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ORIGINAL ARTICLE

Molecular Diversity of Antagonistic *Streptomyces spp.* against *Botrytis allii*, the agent of onion gray mold using Random Amplified Polymorphic DNA (RAPD) Markers

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As an aim in sustainable agriculture, biological control of plant diseases has received intensive attention mainly as a response to public concern about the use of chemical fungicides in the environment. Soil Actinomycetes particularly *Streptomyces* spp. enhance soil fertility and have antagonistic activity against wide range of plant pathogens. To investigate for biocontrol means against the pathogen, 30 isolates of Actinomycetes have been isolated from agricultural soils of Kerman province of Iran and assayed for antagonistic activity against *Botrytis allii*, the agent of onion gray mold. RAPD DNA analysis has been used to determine the relatedness of active and non-active isolates based on their RAPD-PCR fingerprints. PCR amplifiable DNA samples have been isolated using the CTAB method and amplified fragments have been obtained from 5 random 10-mer primers. Different DNA fingerprinting patterns have been obtained for all of the isolates. Electrophoretic and cluster analysis of the amplification products has revealed incidence of polymorphism among the isolates. A total of 138 bands, ranging in size from 150-2800 bp, have been amplified from primers which 63.7% of the observed bands have been polymorphic. Genetic distances among different varieties have been analyzed with a UPGMA (Unweighted pair-group method, arithmetic average)-derived dendrogram. Resulting dendrogram has showed from 0.65 to 0.91 similarities among varieties and divided the isolates into five major groups. Isolates which haven't had any antagonistic activity against *B. allii* have been separated into a group and other isolates classified into four groups. The results indicate that RAPD is an efficient method for discriminating and studying genetic diversity of *Streptomyces* isolates.

Key words: biological control, Streptomyces, Botrytis allii, random amplified polymorphic DNA (RAPD)

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The advent of molecular biology in general and the polymerase chain reaction in particular have greatly facilitated genomic analysis of microorganisms, provide enhanced capability to characterize and classify strains, and facilitate research to assess the genetic diversity of populations (Louws *et al*.,1999), Various molecular techniques such as repetitive element PCR (Rep-PCR), restriction fragment length polymorphism (RFLP) or random amplified polymorphic DNA (RAPD) can be used to discriminate strains. The latter technique is one of the most sensitive DNA fingerprinting methods. It is based on the amplification of distinct genomic sequences using an oligonucleotide of arbitrary sequence (Martin *et al*., 2000) Genomic fingerprinting assays using RAPD are excellent methodologies for differentiating and tracking specific genetic elements within a complex genome or genomes. These methods were originally developed to identify genetic polymorphisms in plant, fungal, and prokaryotic genomes and are fast and sensitive means for identifying small differences between similar complex genomes (Roberts and Crawford, 2000).

Actiomycetes are the gram positive organisms and have been identified as one of the major groups of soil population. These organisms are assumed to be the transition group between fungi and bacteria. Actinomycetes are also called as antibiotic producing bacteria and such antibiotics are diverse in chemical structure. RAPD analysis has previously been used to determine the relatedness of 73 antibiotic-producing soil *Streptomyces* isolates that were recovered from different soil habitats in Jordan based on their RAPD-PCR fingerprints. Further analysis of RAPD patterns with the UPGMA (Unweighted pair-group method, arithmetic average) resulted in clustering the tested isolates into two main super clusters (Gharaibeh *et al*., 2003). In this study antagonistic activity of 30 *Streptomyces* isolates against Pathogenic fungi, *B. allii,* has been evaluated and RAPD analysis has been used to assay the genetic diversity of tested isolates. RAPD reactions have been performed by 5 primers which showed high polymorphic bands among more than 50 primers. Primer 70-34 (with low DNA G+C content); has previously been used in classification of strain VK-A60^T. According to that research by using primer 70-34 all tested strains generated diverse RAPD banding patterns (Lee *et al*., 2005) and Primers 1.80.5, 2.80.11, 4.80.35.and 4.80.37 have previously been used to evaluate the interspecific, intraspecific and intraclonal differences by RAPD fingerprinting (Martin *et al.,* 2000).

