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DEGRADATION OF PHENOLIC ACIDS AND RELIEF OF CONSECUTIVE MONOCULTURE OBSTACLE OF *REHMANNIA GLUTINOSA* BY THE COMBINATION OF *BACILLU* SSP. AND *PICHIA PASTORIS*

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Abstract

Rehmannia glutinosa (*R. glutinosa*) is a high demand traditional Chinese medicine, but it suffers serious consecutive monoculture obstacle (CMO). The disability of root swelling is one of the negative impacts caused by the *R. glutinosa* CMO and is related to allelopathy exudates, such as phenolic acids. It is thought that a microbe agent could improve plant health by eliminating the unfavorable effect of allelopathy exudates. In previous research, we isolated two phenolic acid-degrading microbes from rhizosphere soil surrounding *R. glutinosa.* These were *Bacillus* sp. and *Pichia pastoris*. This study found that *Bacillus* sp. combined with *Pichia pastoris* could degrade 97.19% ferulic acid and 98.73% hydroxybenzoic acid over 15 days. *R. glutinosa* takes a long growth time (7-8 months) under field conditions. We set up a modified tissue culture model to rapidly detect whether *Bacillus* sp. and *Pichia pastoris* combination could relieve the CMO. The results showed that our tissue culture model effectively simulated the *R. glutinosa* growing process in unplanted or second-year monoculture field. Furthermore, the combination of *Bacillus* sp. and *Pichia pastoris* can significantly relieve the CMO-induced suppression of root swelling. All these results suggested that: 1) The combination of *Bacillus* sp. and *Pichia pastoris* has considerable potential to degrade allelopathy exudates and alleviate the CMO of *R. glutinosa*; 2) Our tissue culture model could be used to quickly screen effective microbes that could alleviate CMO in plants.

Key words: *Rehmannia glutinosa* , Monoculture obstacle, *Bacillus*, *Pichia pastoris*

Introduction

R. glutinosa is a high demand traditional Chinese medicine with many pharmacological functions. *R. glutinosa* in Huai Region in Henan province of China is considered to have special effects and thus assumes great economic values. Unfortunately, this plant suffers from a very serious consecutive monoculture obstacle (CMO), which means that it cannot be cultured in the same field for 8-10 years after the previous crop has been removed. This seriously limits large scale planting in Huai Region (Wu *et al*., 2013). One of the most important aspects of the CMO is the disability of the root swelling, which is responsible for dramatic decline in *R. glutinosa* productivity and quality.

It has been widely accepted that allelochemicals from plant roots can be released into rhizosphere soil, harm plant itself and induce CMO. Therefore, eliminating the unfavorable effect of allelochemicals is a promising way to overcome the CMO of the plant. Several methods, such as appropriate crop rotations, intercropping, fertilization, etc., have been tried, but they have various disadvantages (Xie & Dai, 2015). Microbial communities in rhizosphere soil are thought to contribute to plant growth by eliminating environmental compounds and maintaining a healthy soil (Roesti *et al*., 2006). There are increasing evidences that some rhizosphere microbes, such as *Phomopsis liquidambari* and *Trichoderma harzianum SQR-T037*, have considerable potential to degrade allelochemicals produced by plants (Xie & Dai, 2015; Li *et al*., 2011), which suggests that it is possible to alleviate the CMO of *R. glutinosa* by developing microbial agents. However, it normally required almost one year for *R.glutinosa* from planting to harvesting. Field tests of a microbial agent need at least three years due to various factors. Therefore, it is urgent to establish an efficient and quick method to screen potential microbes that can effectively relieve CMO before they are tested in field trials. In this study, we set a modified tissue culture model to quickly detect the ability of the combination of a *Bacillus* sp. and *Pichia pastoris* (isolated from rhizosphere soil) in reliving CMO of *R. glutinosa*.

