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Original Article

کنترل زیستی *ریزوکتونیا سولانی* توسط سویه های *سودوموناس* جدا شده از ریزوسفر

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چکیده

سابقه و هدف: در حال حاضر لوبیا به صورت گسترده به عنوان یک محصول عمده موادغذایی در بسیاری از مناطق گرمسیری، نیمه‌گرمسیری و معتدل آمریکا، اروپا، آفریقا و آسیا کشت داده می‌شود. مرگ گیاهچه و پوسیدگی بذر از شایع‌ترین و مخرب‌ترین بیماری‌های حبوبات در سراسر جهان است. این بیماری توسط قارچ *ریزوکتونیا سولانی* ایجاد می‌شود. هدف از این تحقیق جداسازی و شناسایی باکتری‌های ریزوسفر جهت کنترل زیستی *ریزوکتونیا سولانی* در لوبیا بود.

مواد و روش‌ها: چهار باکتری جدا شده از خاک ریزوسفر گیاهان لوبیا در مزارع استان لرستان، در شرایط آزمایشگاهی به عنوان آنتاگونیست بالقوه پاتوژن قارچی مورد بررسی قرار گرفت. بازدارندگی از رشد میسلیوم قارچ *ریزوکتونیا سولانی* توسط باکتری‌های ریزوسفر در شرایط آزمایشگاهی مورد آزمایش قرار گرفت. درجه بازدارندگی با اندازه‌گیری میزان هاله در اطراف سویه های باکتریایی تعیین شد.

یافته ها: تعیین توالی ژن *16S rRNA* و مقایسه آن با پایگاه داده ژن، نشان داد که سویه های آنتاگونیست متعلق به گونه‌های *سودوموناس ائروجینوسا*، *سودوموناس پوتیدا*، *سودوموناس مانتلی* بودند. تمامی سویه ها اثر کنترلی قابل توجهی در مهار قارچ *ریزوکتونیا سولانی* داشته و منجر به کنترل بیش از ۴۰ درصد در PDA شدند. نتایج نشان داد که بیش‌ترین بازدارندگی مربوط به *سودوموناس پوتیدا* و کم‌ترین مربوط به *سودوموناس ائروژینوسا* بود.

نتیجه‌گیری: پوسیدگی ریشه رایزوکتونیایی یک بیماری بسیار مخرب در بسیاری از مناطق جهان است. استفاده از کنترل بیولوژیکی بر اساس میکروارگانیسم‌ها به عنوان یک جایگزین قدرتمند برای کنترل شیمیایی پوسیدگی ریشه رایزوکتونیایی محسوب می‌شود.

واژگان کلیدی: باکتری‌های ریزوسفر، مرگ گیاهچه، پوسیدگی بذر، سویه های آنتاگونیست.

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Biological control of *Rhizoctonia solani* by *Pseudomonas* strains isolated from the rhizosphere

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Abstract

Background & Objectives: Bean (*Phaseolus vulgaris* L.) is now widely used as a major food product in many tropical areas and semi-temperate and temperate areas of America, Europe, Africa as well as Asia. Damping off and seed rot are the most frequent and damaging diseases of the legumes worldwide which are caused by *Rhizoctonia solani*. The purpose of this study was isolation and characterization of rhizosphere bacteria for the biocontrol of the *R. solani* in the bean.

Material & Methods: Four isolated bacteria from bean plants rhizosphere soil in Lorestan province farms were evaluated *in vitro* as a potential antagonist of the fungal pathogen. *In vitro* inhibition of *R. solani* mycelium growth by rhizosphere bacteria were tested on rye agar media. The degree of inhibition in each medium was determined by measuring the halo around the bacterial strains.

Results: Sequencing of *16S rRNA* and its comparison with Gen Bank sequence database revealed that antagonistic strains belong to *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Pseudomonas monteilii*, and *Pseudomonas putida* species. All the strains significantly inhibited *R. solani* growth, resulting in more than 40% inhibition on potato dextrose agar medium (PDA). The results showed the most inhibition by *P. putida*, and the lowest by *P. aeruginosa*.

Conclusion: Rhizoctonia root-rot is a highly destructive disease in most areas of the world. Biological control using natural microorganisms offers a powerful alternative to chemical control of *Rhizoctonia* root-rot diseases.

