Application of an Endophytic *Bacillus amyloliquefaciens* CC09 in Field Control of *Rehmannia glutinosa* Root Rots Disease

Yarong Xue[1], Lichuan Guo[1], Yunbin Fang[1] and Chanhong Liu[1*]

[1]State Key Laboratory of Pharmaceutical Biotechnology, School of Life Science, Nanjing University, Nanjing 210093, China.

Authors' contributions

This work was carried out in collaboration between all authors. Author CL designed the study and performed the statistical analysis. Authors YX and LG conducted the experiments. Author YF wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aims: To investigates whether a new bio control agent *Bacillus amyloliquefaciens* CC09 can be used to manage the root rot disease of *Rehmannia glutinosa* resulting from continuous cropping.
Study Design: Completely randomized block experiments with 3 replicates were conducted in the field where *R. glutinosa* had been grown for one-year.
Place and Duration of Study: Yangyue village, Linfen city, Shanxi province, from April 11 to September 21, 2010.
Methodology: Four treatments were performed in the trial: T1, seeds were soaked in the bio control agent strain CC09 for 30min; T2: spreading 50 ml of the bio control agent per area right before seed down; T3: seeds were soaked in 1000-fold dilution solution of of 80% carbendazim WP for 30min; T4: seeds were soaked in fresh water for 30min. The incidence of root rot disease (IRRD), disease severity (DS), disease index (DI), relative disease control efficiency (RDCE), the relative yield increase (RYI) and the soil bacterial population were investigated. The dynamics of rif^r strain CC0910 in soil were performed in Petri dishes using the culture dependent method.
Results: Compared with blank control at harvest time, we observed not only 55.5% of disease control efficacy but also 28.6% yield increase in T1 treatment, 43.4% and 39.9%

Corresponding author: Email: chliu@nju.edu.cn;

in T2 treatment. With the aid of rif^r mutant CC0910 and culture-dependent method, we found the soil bacterial populations significant higher in T2 than that in T1 (P<0.05). Moreover, the strain CC09 in soil increased to the maximum in the first few days and then decreased to a relative stable density of 5×10^7 cells g^{-1}.

Conclusion: As the new bio control agent CC09 can maintain high cell density in the soil, thus it could be used to control the root rot disease of *R. glutinosa* caused by continuous cropping.

1. INTRODUCTION

Rehmannia glutinosa L. is a traditional Chinese medicinal plant, which has been used to replenish vitality, strengthen the liver, kidney and heart [1]. It has been also employed for treatment of a variety of ailments like diabetes, anemia and urinary tract problems [2,3]. The productivity and quality of *R. glutinosa* is decreasing due to the continuous cropping, which results in low germination, weak and small plants, more fibrous roots, diseased roots and abnormal enlargement of tuberous roots [4-6]. The disease incidence of *R. glutinosa* could increase by 30% and the yields reduce by 64.3% in a one-year replant field [7]. Because of the severe effect, it was recommended to wait 8-10 years before cultivating *R. glutinosa* again in the same field [8]. To avoid the severe and long-term effects of continuous cropping, *R. glutinosa* has to be cultivated in a virgin field away from its origin, which may result in an adverse impact on the pharmaceutical functions due to variable soil properties and climate conditions.

Although it is an ancient cultivation problem, it is still not clear which factors inhibit the growth and productivity of *R. glutinosa*. It has been suggested that autotoxicity generated by root exudates or plant debris and soil microbial structure disturbance may cause of the continuous cropping problem of *R. glutinosa* [9]. However, there is not enough evidence to support these hypotheses. Recently, we found some allelopathic bacteria such as *Pseudomonas putida* DH03 and *Enterobacter amnigenus* DH05 produce extracellular phytotoxic compounds (allelochemicals) that are toxic to the leaves and roots of *R. glutinosa*; however, the plant composition stimulates the growth of allelopathic bacteria [10]. Based on these results, we believed that allelopathic bacteria are at least one of the causal agents for *R. glutinosa* replant problem. Therefore, any methods targeting the allelopathic bacteria might be effective in protecting *R. glutinosa* from replant root rot.