Material and Methods

High-performance liquid chromatography (HPLC) analysis: Isolated microbes (3×10^5) that degrade phenolic acids were added into the flake containing liquid basal salt medium (250 mL) with or without 500 mg/L ferulic acid or hydroxybenzoic acid. The flake was shaken at 180 rpm/min at 28ºC. After 0, 10 or 15 days, the medium was centrifuged (8000 rpm/min at room temperature for 10 min) to remove the microbial cells, the supernatant was filtered through 0.22 μm filter, and then the ferulic acid and hydroxybenzoic acid contents in the supernatant were detected by HPLC (Agilent 1200, Palo Alto, California, USA). The mobile phase elutions included a two solvent gradient elutions. These were Solvent A (Methanol) and Solvent B (0.1% Phosphoric acid aqueous solution). The gradient program began with 30% A and 70% B from 0 to 8 minutes. This was followed by 40% A and 60% B from 8 to 12 minutes. Then, concentration of A and B was increased and reduced to 45% and 55% for the next 8 minutes, respectively. The column temperature was 25ºC; injection volume was 20 μL; the eluent flowrate was 1.0 mL/min and total run time was 20 minutes. The chromatograms were monitored at a wavelength of 280 nm.

Preparation of the soil extract: Soil samples from unplanted or second-year *R. glutinosa* monoculture fields were obtained from Wuzhi County (113°38′E, 35°10′N; the optimal production areas of *R.glutinosa*), Henan Province, China, during November, 2014. Soil samples weighing about 200 g were collected from the top 5-10 cm soil layer of the unplanted fields or from the soil in the *R. glutinosa* rhizosphere of the second-years monoculture fields (about 20 m^2 per field) at five random locations. Three replicate samples were placed into a sampling bag and evenly mixed. Under sterile conditions, samples were brought to the laboratory, naturally air-dried and sieved through a 0.2-mm mesh.

Two hundred g soil samples were soaked in 1000 mL distilled water. Then, the mixed dilution was boiled for 3 h and left for 24 h. These steps were repeated three times. The extract was filtered sequentially through filter paper and filters with a pore size of 5 and 0.45 μm in order to remove particulate matter. After preparation, the extract was sterilized by autoclaving at 121°C for 15 min and stored at 4 °C until needed.

Tissue culture: Root tubers of *R. glutinosa* (Wen 85-5) were surface-sterilized in a 75% alcohol solution for 30 sec prior to immersing in $Hgcl_2$ for 10 min. Then they were rinsed three times with sterile distilled water, divided into segments and placed into the basal medium which consisted of 1/2 MS major salts supplemented with 0.5 mg/L benzyladenine (BA). The pH was adjusted to 5.7-6.0 before the medium was solidified with 0.7% (m/v) Bacto agar. The aseptic seedlings with the root removed were transferred to propagation medium (MS medium supplemented with 1 mg/L BA, 0.1 mg/L naphthalene acetic acid [NAA]). After 7 days, seedlings, after their roots again were cut off, were transferred to root-inducing medium (1/2MS+indoleacetic acid [IBA] 1 mg/L). After root regeneration, intact seedlings were explanted to swollen root-inducing medium (MS+6-BA 2 mg/L+NAA 0.1 mg/L+5% sucrose). The number and diameter of root were monitored at 7, 14, 21, or 28 days. Three independent experiments were undertaken. The effect of soil extract with or without microbe treatment to stimulate root swelling was measured after the water in medium was replaced with the soil extracts from different treatments.

Results and Discussion

The combination of *Bacillus* sp. and *Pichia pastoris* effectively degraded phenolic acids: Some phenolic acids, such as ferulic acid and hydroxybenzoic acid, are believed to play important roles in *R. glutinosa* CMO (Li *et al*., 2012). In previous research, we isolated two microbes from rhizosphere soil surrounding *R. glutinosa* -- *Bacillus* sp. and *Pichia pastoris* (Genbank accession numbers: KR982689 and KR982690, respectively), which possess the ability to grow on solid basal salt medium plate supplemented with 500 mg/L ferulic acid or hydroxybenzoic acid as carbon and energy sources (Chen *et al.*, 2015).

In this study, we further tested the ability of *Bacillus* sp. and *Pichia pastoris* to degrade ferulic acid and hydroxybenzoic acid by HPLC analysis as described in material and methods. The results showed that the concentrations of ferulic acid and hydroxybenzoic acid after 15 day in *Bacillus* sp.-treated group and *Pichia pastoris*-treated group fell from 500 mg/L at 0 day to 178.62 mg/L and 148.35 mg/L, 117.65 mg/L and 99.93 mg/L, respectively. The degrading rates of ferulic acid and hydroxybenzoic acid after 15 day in *Bacillus* sp.-treated group and *Pichia pastoris*-treated group were 64.32% and 70.23%, 76.47% and 80.14%, respectively.

