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Bacillus species as the most promising bacterial biocontrol agents in rhizosphere and endorhiza of plants grown in rotation with each other

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Abstract In this study, a total of 550 cultivable bacterial isolates were isolated from rhizosphere and endorhiza of rice, Berseem clover, and oilseed rape grown in rotation with each other. The potential of antifungal activity of all isolates against five rice pathogenic fungi was investigated under in vitro conditions. Of 550 isolates, 139 inhibited the mycelial growth of at least one fungal rice pathogen. The results also showed that rhizosphere and endorhiza of every third plant (three studied plants) harbored the bacteria (139 isolates) with good potential for inhibiting fungal rice pathogens in vitro. Based on biochemical tests and by comparison of 16S rDNA sequences, of the superiors six endophytic and rhizosphere isolates, which showed strong inhibitory effects against the mycelial growth of all the five fungal rice pathogens (*Magnaporthe oryzae, M. salvinii, Fusarium verticillioides, F. fujikuroi,* and *F. proliferum*), were identified. Two isolates REN_4 and CEN_2, isolate CEN_6, isolate CEN_3, and two isolates REN_3 and CEN_5 were closely related to *Bacillus mojavensis*, *B. amyloliquefaciens*, *B. subtilis*, and *B. cereus* respectively. Strains REN_4 and REN_3 were obtained from rhizosphere and endorhiza of rice, while strains CEN_6 and CEN_2, and strains CEN_5 and CEN_3 were isolated from rhizosphere and endorhiza of clover and oilseed rape respectively. Therefore, it can be concluded that plants cultivated in rotation with rice and grown on the same soil harbor protective bacteria such as genus *Bacillus* and that may be potential reservoirs of biocontrol agents for control of the rice pathogenic fungi tested in this study.

Keywords Biological control · *Bacillus* species · Fungal rice pathogens · Plants rotated with rice

Introduction

The enormous diversity of microorganisms in rhizosphere and endorhiza of different plants has been reported (Etesami and Alikhani 2016b; Pieterse et al. 2014), which are crucial for plant growth and health, and without them, the plants would subsequently die (Stang and Tkachuk 2013). In addition, this complex microbial community associated with plant is considered as the second genome of the plant (Pieterse et al. 2014). Plants establish a close relationship with the microorganisms that reside in the soil. There are three basic categories of microbial interactions based on ecology namely neutral, negative and positive interactions generally exist between microorganisms and plants (Whipps 2001). There is some experimental evidence that shows that plants are able to control the composition of their microbiome and recruit protective microorganisms effective in repressing pathogenic microorganisms in their rhizosphere or endorhiza (Pieterse et al. 2014). One of the most important groups of microorganisms associated with different plants is bacteria. Some of

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these bacteria, plant growth promoting rhizobacteria (PGPRs), live in the vicinity of root (rhizosphere) or on the surface of root (rhizoplane), while some dwell inside plant tissues (endophytic bacteria) (Azevedo et al. 2000; Petrini et al. 1989; Sturz et al. 2000). 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 one of the most main constraints and yield limiting factors in the production of rice, causing 5% losses in yield (Song and Goodman 2001). Berseem clover (*Trifolium alexandrinum* L.) and oilseed rape (*Brassica napus* L.) are cultivated in rotation with rice in Iran.

Bio-control using antagonistic endophytic and rhizosphere bacteria has been considered as an alternative strategy to agrochemicals, which are harmful to human health and the environment (Compant et al. 2010). There are many studies which showed that rice (Adhikari et al. 2001; Chaiharn et al. 2009; Garcia et al. 2015; Hossain et al. 2016; Shrestha et al. 2016; Vasudevan et al. 2002), *Brassica* species (Card et al. 2015; Danielsson 2008; Zhang et al. 2014), and clover (Sturz et al. 1997) harbor endophytic and rhizosphere microorganisms with antagonistic activities. Although biocontrol agents for fungal rice pathogens in rice have been reported, it has not been known whether plants cultivated in rotation of rice would also recruit the bacteria with similar biological control for these pathogens. To the best of our knowledge, this is the first work performed on evaluating bacterial bio-control agents of rice and the plants grown in its rotation.

Keeping in view the above discussion, the present study was designed to evaluate cultivable rhizosphere and endophytic bacteria isolated from plants (rice, berseem clover, and oilseed rape) grown on the same soil for biological control of fungal rice pathogens.