Generally, biocontrol agents work by competition, antibiotics, parasitism and induced resistance, resulting in the decrease of the harmful microbial population. Among many organisms suited for biocontrol of soil borne diseases, the root-colonizing bacteria *Pseudomonas fluorescens*, *Bacillus subtilis* and *B. amyloliquefaciens* are the most used species for controlling of soybean, cotton, potato, tomato and pepper diseases [11,12]. However, biocontrol agent used for control of *R. glutinosa* diseases or other replant diseases have not been reported yet. In this study, we report a new biocontrol agent, an endophytic bacterium *B. amyloliquefaciens* CC09 isolated from the Chinese medicinal herb *Cinamonum camphora* with broad antimicrobial activity [13], which can be used for field control of *R. glutinosa* root rot diseases caused by continuous cropping by seeds or soil treatments.

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Culture Conditions

Endophytic *B. amyloliquefaciens* CC09 (CGMCC No.4669), originally isolated from the medicinal herb *C. camphora*, was used as a biocontrol agent to control *R. glutinosa* root rot disease. A spontaneous rifampicin resistant (rif^r) mutant CC0910, which was isolated from wild type strain CC09 by plating the strain on medium containing 100µgml^{-1} of rifampicin, was used to test the survival ability of the strain in soil. Both strain CC09 and CC0910 showed the same *In vitro* antifungal activities [14] and were cultured in LB medium (Yeast extract 5gL^{-1}, Tryptone 10g L^{-1}, NaCl 10gL^{-1}, pH7.0) with or without supply of the antibiotics and stored at -80°C.

2.2 Preparation of Biocontrol Agent

Ten µl of stock population of strain CC09 were inoculated into a 15ml Falcon tube containing 4ml of LB, incubated at 32°C for 12h, and then transferred to two 1L Erlenmeyer flasks containing 200ml of LB and incubated at a rotary shaker at 32°C, 120rpm for 36h. Total 400ml of the culture broth of strain CC09 were centrifuged at 3500 g for 20min and then the pellet was washed twice using sterile water. The collected cells were re suspended in sterile physiological saline to make the bio control agent with the cell density of approximately 5.8×10^8CFUml^{-1}. The same procedure was used to prepare the bio control agent of rif^r mutant CC0910.

2.3 Field Trial Design

Completely randomized block experiments with 3 replicates were conducted in the center of a 300m^2 field where *R. glutinosa* had been grown for one-year, at Yangyue village, Linfen city, Shanxi province on April 11, 2010. Each replicate had 4 rows in area of 0.8m^2 (0.8m×1.0m) and each row planted 60 fragments of *R. glutinosa* roots (seeds). Distance between two fragments was 15cm, and 25cm between two rows. Four treatments were performed in this trial: T1, seeds were soaked completely in the bio control agent strain CC09 for 30min; T2: spreading 50 ml of the biocontrol agent strain CC09 per area in the ditch right before seed down; T3: seeds were soaked in 1000-fold dilution solution of synthetic pesticide of 80% carbendazim WP (Tianjin Siterui Chemical Co., Ltd., China) for 30 min (positive control); T4: seeds were soaked in fresh water for 30 min which was served as negative control. The cultivar of *R. glutinosa* was Beijing No. 2, which was one of the dominant cultivars in Shanxi. Normal cultivation methods were conducted during the growth periods of *R. glutinosa*.

2.4 Population Dynamics Determination of Bio Control Agent in Soil

Sixty g of *R. glutinosa* replanted one year soil were aseptically added to a Petri dish (9cm diameter). Two ml of the bio control agent CC0910 were evenly sprayed on the soil surface and then mixed completely to get final cell numbers of 1×10^7cellsg^{-1}. The same amount of the sterile physiological saline was used and treated as a negative control. Each treatment with 3 replicates was incubated at 25°C. Five g of the soil sample were taken at 1, 3, 6, 12 and 24d after treatment, put into a 100ml flask containing 50ml of sterile water and 2g sterile glass beads, shaken at 4°C for 30min, and then used to count the colony formation unit

(CFU) of strain CC0910 in LB medium containing 100µgml^{-1} of the rifampicin using the series dilution plating method [15].

