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Evaluation of Gram-positive rhizosphere and endophytic bacteria for biological control of fungal rice (*Oryzia sativa* L.) pathogens

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Abstract Gram-positive bacteria isolated from the rhizosphere and inside the roots of rice were characterized for plant growth promoting (PGP) traits and antifungal activity against some rice plant pathogenic fungi of rice. The results showed the endophytic and rhizosphere isolates had different PGP traits and antifungal activity. Only one rhizosphere isolate and one endophytic isolate showed highly inhibitory effects against the mycelial growth of all fungal rice pathogens tested in this study. The best bacterial isolates, based on multiple PGP traits and inhibitory effects against the mycelial growth of all fungal rice pathogens, were identified. Based on biochemical tests and by comparison of 16S rDNA sequences, the endophytic isolate REN_3 and the rhizosphere isolate REN_4 were closely related to *Bacillus cereus* and *Bacillus mojavensis* respectively. The broad-spectrum antifungal strains, the REN_3 and REN_4 isolates analyzed here, exert multiple PGP and antagonistic activity and represent an excellent option to be used as either potent bio-promoting or bio-control agents in rice under in vitro conditions. This application may help to minimize dependence on pesticides, which have adverse effects on the environment, finally leading to have sustainable environments. In conclusion, the results of antifungal activity showed rice harbors bacteria with a good potential in biocontrol of rice fungal pathogens.

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Introduction

Rice (*Oryzia sativa* L.) is the major food crop, which feeds half of the population in the world, especially in Asia, Latin America, and Africa. Diseases are considered a main constraint and yield limiting factor in the production of this crop, causing 5 % losses in yield (Song and Goodman 2001). More than 70 diseases caused by fungi, bacteria, viruses or nematodes have been recorded on rice (Manandhar et al. 1998). Although, resistant cultivars and the application of pesticides have been practiced as a means of pathogens control, the useful life span of many resistant cultivars is only a few years due to the breakdown of the resistance in the face of high pathogenic variability and to public pressure to develop production systems favorable to the environment (Whipps 2001). The use of pesticides is also costly as well as environmentally undesirable. It is the principle of the Integrated Pest Management (IPM) to maintain the pest damage under an economical permitted level. Therefore, there is a need to develop strategies to provide durable resistance that are useful over a broad geographic area (Manandhar et al. 1998). Interest in biological control has increased over the past years, driven by the need for alternatives to chemicals. In this respect, antagonistic bacteria

provide an environmentally sound alternative to protect plants against attack by fungal pathogens (Lugtenberg et al. 2001). Much research work has been performed on the Gram-negative bacteria, while the research on the Gram-positive bacteria has ever been limited to the *Bacillus* spp. (Li et al. 2003). However, there has hardly been a systematic study on the Gram-positive bacterial composition, pathogenicity, and the antagonistic effect against some of the major fungal pathogens causing some diseases of rice in Iran. In addition, the ability of some Gram-positive bacteria to form endospores allows them to survive in a wide range of environmental conditions such as elevated temperature and high concentrations of chemicals, and facilitates the formulation of commercial products over the non-spore formers such as *Pseudomonas* (Pérez-García et al. 2011). Moreover, the shelf- life of biological products based on bacterial spores can be 1–3 years (Kumar et al. 2012). Hence, the present study was designed to isolate and characterize cultivable Gram-positive bacterial rhizosphere and endophytic isolates from rice plant having PGP and antagonistic traits so that they can be exploited as a potential bio-inoculant for rice.

Materials and methods

Isolation of endorhiza and rhizosphere bacteria

Rhizosphere soil and roots of rice plants (*Oryzia sativa* L., Cv, *Gohar*) were collected from the Dashte Naz research farm (36° 37′ North, 53° 11′ East, and about 16 m above sea level) in Iran. Endorhiza and rhizosphere bacteria were isolated as previously described by Etesami et al. (2014b). Bacterial isolates identified as individual colony-forming units (CFU) were selected and sub-cultured onto nutrient agar (NA). Similar bacterial isolates were grouped based on phenotypic characteristics such as shape, motility, color, rate of growth, culture morphology, and Gram-staining reaction, as there has not been a possibility of obtaining repeated strains in the collection, and stored (maintained on the respective slants) in a refrigerator at 4 °C for further studies. For long-term storage, bacterial cultures were maintained at −80 °C in nutrient broth (NB) that contained 20 % glycerol.