Materials and methods

Rhizosphere soil and roots (15 samples) of the healthy plants of rice (*Oryzia sativa* L., Cv, *Gohar*), oilseed rape (*B. napus* L., Cv, *Hyula*), and berseem clover (*Trifolium alexandrinum* L.) at flowering were randomly collected from the research farm (36° 37′ North, 53° 11′ East, and 16 m above sea level) of the Dashte Naz in Mazandaran province, Iran. The growing season was from April to September for rice and from September to March for clover and oilseed rape. The order of cultivation was as rice– clover rotation in the first year and as rice– oilseed rape rotation in the second year. Rhizosphere and endophytic bacteria of rice (in the first year), oilseed rape, and clover were isolated as previously described by Etesami and Alikhani (2016b, 2017) and Etesami et al. (2013) respectively.

Rice pathogenic fungi, *Magnaporthe oryzae, M. salvinii, Fusarium verticillioides, F. fujikuroi,* and *F. proliferum*, used in this study are of the most important rice pathogenic fungi in Iran. Potato dextrose agar (PDA) was used for the cultivation of the five rice pathogenic fungi. For preparation of fungal inoculum, each of these fungi was grown on PDA medium and incubated for 7 days at 28 ± 2 °C. Preparation of bacterial inocula was performed according to Etesami and Alikhani (2016b). The bacterial cultures were standardized to 5×10^8 CFU ml^{-1} and used for all following assays.

For in vitro antagonism assay, all bacterial isolates were evaluated for dual culture antagonism assays according to the method described by Whipps (1987). This assay was done in triplicate and was repeated at least twice. Results reported are the mean % inhibition of the growth of the corresponding pathogenic fungus in the absence of the antagonistic bacterial isolate. The % inhibition of the fungal growth due to the presence of antagonistic bacterial agent was computed according to following formula: $\% \text{ Inhibition} = [(C - T)/C] \times 100$;where, C is the radial distance grown by pathogenic fungus in control plates (without antagonist) and T is the distance grown on a line between the inoculation places of fungus and the antagonist isolate as an inhibition value.

Phenotypic identification and the molecular identification (16S rDNA gene sequences) of the most promising endophytic and rhizosphere isolates were performed as described by Holt et al. (2010) and Tindall et al. (2007), and Etesami et al. (2014) respectively. The nucleotide sequences were deposited in the NCBI database to get accession number.

Assays of production of diffusible and volatile antibiotics were performed only on the most promising antagonistic endophytic and rhizosphere strains isolated from three plants (rice, clover, and oilseed rape). These assays were performed according to the methods described by Whipps (1987). Both assays were performed in triplicate and repeated at least two times and % inhibition was calculated as described above.

Table 1 Frequency and population density of the bacterial isolates obtained from the rhizosphere and endorhiza of rice and the plants cultivated in its rotation, Berseem clover and oilseed rape

Plant	Isolate source	No. of total isolates	Mean population density (CFU g^{-1} root fresh weight or soil) ± SE
Rice	Rhizosphere	120	$(2.6 \pm 1.22) \times 10^6$
	Endorhiza	80	$(9.5 \pm 2.12) \times 10^5$
Clover	Rhizosphere	120	$(7.6 \pm 1.23) \times 10^6$
	Endorhiza	80	$(2.4 \pm 1.02) \times 10^5$
Oilseed rape	Rhizosphere	90	$(1.3 \pm 1.22) \times 10^6$
	Endorhiza	60	$(2.1 \pm 1.14) \times 10^5$

Since four strains out of the six selected superior strains were isolated from the plants of clover and oilseed rape, it was essential to test whether these isolates would be pathogenic to rice plant (Cv, *Gohar*). This assay was performed under controlled conditions as previously described by Etesami and Alikhani (2016a). The statistical design selected for this experiment was a randomized complete design (RCD). This experiment was repeated twice with six replicates of each treatment. After 20 days of inoculation, the effect of the bacterial treatments on rice shoot (stem plus leaf) dry weight and root fresh weight was measured and recorded. These seedlings were also considered in terms of signs of pathogenicity (e.g. lesion formation or wilting). In addition to this assay, parallel tests with 100 seeds for each treatment were conducted to evaluate the potential influence of the bacterial broth cultures on seed germination of this cultivar. After 7 days, the germinated seeds were counted and the germination ratio was calculated.

The antagonistic superior strains were inoculated on rice seedlings (Cv, *Gohar*) to study rhizoplane colonization of these bacteria according to Etesami and Alikhani (2016b). This experiment was arranged in a completely randomized design with eight replications within laboratory tubes and reiterated two times for 20 days. The viable plate count method was used to measure the bacterial population on roots (CFU g^{-1} root).