2.5 Investigation

The incidence of root rot disease (IRRD), disease severity (DS), disease index (DI), relative disease control efficiency (RDCE) and the yield of each block were investigated during harvest on Sep. 21, 2010. IRRD was measured as the percentage of necrotic roots in relation to the total roots (numbers of necrotic roots/numbers of total roots×100). Based on the percentage of necrotic area to the whole root surface, the disease was roughly classified into 6 grades (S_{0-5}), S_0=0, S_1<20%; S_2=21-40%, S_3=41-60%, S_4=61-80%, S_5>80%. The DS, DI and RDCE were calculated according to the following equations: DS(%)=[$\sum n_i DS_i/N$]×100; DI(%)=$\sum n_i DS_i/(N \times 5)$×100; RDCE(%)=$\sum(DI_{ck}-DI_{agent})/DI_{ck}$×100, where i is the DS grade from 0 to 5, n is the number of samples, N is the total roots being investigated, DI_{ck} and DI_{agent} mean the DI in the T4 and T1-T3 treatments respectively. The relative yield increase (RYI) was calculated according to the following equation, RYI (%)=[(yield of T1 or T2 or T3)-(yield of T4)] / (yield of T4)×100.

In addition, 3g of the field soil under the surface of 2cm were collected from 3 sites in each block when the *R. glutinosa* was harvested, put in sterile tubes and transferred to the laboratory for cell counts of bacterial population in the soil bacterial population able to growth in LB medium at 32°C for 48h using the series dilution plating method [15]. Each experiment had 3 replicates.

2.6 Data Analysis

All data were analyzed with GraphPad Prism (version 3.02) software to estimate the significance of the differences (P<0.05) using one-way ANOVA and the Dunnett test.

3. RESULTS AND DISCUSSION

3.1 Efficiency of Bio Control Agent in Control of *R. glutinosa* Root Rots Disease

Fig. 1 shows the IRRD of the four treatments. Although the average IRRD among the various treatments was not statistically significant (P>0.05), the IRRD value was still lower for T1, T2 and T3 than the negative control T4. However, compared with T4, the DS and DI were significantly reduced in T1, T2 and T3 (P<0.05). Moreover, the DS and DI in T1 were not significantly different from that of positive control T3 (P>0.05), which indicated that the biocontrol agent strain CC09 exhibits great potential for the efficient control of *R. glutinosa* root rot diseases Fig. 1. Although the RDCE in T1, T2 and T3 was not very high with average of 55.5%, 43.4% and 61.1% respectively, the RYI was 28.6%, 39.9% and 27.1% respectively Fig. 2. Particularly, the RYI in the T2 was significant higher than that in the other treatments (P<0.05).
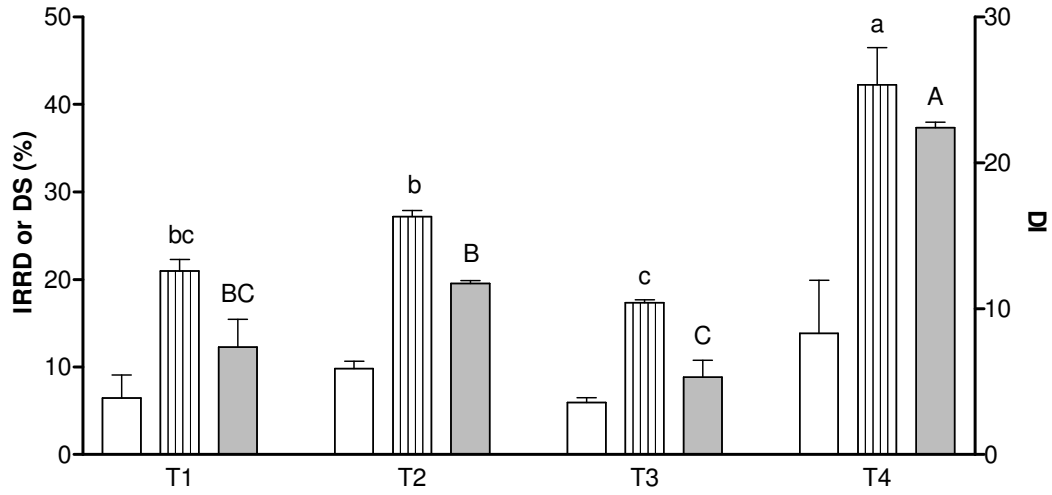
Fig. 1. IRRD (Incidence of necrotic roots, open bar), DS (disease severity, vertical line bar) and DI (disease index, filled bar) of *R. glutinosa* under different treatments (T1-T4)