MATERIALS AND METHODS

Soil sampling and isolation of *Streptomyces* isolates:

Soil samples were collected from grasslands, orchards and vegetable fields in different localities of Kerman province, Iran. Several samples randomly were selected from mentioned localities using an open-end soil borer (20 cm in depth, 2.5 cm in diameter) as described by Lee and Hwang (Lee and Hwang, 2002). Soil samples were taken from a depth of 10-20 cm below the soil surface. The soil of the top region (10 cm from the surface) was excluded. Samples were air-dried at room temperature for 7-10 days and then passed through a 0.8 mm mesh sieve

and were preserved in polyethylene bags at room temperature before use. Samples (10 g) of air-dried soil were mixed with sterile distilled water (100 mL). The mixtures were shaken vigorously for 1 h and then allowed to settle for 1 h. Portions (1 mL) of soil suspensions (diluted 10^{-1}) were transferred to 9 mL of sterile distilled water and subsequently diluted to 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Inocula consisted of adding aliquots of 10^{-3}-10^{-6} soil dilutions to autoclaved CGA (1 mL^{-25} CGA) at 50°C ere considered for each dilution. Plates were incubated at 30°C for up to 20 days. From day 7 on, Actinomycetes colonies were isolated on CGA, incubated at 28°C for one week and stored refrigerated as pure cultures before use. For screening studies 50 pure Actinomycetes isolates were collected.

Screening procedure and *in vitro* antifungal bioassays:

A- Agar disk-method: Each *Streptomyces* isolate was smeared on CGA medium as a single streak and after incubation at 28°C for 4-6 days, from well-grown streaks 6 mm Agar disks of *Streptomyces* colony mass was prepared by using sterile cork borers. Disks were then aseptically transferred to PDA plates having fresh lawn cultures of *B. allii* isolate. Controls included using plain disks from CGA medium. Plates were incubated at 24°C for 4-6 days and bioactivity was evaluated by measuring the Diameter of Inhibition Zones (DIZ, mm) (Aghighi *et al*., 2004).

B- Dual Culture Bioassay: Disks of Fungal mycelium (diameter of 6mm) prepared from growing margin of cultures of test isolate and placed in the center of PDA plates, and at 30 mm distance from it, the *Streptomyces* disks (prepared as mentioned) were placed. Plates incubated at 27°C for 2-3 weeks (Aghighi *et al*., 2004). Antifungal activity was indicative as mycelial growth of *B. allii* was prohibited in the direction of active Actinomycetes isolates. The level of inhibition at dual cultures was calculated by subtracting the distance (mm) of fungal growth in the direction of an antagonist colony (γ) from the fungal growth radius ($\gamma_\circ$) of a control culture to give $\Delta\gamma = \gamma_\circ - \gamma$. The ratings used were modified from those of Lee *et al.* where Δ γ: 5-9 mm, + (weak inhibition); Δ γ: 10-19 mm, ++ (moderate inhibition); and Δ γ>20 mm, +++ (strong inhibition) (El-Tarabily, *et al.,* 2000 and Lee *et al.,* 2002)

CTAB procedure for the isolation of genomic DNA

Actinomycetes were grown in liquid culture at 28°C to late exponential phase. A modified version of the procedure of Tripathi and Rawal (1998) was used for bacterial DNA isolation. Mycelia or spores from species of *Streptomyces* were spun down and resuspended in 1 ml Tris-EDTA (TE) buffer after growing in CG liquid medium for 5 days at 28°C. TE buffer was removed after centrifugation (3000· g for 4 min at room temperature), and the pellet was resuspended in 500 ml TE containing 100 mg/ml lysozyme. After incubation at 55°C for 1 hours, 60μl of 10% sodium dodecyl sulfate (SDS) and 10 μl of Proteinase K (with a final concentration of 20 mg/ml) were added to this mixture and the mixture was incubated in a 65°C water bath for 10 min. 200 μl of 5 M NaCl was added with gentle mixing to avoid shearing the DNA. 130 μl of 10% (w/v) CTAB in 0.7 M

NaCl (CTAB/NaCl solution) was added and incubated for 10 min at 65°C. After the addition of CTAB, all the steps were carried out at room temperature.500 µl of chloroform/isoamyl alcohol (24:1, by vol) was added, and upper aqueous phase was recovered after centrifugation (14000· g for 5 min at room temperature) and supernatant transferred to a fresh tube. 0.6 volume of isopropanol was added and DNA spooled out after 10 min. alternatively, it was recovered by centrifugation at 12,000 g for 10 min. The pellet was washed twice with 70% (v/v) ethanol, vacuum dried and dissolved in 2 ml TE buffer (10 mM Tris/HCl and 1 mM EDTA, pH 8.0).