Analysis of variance (ANOVA) was performed on data, and means were compared by the Tukey's least significant difference test at 5% probability level using the SAS (V. 8) software package (SAS Institute, Cary, NC, USA). Data reported are means ± the standard error of the mean (SE). After log transformation of individual estimation, the population densities of the antagonistic bacterial strains were calculated and reported.

Results and discussion

A total of 550 bacterial isolates with different phenotypes were isolated from the rhizosphere and surface-sterilized roots of rice, clover and oilseed rape grown in rotation with rice. Frequency and population density of the bacteria isolated from the rhizosphere and endorhiza of rice and the plants cultivated in its rotation are shown in Table 1. The isolate source of the 60% of isolates was from rhizosphere and the rest were from endorhiza of every third plant (three studied plants). Almost the percent of the Gram-negative and Gram-positive isolates was the same. Of the 550 isolates, 139 isolates were

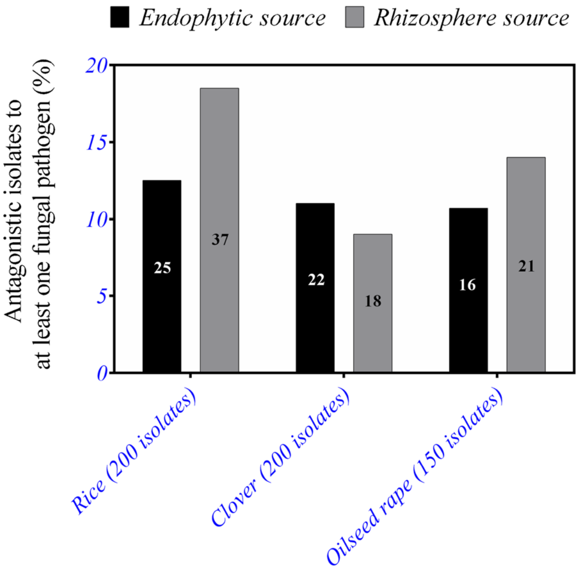

Fig. 1 Frequency (%) of bacterial isolates antagonistic to at least one pathogenic fungus isolated from the rhizosphere and endorhiza of rice and the plants cultivated in its rotation, Berseem clover and oilseed rape. The numbers shown on Fig. are the number of isolates

positive in terms of inhibitory against at least one pathogenic fungus. Frequency of bacterial isolates antagonistic to at least one pathogenic fungus is shown in Fig. 1. The portion of antagonists varied for bacterial isolates with different origins, 45% of the bacterial isolates showing antagonistic effect was the endophytes obtained from the root tissues of three plants, while 55% of which was obtained from rhizospheres of these plants. As shown in Fig. 1, the number of antagonistic isolates found in rhizosphere of plants was higher than the number of these isolates in endorhiza of plants, except for clover. Dual culture assay for in vitro inhibition of mycelia of *F. fujikuroi* by some of isolates is shown in Fig. 2. The surfaces of the control plate (not treated with the isolated bacterium) were almost completely covered by the pathogen (Fig. 2a). Non-antagonists were covered with the hyphae of pathogenic fungi (Fig. 2b). Some of the isolates (unidentified isolates) were able control the hyphal growth of fungal pathogens to a certain extent but not completely (Fig. 2c, d), while the superior isolates (*B. cereus* REN_3, *B. mojavensis* REN_4, *B. mojavensis* CEN_2, *B. amyloliquefaciens* CEN_6, *B. subtilis* CEN_3, and *B. cereus* CEN_5) inhibited the hyphal growth of fungal pathogens completely (see Fig. 2e for *B. subtilis* CEN_3 and Fig. 2f for *B. cereus* CEN_5). Frequency (%) of the antagonistic endophytic and rhizosphere bacterial isolates isolated from rice, clover, and oilseed rape is shown in Fig. 3. For every third plant, frequency of growth inhibition of these fungi by endophytic isolates was more than rhizosphere ones. However, the number of both rhizosphere bacterial isolates and endophytic bacterial isolates suppressing the mycelial growth of fungal rice pathogens was reduced in the order; *M.salvinii*>*M.oryzae*>*F.verticillioides*>*F.fujikuroi*>*F.proliferum* (Fig. 3a, b, c).