T1, T3 and T4 indicate that the seeds were soaked in biocontrol agent strain CC09, 1000-fold dilution solution of 80% carbendazim and fresh water for 30 min, respectively. T2 indicates that the soil was treated by strain CC09.
a, b and c indicate DS and A, B and C represent DI that show significant difference among the given treatments with P<0.05 in one-way ANOVA and Dunnett tests
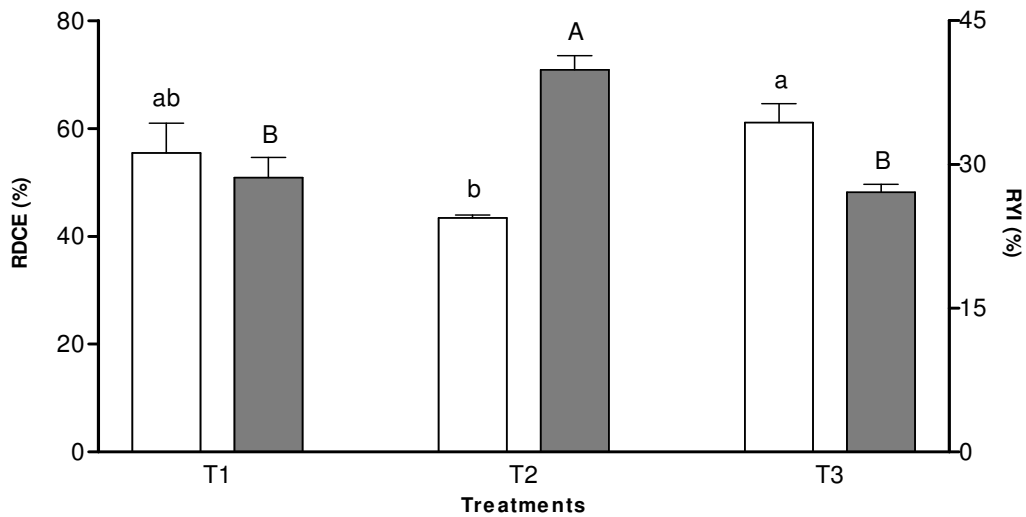

Fig. 2. RDCE (relative disease control efficiency, open bar) and RYI (relative yield increase, filled bar) of *R. glutinosa* in different trials (T1-T3)

T1-T3 is same to that described in Fig.1.
a & b indicate RDCE and A & B represent RYI that show significant differences among the given treatments with P<0.05 in one-way ANOVA and Dunnett tests

Although statistically analysis showed that the IRRD, DS, DI and RDCE in T2 were not significantly different from those in T1, however, the RYI in T2 was significantly higher than that in T1 Fig. 2.The higher RYI in T2 indicated that the CC09 strain might have the capacity to promote plant growth or/and increase in the population of other beneficial microbes in the soil, which may play a direct or indirect role in enhancement of the plant growth. However, the exact mechanism of the increase in both the soil bacterial population and the yields caused by T2 treatment needs to be further studied.