Previous reports indicated that combinations of biocontrol agents could result in more effective and robust control of plant diseases (Hamidreza *et al*., 2013). Therefore, we investigated the ability of a *Bacillus* sp. and *Pichia pastoris* combination to degrade ferulic acid and hydroxybenzoic acid. As shown in table 1, along with the increase of times, peak areas of ferulic acid and hydroxybenzoic acid dramatically decreased. Under the same conditions, the combination treatment reduced ferulic acid and hydroxybenzoic acid concentrations to 71.1 mg/L and 14.05 mg/L, 23.40 mg/L and 6.35 mg/L by 10 day and 15 day, respectively. The degradation rates at 10 day and 15 day reached 85.66% and 97.19% for ferulic acid, 95.32% and 98.73% for hydroxybenzoic acid, respectively, indicated that the combination was more efficient in degrading ferulic acid and hydroxybenzoic acid than the individual treatments.

Table 1. The ability of *Bacillus* sp. and *Pichia pastoris* combination to degrade ferulic acid and hydroxybenzoic acid.

Samples	Appearance time (min)	Peak high (mAU)	Peak area (mAU*s)	Degradation rate (%)
Ferulic acid				
0 day	6.996	321	11800	
10 day	7.897	46	2395	85.66
15 day	7.961	8.89	292.8	97.19
Hydroxybenzoic acid				
0 day	12.135	791	29000	
10 day	12.979	37	4349	95.32
15 day	13.014	10	282	98.73

A tissue culture model to simulate the growing process of *R. glutinosa*: Considering that the *R. glutinosa* root growth and swelling requirea long period (7-8 months) under field conditions, we created a tissue culture model to quickly determine whether the consecutive monoculture soil could suppress the *R. glutinosa* root growth and swelling. We firstly prepared sterile extracts of unplanted and second-year monoculture soils to respectively replace the water in the medium of tissue culture model as described in materials and methods. The results showed that the *R. glutinosa* growing on medium containing second-year monoculture or unplanted soil extracts did not show any significant differences in the average root number during the tissue culture process, although the former showed a slight reduction (Fig. 1A). However, compared with the unplanted soil extracts, the soil extracts from second-year monoculture field resulted in reduction of average root diameter by 4.1-fold, 5.0-fold, 5.5-fold and 5.9-fold, respectively, at 7, 14, 21 and 28 days (Fig. 1B). Furthermore, *R. glutinosa* growing on medium containing second-year monoculture soil extracts began to die at 7 days and by 28 days, the dead rate increased to 80.77%. All these results indicated that the extracts from second-year monoculture soil significantly suppressed the *R. glutinosa* growth and root swelling, which were consistent with traditional agriculture practices. Thus, our tissue culture model could simulate the *R. glutinosa* growing process in unplanted or second-year monoculture field.

The combination of *Bacillus* sp. and *Pichia pastoris* effectively stimulate *R. glutinosa* root swelling: Tissue culture model was used to detect the effect of the *Bacillus* sp. and *Pichia pastoris* combination on root swelling of *R. glutinosa*. The extracts (250 ml) taken from the second-year monoculture soil was first treated with the two microbe combination (3×10^5, respectively) for 7 days with shaking, and then these microbes were removed with a 0.22 μm filter. The microbes-treated soil extracts were detected in the *R. glutinosa* tissue culture model using exactly same procedure as described above to test the relief effects of the microbial combination. The results from tissue culture showed that *R. glutinosa* growing on medium containing unplanted soil extracts or microbes-treated soil extracts exhibited root swelling at 7 days, although the number of swollen root is slight higher in the former than in the latter (Fig. 2a, a', c, c'). By 28 days, the swollen root number and size of *R. glutinosa* growing on the two media had no obvious difference (Fig. 2a1, a1', c1, c1'). In contrast, *R. glutinosa* growing on medium containing second-year monoculture soil extracts that were not treated with the two microbes had a higher death rate and no obvious root swelling throughout entire tissue culture process was observed (Fig. 2b, b', b1, b1'). Taken together, the combination of *Bacillus* sp. and *Pichia pastoris* isolated from rhizosphere soil could effectively degrade ferulic acid and hydroxybenzoic acid, and significantly relieve theCMO-induced suppression of root swelling.