Only one endophyte and one rhizosphere isolate from each plant that were antagonistic to all the five rice pathogenic fungi were selected and identified. The 16S rDNA gene identification revealed that the endophyte REN_3 and the rhizosphere isolate REN_4, isolated from endorhiza and rhizosphere of rice plant, were closely related to *B. cereus* and *B. mojavensis* respectively, while the endophyte CEN_2 and the rhizosphere isolate

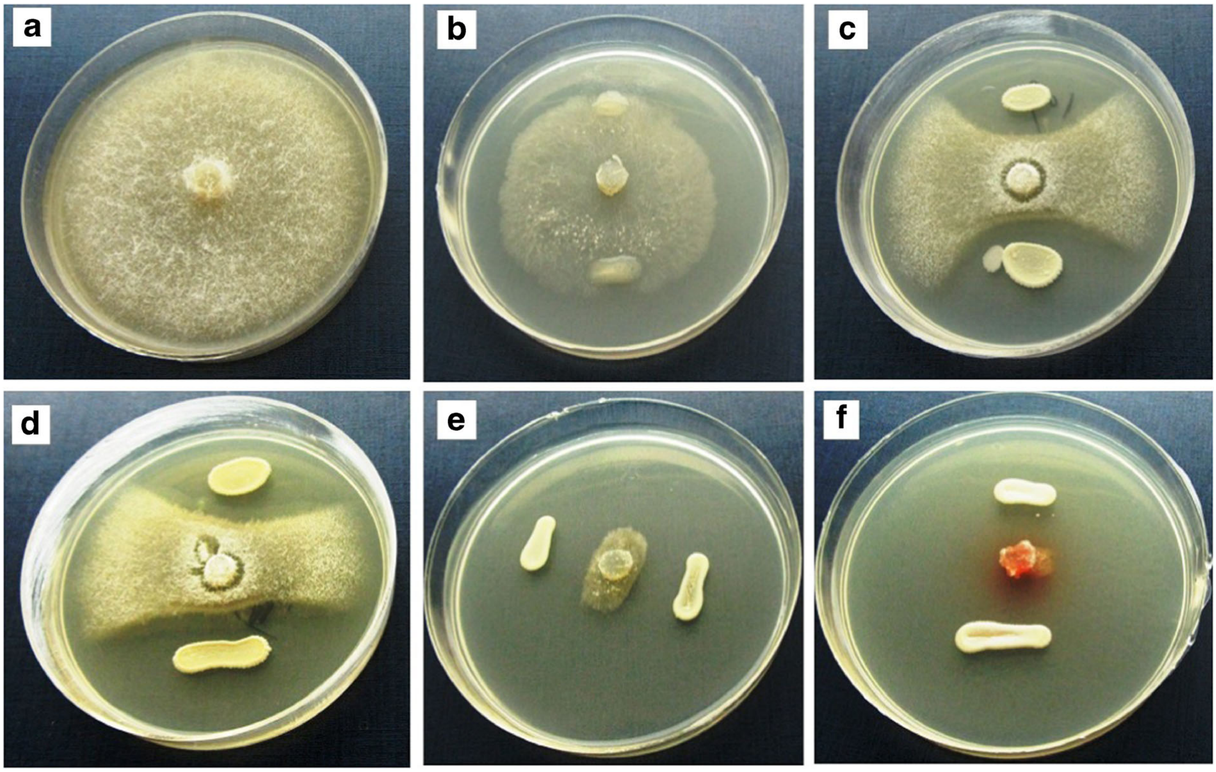

Fig. 2 Dual culture assay for in vitro inhibition of mycelia of *F. fujikuroi* by bacterial isolates grown on PDA for 7 days. **a** control; **b** non antagonistic isolate (unidentified isolate); **c** and **d**, antagonistic isolates (unidentified isolates); **e** and **f**, antagonistic superior isolates (**e**, *B. subtilis* CEN_3 and **f**, *B. cereus* CEN_5)