Like *B. subtilis*, *B. amyloliquefaciens* is another important biocontrol agent that has been widely used in plant disease control. Based on genomic analysis, *B. amyloliquefaciens* FZB42 and *B. subtilis* 168 share about 50% (3271 genes) of their homology genes [16]. *B. amyloliquefaciens* possesses not only many of the advantages that *B. subtilis* has, such as great environmental adaptability, strong antimicrobial activity, but also some special characteristics that *B. subtilis* does not have, such as plant growth promotion and larger proportion of genes (7.5% to the total genes) encoding secondary metabolites biosynthases [16]. Due to these additional advantages, several *B. amyloliquefaciens* strains, such as FZB42, LX11, TB-2, IMAUB1034 and YN-1 have been tested and applied to control diseases caused by *Xanthomonas oryzae*, *Phytophthora* sp., *Rhizoctonia* sp.[17-20] Our previous studies also proved that strain CC09 isolated from the healthy stem of *C. camphora* exhibited broad antimicrobial spectrum against many plant pathogens such as *Phytophthora capsici*, *Fusarium graminearum*, *Alternaria alternata* and *Rhizoctonia solani* [14]. Here, we first demonstrated that strain CC09 can be used as an effective biocontrol agent against *R. glutinosa* root rot disease. To our knowledge, this is the first report about *R. glutinosa* root rot disease control by application of biocontrol agents.

B. amyloliquefaciens is a plant root-colonizing bacterium, which has the ability to stimulate plant growth and suppress phytopathogens. The commercialized *B. amyloliquefaciens* FZB24 shows great potential in plant-growth promotion and disease control of stem canker and black scurf in the field trials on potatoes resulting in yield increase of 7.5–10% [21]. It has been reported that the disease control mechanism of FZB42 could be attributed to the suppression of the competitive plant-pathogenic microflora within the rhizosphere by secreted antifungal and antibacterial lipopeptides and poly- ketides [21]. Due to production of cycle lipopeptide Iturin A and surfactins [14], we speculated that *B. amyloliquefaciens* CC09 may have the same disease control mechanism to the strain FZB42.

3.2 Effect of Bio Control Agent on Soil Bacterial Populations

Fig. 3 shows the cell numbers of bacteria in the soil under the given treatments. The effect of the bio control agent on the soil bacterial population was dependent on its application. The bacterial population was increased ($P<0.05$) in T2 (spray treatment) but not ($P>0.05$) in T1 (seed treatment) compared T4. The cell numbers of bacteria in T2 were 24.4×10^6CFUg^{-1}, about 2.6-, 2.5- and 1.8-fold as high as those in T1, T3 and T4, respectively. The higher cell density in T2 relative to the other treatments, especially to the T1 treatment was probably caused by the higher inoculated strain CC09 in the soil or/and the CC09 strain that led to an increase in the population of other bacteria in the soil. Although we did not compare the inoculated cell numbers of strain CC09 in the field between T1 and T2, we believed that the abundance of inoculums applied to the soil by T2 treatment (spraying soil: 50 ml per area) must be much higher than that by T1 treatment (soaking seeds).
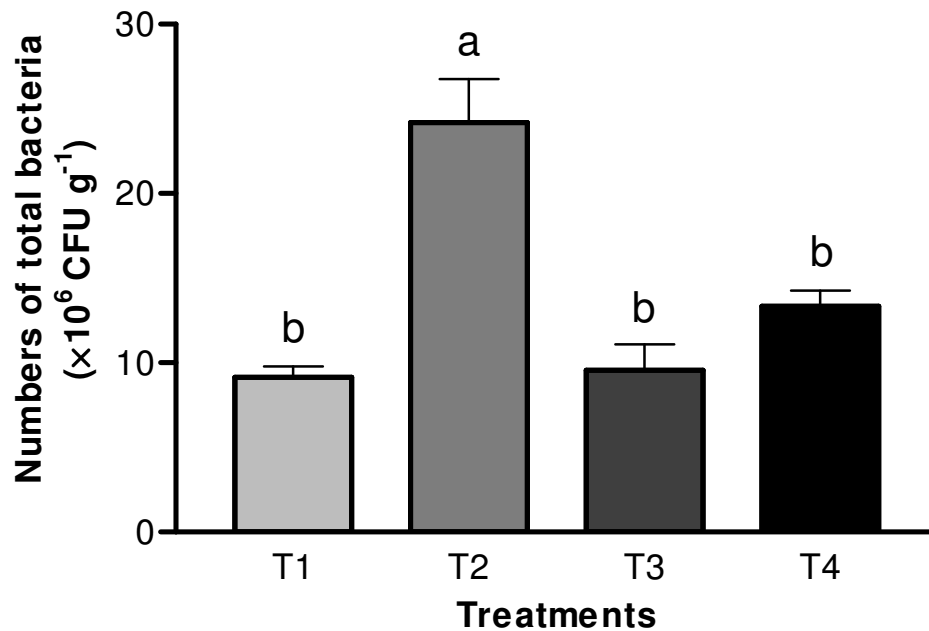
Fig. 3. Abundance of total soil bacteria in different trials (T1-T4)
a & b indicate the abundance of total bacteria shows significant differences among the given treatments with P<0.05 in one-way ANOVA and Dunnett tests