Keywords: Rhizosphere bacteria, Damping off, Seed rot, Antagonistic strains.
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Introduction

Rhizoctonia solani is a widespread soil-borne fungus comprising plant parasitic or saprophytic strain (1). The species affects many important agricultural crops worldwide, causing diseases such as the black scarf, crown rot and bottom rot (2). Pathogen control is difficult because of its wide host range, and ability to survive as sclerotia under adverse environmental conditions.

Biological control using microorganisms that can suppress or antagonize plant pathogens is being considered as a substitute or a supplement to reduce the use of chemical pesticides (3). Bean damping-off caused by *R. solani* Kühn (AG-4) as an important soil-borne plant pathogen damages a wide range of host plants worldwide (4). In Iran, it causes a major constraint to common bean production (5).

Controlling the soil-borne pathogens is difficult because of their ecological behavior, extremely broad host range, and the high survival rate of resistant forms under different environmental conditions. Many research studies have shown that biological control offers an environmentally friendly alternative to protect plants from soil-borne pathogens (6-8). Hence, introducing beneficial bacteria into the rhizosphere, most notably the use of plant growth promoting rhizobacteria (PGPR) for the promotion of crop growth, bioremediation, and biocontrol has been of great interest to many microbiologists (9-11).

Because of the importance of bean damping-off caused by *R. solani* (AG-4) in Iran, and the possibility of the affection of *Rhizosphere* bacteria isolates as its biological control, this study was commenced with the biocontrol efficiency of some Rhizosphere bacteria isolates as biocontrol agents of *R. solani*.

Material and Methods

Fungal material

The isolates of *R. solani* used in this study were obtained from Lorestan province. Fungal isolates were taken from the root and lower hypocotyl samples presenting reddish-brown, elongate, cancerous lesions.

Isolation of antagonistic bacteria

For isolation of bacteria, roots of healthy Lorestan province beans were excised and placed into 10 mL of a sterile 0.9% NaCl solution, and vortexed for 10 min in order to detach the associated rhizosphere soil. Serial dilutions of the resulting root wash were placed on nutrient agar (NA-Merck, Germany), supplemented with ampicillin (100 mg/mL) (12). Plates were incubated for 24-48 h at 28°C. To obtain the most abundant bacteria from each sample, selection of strains with different colony morphology was performed from the highest dilutions. The isolates were stored in 20% (v/v) glycerol solution at -80 °C.

Evaluation of the strains for in vitro biological control

The in vitro inhibition of *R. solani* mycelium growth induced by 26 rhizosphere bacterial isolates were tested on rye media (13). For each bacterial isolates, 1 ml (108 CFU/ml) of the rhizospheric bacterial suspension was poured on the margin of rye media plates, and a 6 mm *R. solani* agar disc from fresh PDA culture was placed at the other marginal side, and subsequently incubated at 27°C for seven days.

The radii of the fungal colonies towards and away from the bacterial colony were noted. The degree of inhibition in each medium was determined by measuring the halo around the bacterial strain without fungal growth. Three replicates were considered for the value of the inhibition halo. Isolates that had the most mycelial growth inhibition were selected for further study.

Identification of antagonistic bacteria

Different phenotypic features were assessed including the Gram stain, catalase test, levan test, gelatinase test, oxidase test, acids production from 1% glucose in oxidation/ fermentation (OF) basal medium, as well as utilization of sole carbon and nitrogen sources (14). Molecular identification of antagonistic strains that were used in this study was made by sequencing the *16S rRNA* gene.

Amplification was carried out by PCR with 10f (5'-AGTTTGATCATGGCTCAGATTG-3'), 1507r (5'-TACCTTGTTACGACTTCACCCCAG-3') primers. Standard PCR conditions included 1 min DNA denaturation at 94°C, 1 min annealing at 53°C, and 1 min extension at 72°C for 35 cycles. PCR base solution was prepared using a mixture of deionized water (16.95 µl), PCR buffer (2.5 µl), MgCl2 (0.75 µl), dNTPs (0.5 µl), primer F/R (2 µl), and DNA Taq polymerase (0.3 µl). The sequencing of PCR products was carried out in CinnaGen Company.