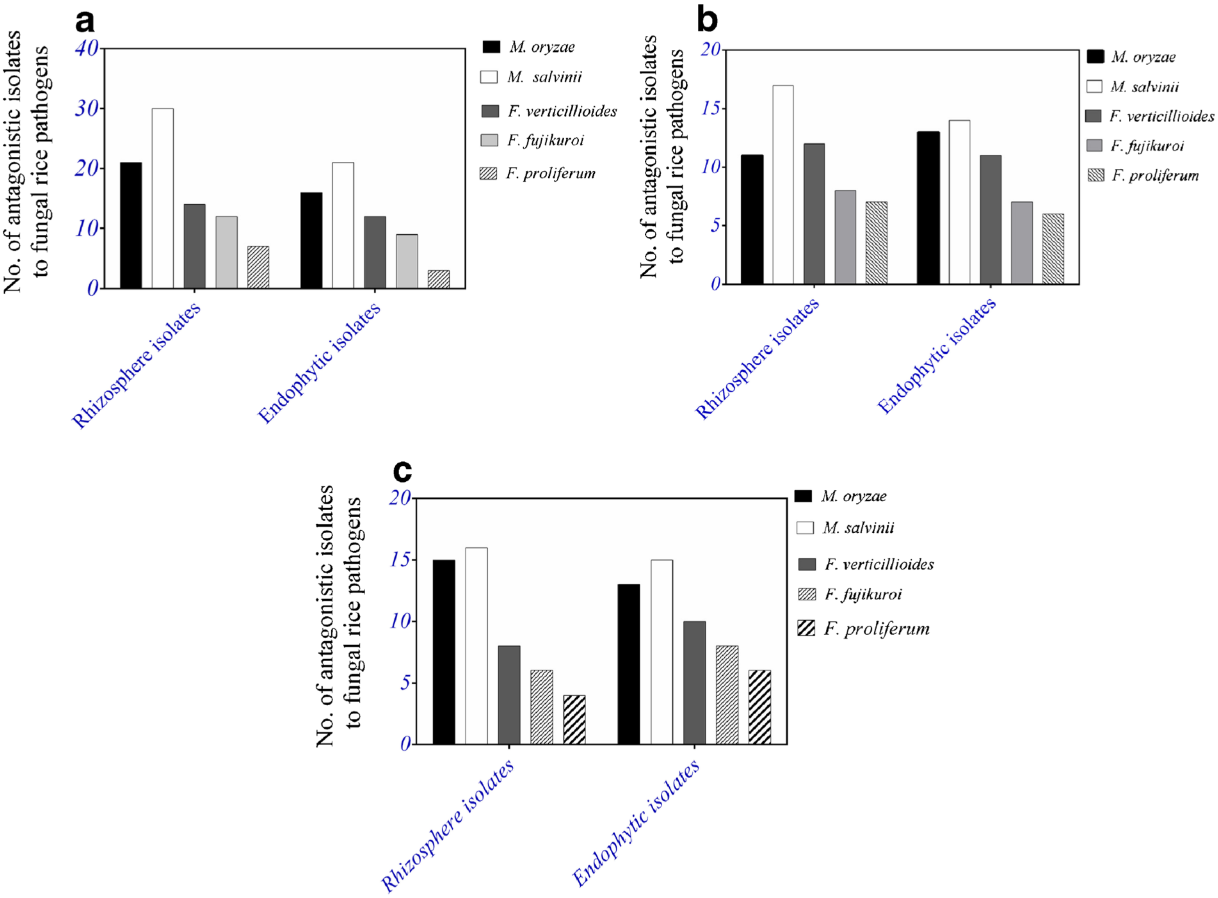

Fig. 3 Number of the antagonistic endophytic and rhizosphere bacterial isolates to fungal rice pathogens by dual culture assay, isolated from rice plant (**a**) and plants cultivated in its rotation, clover (**b**) and oilseed rape (**c**)

CEN_6, isolated from endorhiza and rhizosphere of clover plant, were closely related to *B. mojavensis* and *B. amyloliquefaciens* respectively. In addition, the endophyte CEN_3 and the rhizosphere isolate CEN_5, isolated from endorhiza and rhizosphere of oilseed rape plant, were closely related to *B. subtilis* and *B. cereus* respectively. The nucleotide sequences determined in this work have been deposited in Gen Bank database with accession numbers KF822666, KF822667, KF822668, KF822672, KF822673, and KF731834 for strains REN_3, REN_4, CEN_3, CEN_5, CEN_6, and CEN_2 respectively.

When the effect of diffusible and volatile antibiotics of the superior strains on fungal pathogens was tested, these strains showed the inhibitory effect similar to those obtained from dual culture antagonism assay on all five fungal pathogens 7 days after of incubation (DAI). Dual culture antagonism assay and the production of diffusible and volatile antibiotics by the superior strains are shown in Fig. 4. Among *Bacillus* species assayed in this study, *B. subtilis* CEN_3 showed more inhibitory effect compared to other *Bacillus* species. In general, the maximum growth inhibition of these fungi was recorded among the endophytic strains after 7 days after inoculation as compared with rhizosphere ones.

All bacterial strains ($p < 0.01$) increased root fresh weight (11–33%) and shoot dry weight (5.8–28.2%) of inoculated plants after 20 days as compared to the control (Fig. 5a, b). In addition, significant differences were observed in germination by bacterial cultures in inoculated seeds of rice after 7 days after inoculation ($p < 0.01$) (Fig. 5c). The roots and rice seedlings inoculated with all of these strains appeared healthy and without obvious symptoms of disease such as lesion formation or wilting.

