium was filtered with multi-layer gauze and drought with sterile filter paper. 50 mg fresh mycelium, 600 μL 2 × CTAB extraction buffer (containing 0.7 M NaCl, 100 mM Tris-HC1 pH 8.0, 20 mM EDTA, 10 g/L PVP-360, 20 g/L CTAB, β-mercaptoethanol final concentration = 0.1%) and 100 mg sterile quartz sand (diameter: 0.180 mm–0.250 mm, Kermel) were mixed and then ground thoroughly in a mortar. The homogenates were transferred into a 1.5 mL centrifuge tube, kept at 65 °C for 30 min

and fully mixed with an equal volume of chloroform/isoamyl alcohol mixture [(chloroform): V (iso-amyl alcohol) = 24:1]. The mixture was centrifuged at 4 °C under 8000g for 5 min. The supernatant was transferred into a new tube, mixed with 10% of 3 M NaAc solution (pH 6.0) and 2.5 vols of frozen ethanol precipitation and centrifuged at 4 °C 10,000g for 5 min. The pellet was collected, washed 3 times with 70% ethanol solution, dried and then stored at −20 °C after fully dissolved with 50 μL ddH$_2$O.

Previously reported PCR primers are used (Matsumoto 2002). Primers 5′-CTCAAACAGGCATGCTC-3′ (forward) and 5′-CAGCAATAGTT-GGTGGA-3′ (reverse) were used to identify the subgroup AG1-IA. Reaction system including 12.5 μL Mix (Guangzhou Dongsheng Company), 0.5 μL primer each, 0.5 μL Taq enzyme, 1 μL DNA as template, 10 μL ddH$_2$O, a total of 25 μL. Reaction program: 94 °C for 4 min, denaturation at 94 °C for 1 min, annealing at 54 °C for 2 min, extension at 72 °C for 3 min, repeat for 30 cycles. After electrophoresis in a 1% agarose gel, the PCR product was stained with EB and analyzed by gel imaging system.

2.3. Random amplified polymorphic DNA (RAPD) assay

Chromosome DNA extraction was carried out as described above. Total 220 identified *R. solani* strains, 20 from each 11 provinces, were selected for analysis.

Total 100 single-stranded primers with 10 bases each were randomly designed. One strain was selected from each province to measure the polymorphic rate of these primers. All primers were classified by each polymorphic rate calculated as amplified polymorphic bands/total number of bands. 20 primers with high polymorphic rate were selected for further study (Fig. S2).

For the phylogenetic tree construction, bands successfully amplified were recorded as "1", otherwise recorded as "0". Nysis 2.10 data analysis software is used to calculate the rate of polymorphism.

2.4. Bio-control agents screening for antagonism towards R. solani

Bacterial strains from BCAs pool of our lab were cultured in LB medium. Single colony of each strain was then picked with sterile toothpick and inoculated 3 cm from the center of the plate along the vertical lines. One plate was left as a control with no bacteria inoculated. Pathogenic fungal hyphae disk (4 mm diameter circle taken from the edge of the culture to obtain a strong vitality) formerly cultured on PDA medium was placed at the center of each plate. The co-culture of bacteria and fungi was incubated at 25 °C. Inhibition zones were recorded.

2.5. In vitro detection of extracellular hydrolytic enzymes activities and siderophores production

All bacterial isolates were tested for in vitro activities of their extracellular metabolites (cellulase, chitinase, glucanase, protease and siderophores), which were indicated by distinct circular hyaline zones around bacterial colonies on specific agar media. Cellulose activity was determined as described by Ghose (1987), chitinase activity was tested in minimal medium (Chernin and Chet, 2002), and glucanase activity was detected according to Fan et al. (2002). 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 in detection of protease activity, which shows casein degradation. Siderophores production was determined as previously described (Shin et al., 2001).

2.6. Greenhouse test

The greenhouse assay included 14 bio-control treatments, a validamycin control and a water control. In each treatment, 264 rice plants were used in testing their bio-control efficacies against sheath blight