Bio control agents suppress the diseases mainly through competition, antibiotics and induced resistance. *B. amyloliquefaciens* has been reported to have the ability to secrete extracellular antimicrobial metabolites, induce disease resistance and enhance plant growth [14,16,22]. For example, *B. amyloliquefaciens* strain KPS46 has proved to be an inducer of systemic resistance against *Xanthomonas axonopodis* pv. *glycines*, a causal agent of soy bean bacterial pustules, as well as a biocontrol agent to control multiple pathogens causing either foliar or root diseases of various plants [22]. In this study, we demonstrated that the DS and DI were decreased and the yield was increased at T1 and T2. The effect of the biocontrol agent CC09 on the root diseases was probably dependent on the extracellular antimicrobial substances or species competition [14]. However, it has been reported that some bacteria in the soil are beneficial to *R. glutinosa*, but others are inhibitory or lethal to the plant [23]. Therefore, strain CC09 could act as a beneficial bacterium and exert its biocontrol ability by replacing or/and inhibiting the pathogenic harmful bacteria resulting in a relative health soil ecosystem. Keeping high populations of the beneficial bacteria including strain CC09 and suppressing the harmful allelopathic bacteria is a way to prevent the *R. glutinosa* from the continuous cropping problem.

3.3 Population Dynamics of the Bio control Agent in the Soil

With the aid of rif^r strain CC0910, the population dynamics of the bio control agent in the soil was investigated. Result indicated that the cell numbers of the bio control agent in the soil increased to the maximum of $9.1 \times 10^7 CFUg^{-1}$ on the third day, then declined linearly to $6.6 \times 10^7 CFUg^{-1}$ on the twelfth day and stayed $4.8 \times 10^7 CFUg^{-1}$ for the remaining 24 days Fig. 4. None rif^r CC0910 colonies were observed in the negative control.
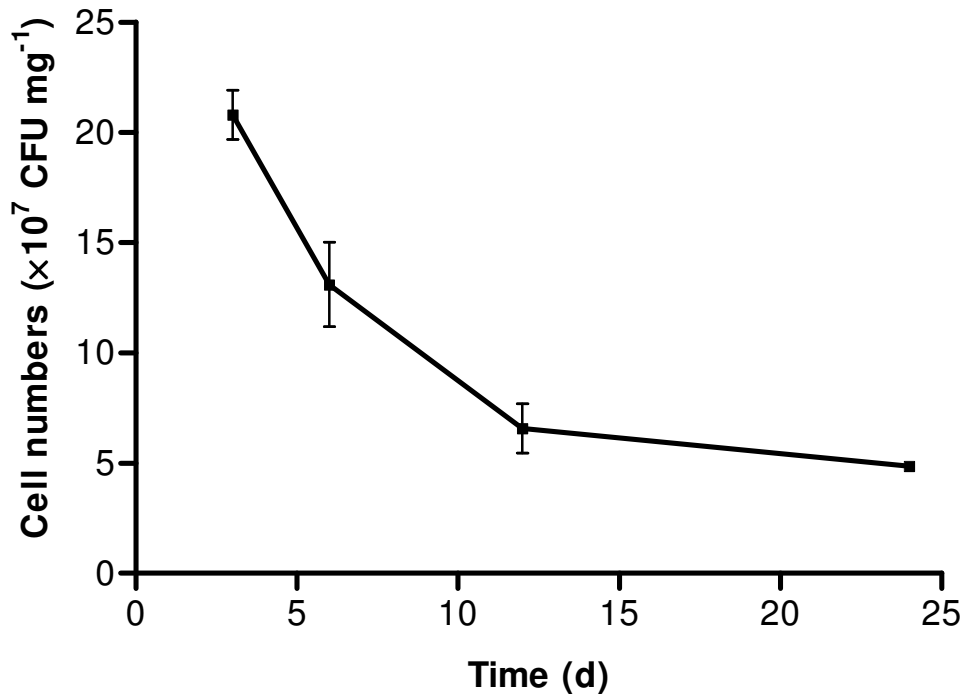
Fig. 4. Cell numbers of the strain CC0910 in the soil at various sampling days