The levels of rhizoplane colonization by *Bacillus* species 20 days after inoculation on rice seedlings are given in Fig. 6. These strains colonized the exterior of

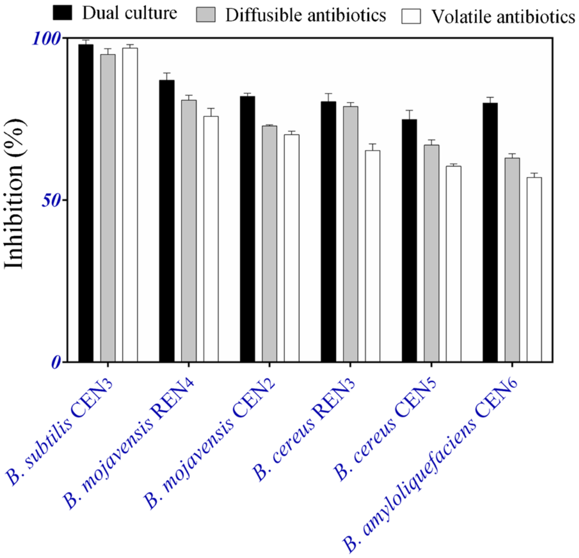

Fig. 4 Dual culture antagonism assay and assay of the production of diffusible and volatile antibiotics by the superior strains isolated from endorhiza and rhizosphere of rice (endophytic strain REN_3 and rhizosphere strain REN_4), Berseem clover (endophytic strain CEN_2 and rhizosphere strain CEN_6) and oilseed rape (endophytic strain CEN_3 and rhizosphere strain CEN_5)

roots of seedlings, as suggested by re-isolation of the inoculated strains. The population density of the strains re-isolated was different ranging between 4.4 and 8.3 log10 CFU g^{-1} root. The highest colonization level was observed at the seedlings inoculated with *B. subtilis* CEN_3. Any rhizoplane colonization on the roots of un-inoculated control plants was not observed. Throughout the study, both control and inoculated seedlings were without any visible disease symptoms.

In this study, we showed that the plants cultivated in rotation with rice and grown on the same soil harbor protective bacteria such as genus *Bacillus*. In addition, considerable number of the isolates obtained from each plant was antagonistic to rice fungal pathogens (62 out of 200 isolates obtained from rhizosphere and endorhiza of rice, 40 out of 200 isolates obtained from rhizosphere and endorhiza of clover, and 37 out of 150 isolates obtained from rhizosphere and endorhiza of oilseed rape) (Fig. 1).

Under present study, six isolates (one rhizosphere isolate and one endophytic isolate from each plant) were identified, which strongly suppressed the growth of all the five pathogenic fungi tested in this study. All of the isolates were closely related to the genus *Bacillus*. Many studies have reported different *B. subtilis* strains, *B. cereus*, *B. amyloliquefaciens, B. mojavensis,* and *Bacillus* sp. as promising biocontrol agents against different fungal pathogens (Arguelles-Arias et al. 2009; Bacon and Hinton 2002; Chen et al. 2010; Chowdhury et al. 2015; Correa et al. 2009; Hossain et al. 2016; Kim et al. 2008; Kumar et al. 2012; Li et al. 2012; Shrestha et al. 2016).

The ability of bio-control agents to efficiently colonize surfaces of plant roots is a prerequisite for phytoprotection (Arguelles-Arias et al. 2009). In this study, all superior strains, both strains obtained from rice and those obtained from clover and oilseed rape were able to colonize roots of rice seedlings in vitro.

That the strains isolated from clover and oilseed rape were also able to colonize rice seedling suggests that the root exudates of these cultivars did not have any inhibitory effect on these strains. It has been found that plant roots can also secrete secondary metabolites that influence the microbial composition of the rhizosphere (Chaparro et al. 2013; Chaparro et al. 2014) and inhibit growth of specific microbes in the rhizosphere (Bais et al. 2002; Zhang et al. 2011). In addition, potentially antimicrobial mixtures and the specific substrates released by plant make particular microbial inhabitants of the rhizosphere or endorhiza (Badri et al. 2013; Chaparro et al. 2013), which allow some microbes to colonize roots. Therefore, it can be concluded that the root exudates of the plants tested in this study presumably did not have any inhibitory effect on antagonistic bacteria to fungal rice pathogens especially *Bacillus* species. On the other hand, these bacteria were also able to colonize the rhizoplane of rice seedlings substantially, which proved them as potent biocontrol agents. Hence, these strains may be used as antagonist against a range of phytopathogenic fungi that infect rice plant.