Multiple sequence alignment of 16S rDNA sequences of antagonistic strains and those of related bacteria was performed with the Clustal W program of DDBJ. An evolutionary tree for the datasets was inferred by the neighbor-joining (NJ) method of Saitou and Nei (15) using the NJ program of Molecular Evolutionary Genetics Analysis version 5 (MEGA5). The stability of relationships was assessed by performing bootstrap analysis of the NJ data based on 1000 re-samplings.

Production of hydrolytic enzymes

Proteolytic activity was detected by inoculating the strains on a medium composed of 1% casein, and 2.3% agar dissolved in Castaneda medium. Plates were incubated for 48 h at 28°C. Casein hydrolysis was detected by the formation of a whitish around a translucent area, surrounding the colony. To determine the cellulolytic activity, CMC (carboxyl methyl cellulose) was incorporated at 0.1% into the YEMA-0.2% mannitol agar plates. Colonies were grown for three days at 28 °C and washed off with water. The plates were then flooded with 0.1% (wt: vol) Congo Red in water for 15 min, washed for 10 min with 1 M NaCl, and for 5 min with 5% acetic acid. Degradation of CMC was observed as a reduction of staining (16).

Assay of chitinases

Strains were cultured at 28 °C for 4 days on a rotary shaker in 250 mL conical flasks containing 50 mL of chitin–peptone medium (glucose 0.5%, peptone 0.2%, colloidal chitin 0.2%, K_2HPO_4 0.1%, $MgSO_4$-$7H_2O$ 0.05%, and NaCl 0.05%, pH 6.8). The cultures were centrifuged at 12000 g for 20 min at 4°C, and the supernatant was used as enzyme source.

Colloidal chitin was prepared from crab shell chitin according to Berger and Reynolds (17). The reaction mixture contained 0.25 mL of

enzyme solution, 0.3 mL of 1 M sodium acetate buffer (pH 5.3), and 0.5 mL of colloidal chitin (0.1%). The reaction mixture was incubated at 50 °C for 4 h in a water bath.

Chitinase activity was determined by measuring the release of reducing sugars according to Nelson method (18). One unit of chitinase was determined as 1 nmol of GlcNAc released per minute per mg of protein. Protein content in all the samples was determined as described by Bradford using bovine serum albumin as the standard.

Results

Four bacterial strains were obtained from bean roots. Recovered bacterial strains were tested for their antagonistic ability against the phytopathogenic fungi *R. solani*. The result of antagonistic activity of the strains against *R. solani* is shown in Table 1.

Sequencing of *16S rRNA* and its comparison with Gen Bank sequence database revealed that antagonistic strains belong to the species *P. fluorescens*, *P. aeroginosa*, *P. monteilii* (KR527203), and *P. putida* (KR527207). (Figure 1).

Table 1. Effect of bacterial isolates on *R. solani* mycelial growth, measured as diameters of the fungal colonies in the presence of each antagonistic bacterial strain as compared to the control plate.

Bacterial strain	Inhibition percentage of mycelium growth
MO1 *(Pseudomonas putida)*	23.11a
MO2 *(Pseudomonas fluorescens)*	21.24b
MO3 *(Pseudomonas monteilii)*	19.15c
MO4 *(Pseudomonas aeroginosa)*	18. 5c

All the tested strains resulted in more than 40% inhibition of PDA. The effect of bacterial strains on *R. solani* mycelial growth is presented in Table 1. All strains showed protease activity and produced cellulase or chitinase.