As a new biocontrol agent, the population dynamics of strain CC09 in soil have been determined under laboratory conditions. The cell numbers of the bio control agent increased up to the maximum on the third day and reduced thereafter to reach a relative stable population, about $5.0 \times 10^7 CFUg^{-1}$, after 6-12 days, which was similar to the average cells $(1.6 \times 10^8 CFUg^{-1})$ in fresh soil [24]. The high survival ability and long persistence of the strain CC0910 in soil Fig. 4. suggested the biocontrol agent CC09 or CC0910 might have good colonization in rhizosphere and maintain control of *R. glutinosa* replant diseases for many years after the initial release of the strain. This needs to be the subject of future testing.

4. CONCLUSION

Although bio control agents have been widely used for soybean, cotton, potato, tomato, pepper and economical crops, they are rarely used to control or prevent plant diseases caused by continuous cropping. Therefore, this study at the first time to demonstrate that an endophytic bacterium *B. amyloliqufaciens* CC09 can be used as a potential bio control agent to control *R. glutinosa* root rots caused by continues cropping under field conditions using two application strategies: 1) soaking the seeds and 2) spaying the soil with bacteria. The application of this bacterium not only has practical significance for controlling the root diseases of medicinal plant *R. glutinosa* resulting from continuous cropping, but also opens a new window for overcoming replant diseases of other plants.

CONSENT

All authors declare that written informed consent was obtained from School of Life Science, Nanjing University for publication of this report.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Lee BC, Choi JB, Cho HJ, Kim YS. *Rehmannia glutinosa* ameliorates the progressive renal failure induced by 5/6 nephrectomy. J Ethnopharmacol. 2009;122(1):131-135.
2. Oh H. Remophilanetriol: A new eremophilane from the roots of *Rehmannia glutinosa*. Bull Korean Chem Soc. 2005;26(8):1303-1305.
3. Waisundara VY, Huang M, Hsu A, Huang D, Tan BK. Characterization of the anti-diabetic and antioxidant effects of *Rehmannia glutinosa* in streptozotocin-induced diabetic wistar rats. Am J Chin Med. 2008;36(6):1083-1104.
4. Liu HY, Wang F, Wang YP, Lu CT. The causes and control of continuous cropping barrier in Dihuang (*Rehmannia glutinosa* Libosch.). Acta Agriculturae Boreali-Sinica. 2006;21(4):131-132. Chinese.
5. Qu XH, Wang JG. Effect of amendments with different phenolic acids on soil microbial biomass, activity, and community diversity. Appl Soil Ecol. 2008;39(2):172-179.
6. Zhang ZY, Li G, Niu M, Fan H, Li J, Lin W. Responses of physiological ecology and quality evaluation of *Rehmannia gltinosa* in continuous cropping. Zhongguo Zhong Yao Za Zhi. 2011;36(9):1133-1136. Chinese.
7. Qu YQ, Ren DZ, Yao Y. Preliminary report of various cultivars of *Rehmannia glutinosa* resistant to continuous cropping problem. Shaanxi Agri Sci. 2011;(4):103-105. Chinese
8. Wen XS, Yang SL, Wei JH, Zheng JH. Textual research on planting history of *Rehmannia glutinosa* and its cultivated varieties. Chin Tradit Herbal Drugs. 2002;33(10): 946-949. Chinese.
9. Du JF, Yin WJ, Zhang ZY, Hou J, Huang J, Li J. Autotoxity and phenolic acids content in soils with different planting interval years of *Rehmannia glutinosa*. Chin J Ecol. 2009;28(3):445-450. Chinese.
10. Guo LC, Liu CH, Zuo GS, Xue YR, Shuang JL. Mechanism analysis of continuous cropping obstacle of *Rehmannia glutinosa*. Chin Agri Sci Bull. 2012;28(31):227-230. Chinese.
11. Rezzonico F, Zala M, Keel C, Duffy B, Moënne-Loccoz Y, Défago G. Is the ability of bio control *Pseudomonads fluorescent* to produce the antifungal metabolite 2,4-diacetylphloroglucinol really synonymous with higher plant protection? New Phytologist. 2007;173(7):861-872.