According to the reports, genus *Bacillus* has been frequently used as biocontrol agents to promote plant health. Some *Bacillus* sp. can suppress plant pathogen microbes and *Bacillus* populations can be utilized as biofertilizers to improve the bioavailability of essential compounds and increase the supply of mineral nutrients to the host plants (Alejandro *et al*., 2011). Some yeasts, such as *Candida tropicalis* and *Rhodotorula ferulica sp. nov.*, have also been reported to degrade phenol derivatives (Sampaio *et al*., 1991; Phalgune *et al*., 2013). All these data support our opinion that the combination of *Bacillus* sp. and *Pichia pastoris* has great potential in soil remediation and maintaining plant health. However, whether these two microbes can successfully colonize in *R. glutinosa* rhizosphere and improve *R. glutinosa* growth and root swelling of require further large scale planting tests in fields, especially CMO fields. However, this study has provided a quick screening method for candidate microbes.

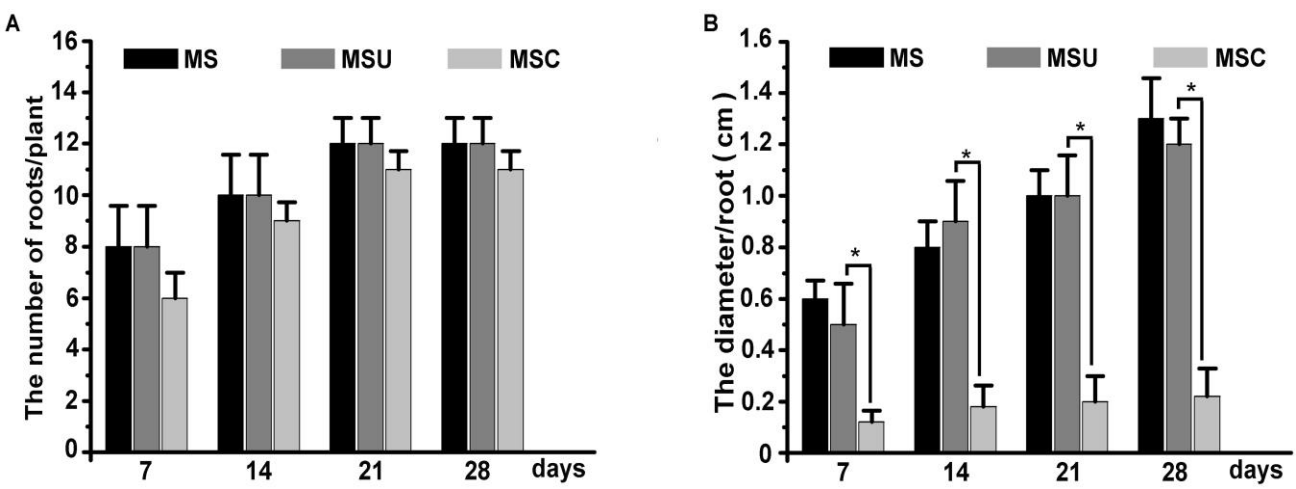

Fig. 1. Effects of soil extracts on the rooting and swelling of *R. glutinosa* at different time intervals of tissue culture. A. The average root number of *R. glutinosa* growing on different media. B. The average root diameter of *R. glutinosa* growing on different media. MS-normal medium; MSU-medium prepared by replacing the water in MS medium with unplanted soil extracts; MSC-medium prepared by replacing the water in MS medium with second year-monoculture soil extracts. At least twenty roots were analyzed per condition in each experiment. Error bars represent the standard error of the mean from three separate experiments per condition. *, $p<0.01$.