It has been proved that plants naturally select PGPRs that are competitively fit to occupy compatible niches without causing pathological stress on them (Yanni et al. 1997). It seems that all *Bacillus* species identified in this study are of this type of PGPRs, which were able to colonize rice seedlings significantly. In addition, ability of *Bacillus* strains to form endospores allows them to survive in a wide range of environmental conditions (Pérez-García et al. 2011). This ability may be another reason for the presence of *Bacillus* species in the rhizosphere and endorhiza of every third plant cultivated in rotation with each other.

Although specific bio-control agents have the ability to protect the plant against different pathogens, their efficacy is mainly influenced by the rest of the microbial

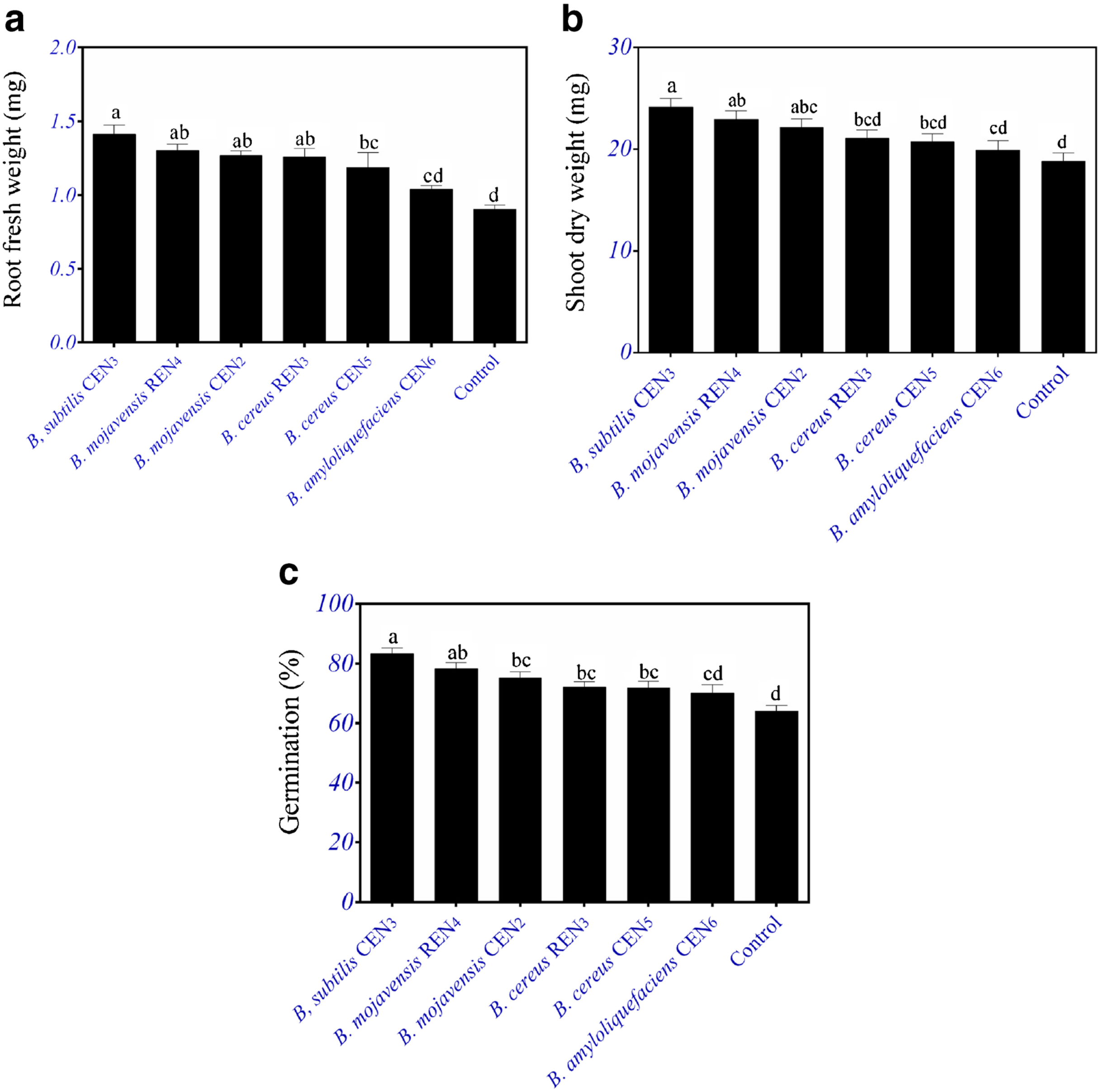

Fig. 5 Effect of inoculation of the superior strains isolated from endorhiza and rhizosphere of rice (endophytic strain REN_3 and rhizosphere strain REN_4), Berseem clover (endophytic strain CEN_2 and rhizosphere strain CEN_6) and oilseed rape (endophytic strain CEN_3 and rhizosphere strain CEN_5) on root fresh weight, shoot dry weight, and seed germination rate (%) of rice seedlings (Cv, *Gohar*). The values are mean of six replicates ± SE. Values followed by the same letter are not significantly different as determined by the Tukey's least significant difference test ($p < 0.01$; $n = 6$)