In vitro antifungal assays

Fungal rice pathogens *Fusarium proliferum*, *Fusarium verticillioides*, *Fusarium fujikuroi*, *Magnaporthe salvinii* and *Magnaporthe grisea* (*M. oryzae*), which are the most important fungal rice pathogens in Iran, were kindly provided by the Laboratory of Phytopathology, Department of Plant Protection, University of Tehran, Iran. The bacterial isolates were evaluated for dual culture antagonism assays, production of diffusible antibiotics, and production of volatile antibiotics as described by Whipps (1987). Each experiment was run in triplicate and was repeated at least two times. Results are expressed as mean % inhibition of the growth of the corresponding fungal isolates in the absence of the bacterium. The percent inhibition of the fungal growth due to the presence of antagonistic agent was calculated with the following formula: $[(C - T)/C] \times 100$, where C is the radial distance grown by fungus in plates without antagonist (a control value) and T is the distance grown on a line between the inoculation positions of the fungal phytopathogen and the antagonist strain (an inhibition value).

Assay for plant growth promoting (PGP) activities

Production of siderophore and indole-3-acetic acid (IAA) (μg ml^{-1}) were determined as described by Schwyn and Neilands (1987) and Patten and Glick (2002) respectively. Assay of phosphate solubilizing activity of the bacteria was determined on Pikovskaya agar (Pikovskaya 1948) and the ability of the bacterial isolates to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase was assessed on minimal media containing ACC as its sole nitrogen source as described by Penrose and Glick (2003).

Identification of the isolates

Phenotypic identification of the most promising antagonistic endophytic and rhizosphere isolates was done as described by Etesami et al. (2014a). The amplification of 16S rDNA gene was done by using universal bacterial primer 1492R (5′-TACGGTTACCTTGTTA CGACTT-3′) and 27F (5′-AGAGTT TGATCTTG GCTCAG-3′) in accordance with the conditions described by Sandhu et al. (2009). The sequence of 16S rDNA gene was determined after genomic DNA extraction and polymerase chain reaction amplification. The PCR conditions were 1 min at 94 °C and then 35 cycles of 30 s at 95 °C, 30 s at 45 °C, and 1 min at 72 °C, and a final 5 min extension at 72 °C.

The PCR products were sequenced using the 27F and 1492R primers. The PCR product was purified and sequenced on a DNA sequencer (Iowa State University, USA) and the resulting sequences were compared with sequences found in Gen-Bank database using Basic Local Alignment Search Tool (BLAST) program at the National Center for Biotechnology Information (NCBI) BLAST server (http://www.ncbi.nih.gov/BlAST/). The nucleotide sequences determined in this study have been deposited in the NCBI database.

Statistical analysis

All the experiments of assaying PGP traits were arranged in randomized complete design with four replications in each treatment and repeated two times. Analysis of variance (ANOVA) was performed, and means were compared by the Tukey's test at 5 % probability level using the SAS (V. 8) software package (SAS Institute, Cary, NC, USA). Before means comparison, normality test was conducted and distribution function for all data was normal. Data were reported as means ± the standard error of the mean (SEM).

Results

Isolation of bacterial isolates

A total of 200 bacterial isolates with different phenotypes were isolated from the rhizosphere and surface-sterilized roots of rice plant (Cv, *Gohar*). Based upon the isolate source, 60 % isolates were isolated from rhizosphere and 40 % from endorhiza. Colony and cell morphology and Gram-staining tests were performed on the isolates. Fifty-three percent of the isolates were Gram-positive and the remainder were Gram- negative. All of the Gram-positive isolates were evaluated for their antagonistic potential against fungal rice pathogens (Table 1).