12. Tan GY, Yang ZL, Yuan ZL, Yang X. Research advances in continuous cropping obstacle in medicinal plants and its management. J Northwest A and F Univ (Nat Sci Ed). 2012;40(4):197-204. Chinese.
13. Liu CH, Wang F, Wang YP, Lu CT. A bio control agent used for plant diseases control and its preparation. Chinese Patent, Authorized No: ZL201110073773.9, June 27; 2012.
14. Cai XC, Li H, Xue YR, Liu CH. Study of endophytic *Bacillus amyloliquefaciens* CC09 and its antifungal CLPs. J Appl Biol Biotechnol. 2013;1:1-5.
15. Davis KER, Joseph SJ, Janssen PH. Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria. Appl Environ Microbiol. 2005;71(2):826-834.
16. Arguelles-Arias A, Ongena M, Halimi B, Lara Y, Brans A, Joris B, et al. *Bacillus amyloliquefaciens* GA1 as a source of potent antibiotics and other secondary metabolites for biocontrol of plant pathogens. Microb Cell Fact. 2009;8:63.
17. Deng JL, Liu HY, Liu YX, Liu XT, Ni YX, Gao SX, et al. Identification of the antifungal substances from *Bacillus amyloliquefaciens* strain YN-1. Acta Phytopathologica Sinica. 2010;40(2):202-209. Chinese
18. Qiu SX, Ruan HC, Song MX, Hu FP. Biochemical mechanisms of the resistance induced by metabolites of endophytic *Bacillus amyloliquefaciens* strain TB2 to suppress pepper fruit *Phytophthora* blight. Chin J Trop Crops. 2010;31(10):1813-1820. Chinese.
19. Zhang ZY, Chen H, Yang YH, Chen T, Lin RY, Chen XJ, et al. Effects of continuous cropping on bacterial community diversity in rhizosphere soil of *Rehmannia glutinosa*. Chin J Appl Ecol. 2010;21(11):2843-2848. Chinese.
20. Zong ZY, Wang HK, Xiao RF, Yan YH. Preliminary research on antifungal bioactive substance of *Bacillus amyloliquefaciens* IMAUB1034. J Tianjin Univ Sci and Tech. 2012;27(1):5-8. Chinese.
21. Choudhary DK, Johri BN. Interactions of *Bacillus* spp. And plants–with special reference to induced systemic resistance (ISR). Microbiol Res. 2009;164:493-513.
22. Buensanteai N, Athinuwat D, Prathuangwong S. *Bacillus amyloliquefaciens* induced systemic resistance against *Xanthomonas axonopodis* pv. *glycines* caused agent soybean bacterial pustule with increased phenolic compounds and phenylalanine ammonia lyase. Proceedings of the 45th Kasetsart University Annual Conference, Bangkok, Thailand, 30 January-2 February. Subject: Plants. 2007:364-371.
23. Wang XC, Wen XS, Yang DK. Effects of inoculation with soil bacteria on growth of *Rehmannia glutinosa* in tube and potted plantlets. Chin Tradit Herbal Drugs. 2009;40(5):797-800. Chinese.
24. Dabek-Szreniawska MD, Hajnos M, Stotzky G, Collins Y, Malicki J. Numbers of culturable bacteria in soil under mineral or organic cultivation: Comparison of Hattori's 'FOR' and standard dilution plate methods. Int Agrophysics. 2006;20(4):277-288.