community (De Boer et al. 2007; Pieterse et al. 2014; Raaijmakers et al. 1995). In addition, the plant genotype and soil type are important drivers of the microbial community composition in the rhizosphere (Garbeva et al. 2008; Viebahn et al. 2005) or endorhiza. Therefore, our results suggest that *Bacillus* species identified in this study existed in sufficiently high numbers in soil to have significant and beneficial effects. In addition, these bacteria could compete effectively with other pathogen-suppressing bio-control bacteria or with commensal microorganisms.

It has been known that bio-control agents such as bacteria may also act synergistically on each other, as apparently non-antagonistic bacterial strains can become

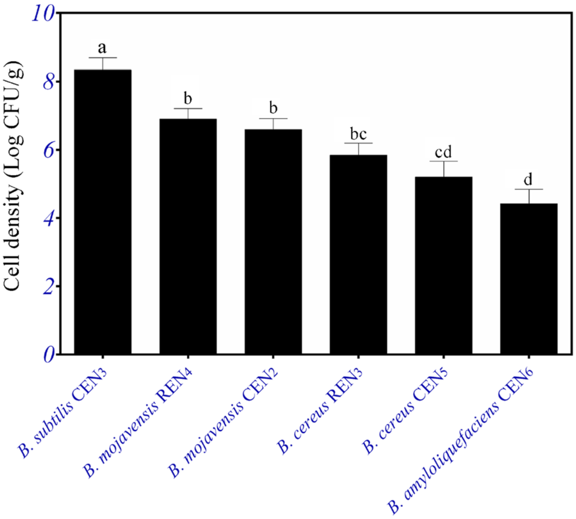

Fig. 6 Population size of the superior strains isolated from endorhiza and rhizosphere of rice (endophytic strain REN_3 and rhizosphere strain REN_4), Berseem clover (endophytic strain CEN_2 and rhizosphere strain CEN_6) and oilseed rape (endophytic strain CEN_3 and rhizosphere strain CEN_5) colonizing on roots (rhizoplane) of rice seedlings (Cv, *Gohar*) grown in tube culture for 20 days after inoculation in Hoagland liquid under gnotobiotic conditions. Values shown are the mean log10 CFU g^{-1} root (FW) ± SE ($n = 8$). Values followed by the same letter are not significantly different as determined by Tukey's least significant difference test ($p < 0.01$; $n = 8$)

antagonistic when grown together with other specific strains (De Boer et al. 2007; Etesami and Alikhani 2016b). This may be the reason for presence of biocontrol agents (*Bacillus* species) both in the rhizosphere and in the endorhiza of the plant species used in this study.

In general, the results clearly showed that rhizosphere and endorhiza of rice plant and the plants cultivated in rotation with it could be potential reservoirs of biocontrol agents (especially Gram-positive bacteria) for control of fungal rice pathogens. It is well known that endophytic bacteria may help in reducing problems associated with the use of synthetic chemicals in agriculture and for managing diseases (Compant et al. 2010; Eljounaidi et al. 2016). This could be due to: (i) being protected from unfavorable environmental conditions (e.g., flooding conditions in rice fields); (ii) having closer relationships with the host than rhizopheric microorganisms; (iii) having multifaceted beneficial effects (antagonistic to several fungal pathogens); and (iv) occupying ecological niches similar to those occupied by plant pathogens. These roles should be taken into account during the screening of effective antagonistic bacteria in inhibiting fungal rice pathogens.

Conclusions

It can be concluded that the plants cultivated in rotation with each other or grown in the same soil with the same microbial community (e.g. pathogenic or beneficial microorganisms) harbor bacteria (e.g. *Bacillus* species) with a good potential in bio-control of rice fungal pathogens. It seems that the presence of *Bacilius* species in rhizosphere and endorhiza of these plants can presumably be a prerequisite for these plants to have a healthy growth in such a soil. Presumably, these plants could select this type of bacteria for a specific function that the core microbiome could not express (i.e., antibiosis against fungal rice pathogens, etc.) or these bacteria had a particular affinity for these plant genotypes. These hypotheses need to be studied further in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest

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