In vitro antagonistic properties

All the 106 Gram-positive strains were screened in vitro for their activities against five fungal rice pathogens. Of the 106 isolates, the 38 isolates were positive in terms of inhibitory against at least one pathogenic fungus. Only one isolate obtained from rhizosphere and one from endorhiza strongly inhibited the growth of all fungal rice pathogens on medium potato dextrose agar (PDA) at 28 ± 2 °C after seven days of incubation (Fig. 1a, b). These isolates exhibited a broad-spectrum antagonism towards all test fungi. In addition, there was a significant difference ($p < 0.05$) among isolates in terms of inhibitory against the fungi (data not shown). The proportion of antagonists varied for bacteria with different origin, 40 % of the bacterial strains showing antagonistic effect were endophytes isolated from the root tissues of plants, while 60 % were isolated from the rhizosphere. In other words, among 39 root endophytic isolates and 67 rhizosphere isolates, only 15 isolates (Fig. 1a) and 23 isolates (Fig. 1b) showed antifungal activities against some fungal rice pathogens respectively. In general, the frequency of both endophytic isolates and rhizosphere bacteria inhibiting the mycelium growth of fungal pathogens decreased in the order; *M. salvinii* > *M. grisea* > *F. verticillioides* > *F.fujikuroi* > *F. proliferum*. On the basis of data obtained, it could be stated that sensitivity of test fungi was also in order of M. *salvinii* > *M. grisea* > *F. verticillioides* > *F.fujikuroi* > *F. proliferum*. The maximum growth inhibition of these fungi was recorded among the endophytic isolates after seven days of incubation in dual culture assay as compared with rhizosphere isolates (Fig. 1a). When the effect of diffusible and volatile antibiotics was tested, these isolates showed the inhibitory effect similar to those obtained from dual culture antagonism assay (data not shown). About 64 % of the 106 isolates showed no activity against each of the fungal rice pathogens. Dual culture assay for in vitro inhibition of mycelia of fungal rice pathogen *F. fujikuroi* by endophytic strain REN_3 is shown in

Table 1 Frequency and population density of bacteria isolated from the rhizosphere and endorhiza of rice grown in the field in Iran at flowering stage

Isolate source	No. of total isolates	No. of Gram-positive isolates	Mean population density (CFU g^{-1} fresh wt or soil) ± SEM
Rhizosphere	120	67	$(2.6 \pm 1.22) \times 10^6$
Root endophytes	80	39	$(9.5 \pm 2.12) \times 10^5$