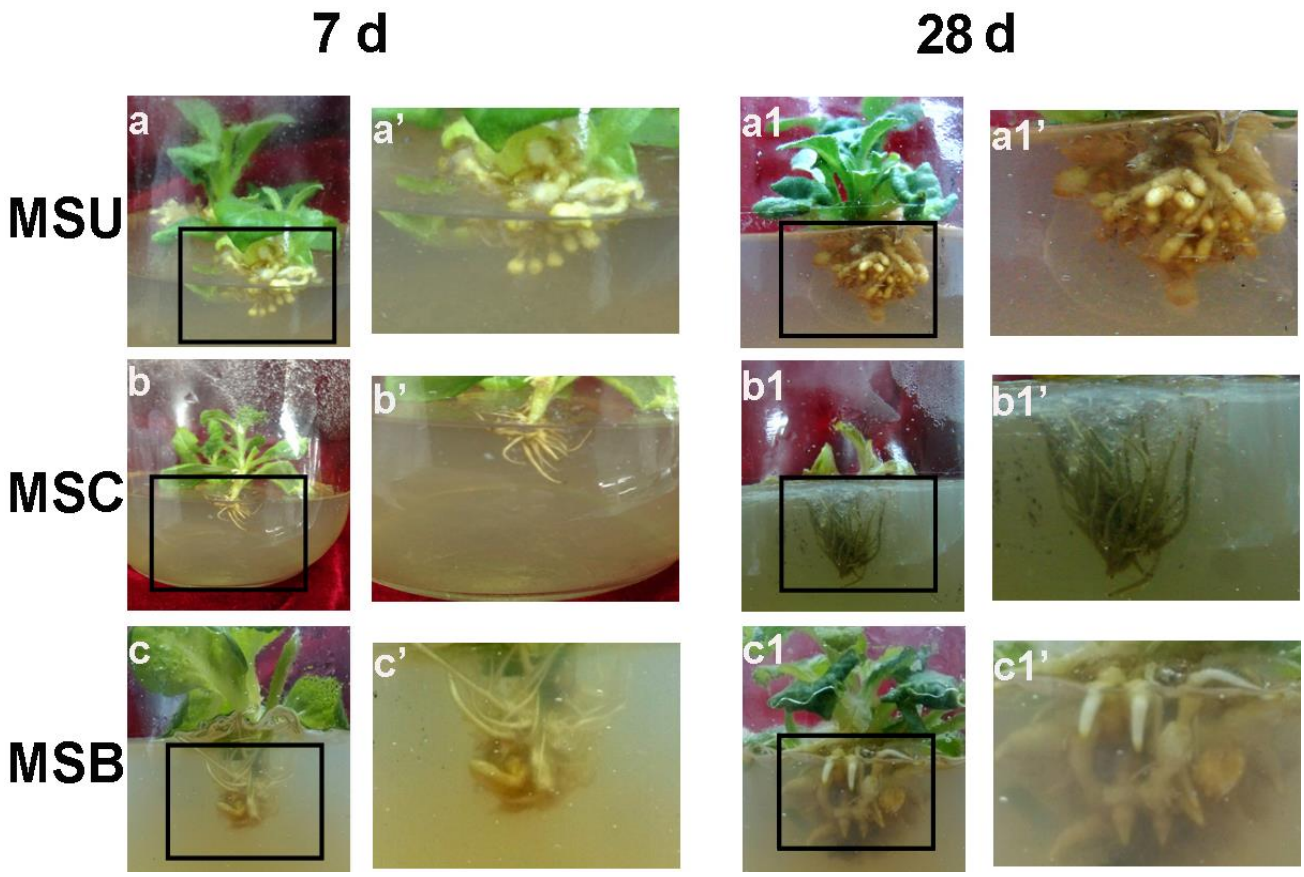

Fig. 2. Effects of second year-monoculture soil extracts treated with the *Bacillus* sp. and *Pichia pastoris* combination on the root swelling of *R. glutinosa* growing on different media at different time intervals of tissue culture. MSU-medium, MSC-medium and MSB-medium were prepared by replacing the water in MS medium with unplanted soil extracts or second year-monoculture soil extracts treated with or without *Bacillus* and *Pichia pastori* combination, respectively. The outlined regions in the a, a1, b, b1, c and c1 are further magnified as a', a1', b', b1', c' and c1', respectively.

Conclusion

Bacillus sp. and *Pichia pastoris* from rhizosphere soil surrounding *R. glutinosa* were isolated and were found to be capable of degrading ferulic acid and hydroxybenzoic. A combination of the two microbes could degrade 97.19% ferulic acid and 98.73% hydroxybenzoic after 15 days in liquid basal salt medium. A tissue culture model of *R. glutinosa* was established to indicate the CMO effects of the consecutive monoculture soil. After the soils were treated with the *Bacillus* sp. and *Pichia pastoris* combination, the suppression of *R. glutinosa* growth and root swelling by consecutive monoculture soil was significantly relieved under tissue culture conditions. This tissue culture model could be used to quickly screen effective microbes that may reduce the CMO affecting *R. glutinosa*, although their ability to colonize the rhizosphere around *R. glutinosa* and their effectiveness in the field needs further investigation.

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