Discussion

R. solani as a soil-borne plant pathogen causes root rot, and damages a wide range of host

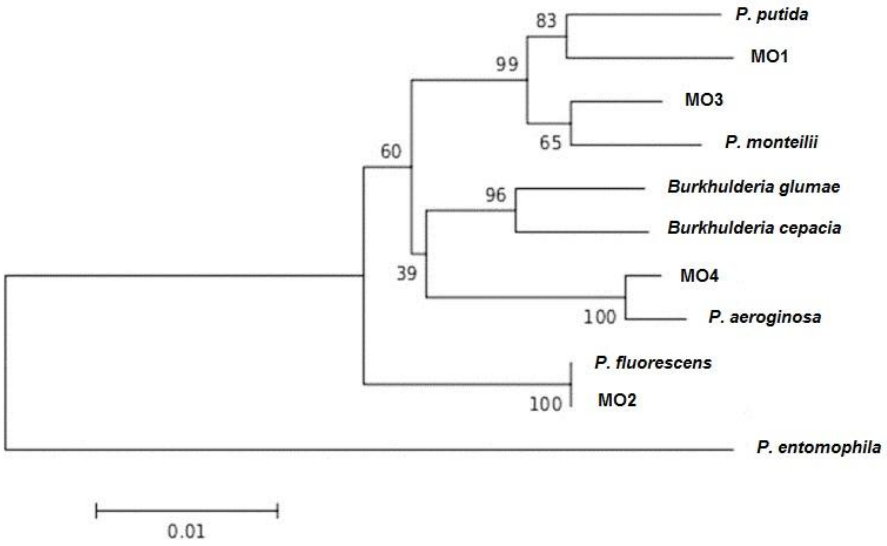

Fig. 1. Neighbor-joining tree based on the *16S rRNA* sequences, showing the relationships between the four representative antagonistic isolates from Iran and type strains (alignment length 1400 bp). Percentage bootstrap is indicated on the internal branches (1000 replicates).

plants. In Iran it causes a major constraint to common bean production. Many *R. solani* hosts plants have been reported including wheat root rot disease. The pathogen reduces plant growth by rotting the roots and thus reducing the ability of the plants to take up water and nutrients (19).

This fungal infection also causes stem canker on underground stems which is more predominant in cool and wet soil conditions. The use of biological control based on natural microorganisms offers a powerful alternative to synthetic chemical control of plant diseases. In fact, the abuse of chemical control agents, such as pesticides or fungicides, to cure or prevent plant diseases has often been reported to bring about a wide array of pernicious effects, particularly on plant, soil, environment, and ultimately, human beings.

The four strains evaluated in this study were isolated from rhizosphere of healthy bean plants from Lorestan province. Rhizobacteria play an important role in identification of potential biocontrol agents, noted that the strains should be from the rhizosphere of the target crop. Rhizospheric *Pseudomonas* strains were reported to be effective for the control of a wide range of fungal and bacterial diseases (20). *Pseudomonas* genus comprises a large group of active biocontrol strains due to their general ability to produce a diverse array of potent antifungal metabolites.

P. fluorescens strains were found to be the most efficient by exhibiting the highest inhibition. Strains of *P. fluorescens* have shown biological control activity against certain soil-borne phytopathogenic fungi. In fact, Marjan et al evaluated the in vitro efficacy of *P. putida* to control *Fusarium* wilt (21). Furthermore, Janisiewicz and Roitman (22) have reported that blue and grey mold of apples and pears can be controlled by *Pseudomonas*.

Scher & Baker (23) demonstrated that application of *P. putida*, a known siderophore producer, makes a soil suppressive to *Fusarium* spp. Various species of *Pseudomonas* have been reported to promote plant growth and suppress disease in plants, but because of the difficulties in their formulation they are still used only on a small scale (24).

Fluorescent *Pseudomonas* strains were also found to be effective against *Sclerotium rolfsii* under greenhouse conditions, in limiting groundnut and collar rot incidence. Enzymatic degradation of the cell wall of fungal pathogens by biocontrol agents has been reported (25). In this work, bacterial isolates showed protease, cellulase, and chitinase activities. In a study by Dilfoza et al., (2013) the biocontrol activity of rhizosphere bacteria in cotton damping-off caused by *R. solani* was assessed. Results showed that *P. extremorientalis* has a great biotechnological potential to simulate plant growth and protect cotton from damping-off disease (26). In another study by Subhashini et al., (2016) biocontrol activity of *R. solani* in Tobacco (*Nicotiana tabacum*) seed beds was examined using *P. fluorescens*. It was clearly indicated that establishment of *P. fluorescens* strains in tobacco rhizosphere is a feasible alternative for the management of *R. solani* symptoms.

P. fluorescens, when applied as a biocontrol agent on tobacco seed beds, shows appreciable increase in the biometric parameters of tobacco seedlings (27).

Conclusion

In this study based on the phenotypic assessment and of comparison *16S rRNA* sequencing results with Gen Bank sequence database, the antagonistic strains were identified as species *P. fluorescens* , *P. putida, P. monteilii,* and *P. aeroginosa*. All the strains resulted in more than 40% inhibition on PDA.

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