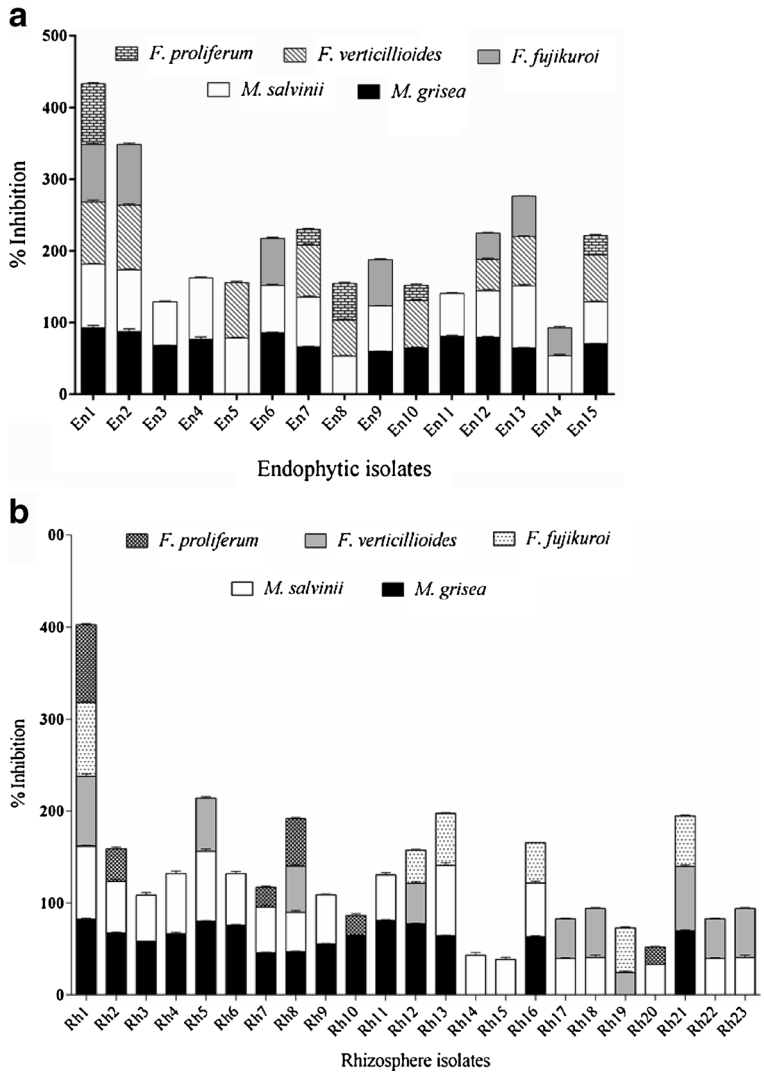

Fig. 1 In-vitro inhibition (Average ± standard error from triplicate samples) of mycelial growth of five fungal rice pathogens by fifteen Gram-positive entophytic isolates (**a**) and twenty three Gram-positive rhizosphere isolates (**b**) isolated from rice plants grown on PDA for 7 days by dual culture assay

Fig. 2. The surface of the control plates (not treated with the isolated bacteria) were almost completely covered by the pathogens (Fig. 2).

PGP characteristics of antagonistic isolates

From 38 antagonistic bacteria (23 antagonistic rhizosphere isolates and 15 antagonistic endophytic isolates), 13 and seven isolates out of the rhizosphere isolates and endophytic isolates produced siderophore as indicated by formation of orange halos around their spots on the chrome azurol S (CAS) agar medium respectively. All these isolates produced IAA ranging from 5 to 25 μg ml^{-1} in the presence of L-Tryptophan (100 μg ml^{-1}). Among the screened isolates, only five endophytic isolates and eight rhizosphere isolates produced intracellular ACC deaminase and six endophytic isolates and seven rhizosphere isolates showed phosphate solubilization. In addition, there was a significant difference ($P < 0.05$) among isolates in terms of the production of PGP traits (data not shown).

Identification of potent isolates

The best bacterial isolates based on their ability to inhibitory activity against all fungal rice pathogens and possessing multiple PGP traits, were identified. Two of the studied isolates were found to possess

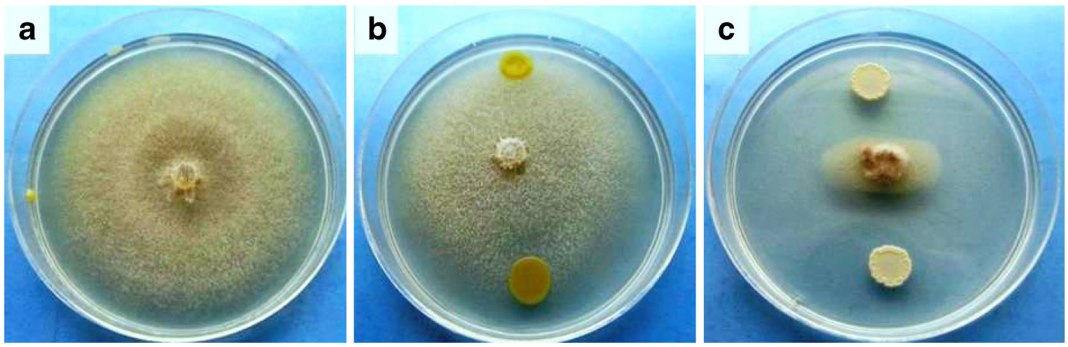

Fig. 2 Dual culture assay for in vitro inhibition of mycelia of *F. fujikuroi* by *B. cereus* REN_3 and isolate REN_2, isolated from inside the root of rice (Cv, *Gohar*), grown PDA for 7 days in Iran. **a**, Control; **b**, Isolate REN_2; **c**, isolate REN_3. Isolate REN_2 resulted in no inhibition zones

strong inhibitory potential on the mycelial growth of all of the studied fungi. Some of the morphological and biochemical traits of the endophytic strain REN_3 and rhizosphere strain REN_4 are shown in Table 2. The 16S rDNA gene identification revealed that the endophyte REN_3 and the rhizosphere isolate REN_4 were closely related to *Bacillus cereus* and *Bacillus mojavensis* respectively. The nucleotide sequences determined in this work have been deposited in Gen Bank database with accession numbers KF822666 and KF822667 for isolates REN_3 and REN_4 respectively.

Discussion

Plant rhizosphere and endorhiza are known to be preferred ecological niches for various types of soil microorganisms due to rich nutrient availability (Bell et al. 1995; Zinniel et al. 2002; Costa et al. 2012; Kuklinsky-Sobral et al. 2004; Etesami and Alikhani 2016a). Rhizosphere and endophytic strains isolated from rice showed PGP traits and antagonistic activity against some of the fungal pathogens of rice. The findings of this study clearly demonstrated the presence of one, or more than one, type of PGP traits and antagonistic

Table 2 Morphological and biochemical traits of the endophytic strain REN_3 and rhizosphere strain REN_4 isolated from inside endorhiza and rhizosphere of rice plant

Isolates	Accession number	Morphological and biochemical traits	Closest relative	Similarity (%)
REN_3	KF822666	Motile , rod, G^+, spore formers , opaque and irregular edged colonies, fast grow, catalase (+), oxidase (+),nitrate reduction (+), indole (−), arginine dehydrogenase (+), urease test (+), amylase production (+), gelatinase production (+), casein hydrolysis (+),citrate (+), KOH test (−), lecithinase test (+), methyl red (−), phosphatase (+),glucose fermentation(+), mannitol fermentation(−), sucrose fermentation(+), arabinose fermentation (−), ammonia production (+),H_2S production (−), fluorescent pigment production (−), growth in NaCl 6.5 % (+), growth at 50 °C (+)	*Bacillus cereus*	99
REN_4	KF822667	Motile , rod, G^+, spore formers , white, dry and fold, opaque and irregular edged colonies , fast grow, catalase (+), oxidase (−), nitrate reduction (+), indole (−), arginine dehydrogenase (+), urease test (−),amylase production (+), gelatinase production (+),casein hydrolysis (+), citrate (+), KOH test (−), lecithinase test (+),methyl red (+), phosphatase (+),glucose fermentation(+), mannitol fermentation(+), sucrose fermentation(+), arabinose fermentation (+), ammonia production (+),H_2S production (−),fluorescent pigment production (−), growth in NaCl 6.5 % (+), growth at 50 °C (+)	*Bacillus mojavensis*	99

activity against some of the fungal rice pathogens in the Gram-positive bacterial isolates. An isolate having multiple PGP characteristics is expected to show better response than those possessing a single PGP trait.

The observation that the frequency of IAA-producing bacteria was higher in isolates than that of other PGP traits suggests that the plant selects for bacteria with those traits or that these bacteria harbor other traits that allow them to more effectively reach and establish themselves in the inner plant tissue (Mendes et al. 2007). These results are similar to results of our previous study performed on berseem clover (Etesami et al. 2014b). In this study, the population density of culturable endophytic bacteria in the endorhiza of rice was less than that of rhizosphere bacteria (Table 1). These results have been reported by Rosenblueth and Martínez-Romero (2004) in *Rhizobium etli* maize populations. The isolation method of bacteria has determined almost equal numbers of Gram-positive and Gram-negative bacteria. As cited in the extensive review of Kobayashi and Palumbo (2000), both Gram-positive and Gram-negative bacterial isolates have been isolated from several tissue types in numerous plant species. Endophytic bacteria are known to control plant pathogenic fungi (Berg and Hallmann 2006; Backman and Sikora 2008; Etesami and Alikhani 2016a, 2016b). Among the isolated strains, two spore-forming *B. mojavensis* REN_4 and *B. cereus* REN_3 strains exhibited broad-spectrum antifungal activity towards all rice plant pathogens used in this study. Ability to perform many PGP traits together suggest the uniqueness of *B. mojavensis* and *B. cereus* and their potential use in developing a cost-effective ecologically-friendly multifunctional biofertilizer for use in rice. Previous studies also reported different strains *B. subtilis*, *B. cereus*, *B. amyloliquoefaciens*, *B. polymyxa*, *B. pumilus*, and *Bacillus* sp. as promising biocontrol agents against different fungal pathogens (Kuarabachew et al. 2007; Aliye et al. 2008; Chen et al. 2010; Kumar et al. 2012; Kurabachew and Wydra 2013; Etesami and Alikhani 2016a). The exact biocontrol mechanisms are largely unknown in many microorganisms. However, it is likely that the most effective biological control strains act via multiple mechanisms. Our results indicate that rhizosphere and endorhiza of rice are potential reservoirs of biological control and PGP agents, which could be used for further biotechnological applications, such as potential microbial biopesticides and bio-fertilizers. We studied cultivable Gram-positive bacteria in these assays, since only cultivable bacteria can be used to develop bio-fertilizer for future research. To the best of the authors' knowledge, this is the first report of the study of all Gram-positive endophytic and rhizosphere bacteria isolated from rice plant for screening in terms of PGP traits and antifungal activity. In this study, it has been shown rhizosphere and endorhiza of rice plant harbor bacteria with multifaceted beneficial effects. Indeed, bacteria having multifaceted beneficial effects can complement each other (Maheshwari et al. 2014). Biological control agents, such as *B. mojavensis* REN_4 and *B. cereus* REN_3, can control disease (primary effect) but will also demonstrate stimulation of rice plant growth (secondary effect) in the absence of a pathogen(Avis et al. 2008; Etesami and Alikhani 2016a). Two spore-forming *B. mojavensis* REN_4 *and B. cereus* REN_3 strains identified in this study exhibited broad-spectrum antifungal activity towards all rice plant pathogens. Their ability to form endospores allows them to survive in a wide range of environmental conditions such as elevated temperatures and high concentrations of chemicals, and facilitates the formulation of commercial products over the non-spore formers (Pérez-García et al. 2011). In addition, the plant provides a ready-made environment for endophytic bacteria so that the biotic and abiotic stresses against colonization of the desired endophytes would be reduced (Newman and Reynolds 2005). Since endophytes are more protected from unfavorable environmental conditions such as flooding conditions in rice fields and establish closer relationships with the host compared to rhizopheric microorganisms, it may be supposed that their influence is rather significant (Garipova 2014). Endophytic bacteria occupy ecological niches similar to that occupied by plant pathogens, and these endophytic bacteria can, therefore, act as biological control agents against pathogens (Hallmann et al. 1997). In general, the potential increased use of these bacteria especially Gram-positive endophytic bacteria afforded by their multifaceted beneficial effects may further help in reducing problems associated with the use of synthetic chemicals in agriculture and managing better disease control.

Conclusions

This study showed rhizosphere and endorhiza of rice plant harbor Gram-positive bacteria with multifaceted beneficial effects. The study indicated that the isolates studied had an excellent potential to be used as biocontrol agents of fungal rice pathogens under in vitro

conditions. For examples, the broad-spectrum antifungal strains, *B. mojavensis* REN_4 and *B. cereus* REN_3 possessed desirable PGP traits and had the innate fungicidal potential to inhibit the growth of all fungal rice pathogens tested in this study under in vitro conditions. However, further evaluation of the isolates exhibiting multiple PGP traits on soil–plant system under greenhouse and field conditions is needed to uncover their efficacy as effective PGPR.

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