PCR Amplification

RAPD-PCR Reaction Mixture: A modified version of the procedure of Kong *et al*. (2001) was used for PCR Amplification. Genomic DNA from bacteria was used to perform PCR reaction. RAPD amplification reaction mixtures consisted of 1 µl of template DNA (50 ng), 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 250 µM of each dNTP, 0.3 U of *Taq* polymerase and 0.8 pM of each oligonucleotide primer in a volume of 25 µl.

Primers: A list of PCR primers and their sequences is given in Table 1. The oligonucleotide primers were synthesized by CinnaGen Co.

Thermal Cycling programmes for Actinomycetes isolates: Amplification was performed with an initial denaturation step of 4 min at 94°C and then 35 cycles of 50 s denaturation at 94°C, 40 s at 47°C for primer annealing, and 2 min at 72°C for primer extension. An 8-min extension and cooling to 4°C completed the reaction sequence.

Electrophoresis: Agarose gel electrophoresis was done essentially as described by Sambrook *et al.* (1989). Approximately 1/10 volume of loading buffer was added to the DNA solutions prior to loading samples into wells. DNA molecules were separated on 1.2% Agarose gels containing 0.5 µg/ml ethidium bromide. Electrophoresis was carried out at 5-10 V/cm. in 1X TBE buffer as a running buffer. DNA was visualized by illumination with long wavelength UV-light (320nm).

RAPD Data Analysis:

Genetic distances among the different varieties were analyzed with a UPGMA-derived dendrogram.

RESULTS

Isolation of *Streptomyces* isolates: A total of 30 different Actinomycetes (*Streptomyces* like colonies) were isolated.

Screening and *in vitro* antifungal bioassays: In screening for metabolites of soil Actinomycetes having antifungal activity against an isolate of the cosmopolitan pathogen, *B. allii*, 30 isolates were assayed from which 4 isolates showed strong inhibition (+++), 7 isolates showed moderate inhibition (++), 9 isolates showed weak inhibition (+) and 10 isolates showed no inhibition zone (-) against pathogenic fungi. Bioassay results of isolates have been showed in Fig. 1 and Table 2.

RAPD Data Analysis: RAPD analysis was performed using primers of various G+C contents and yielded 6–13 bands for each *Streptomyces* strain. A RAPD fingerprint profile of *Streptomyces* isolates using primer 2.80.11 has been shown as example in fig.2. A total of 138 bands, ranging in size from 150-

2800 bp, were amplified from primers. 63.7% of the observed bands were polymorphic. Similarity matrix showed the similarities between varieties ranged from 0.65 to 0.91 (Fig.3). The quota of three main component in making diversity are respectively 12.6, 9.99 and 8.83.

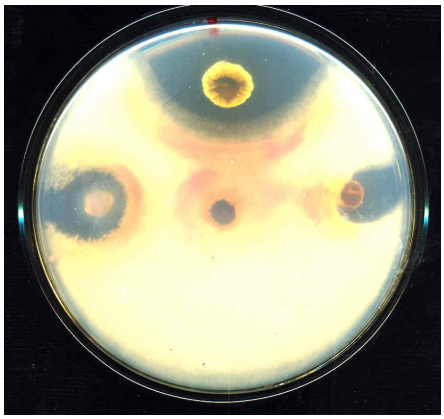

Figure 1. Test result of screening using dual culture bioassay. Center, agar plug of *Botrytis allii* with radial growth; bottom, plain agar plug as control; right, *Streptomyces* isolate No. 356; left, *Streptomyces* isolate No. 412 and top, *Streptomyces* isolate No. 345 showing inhibitory effect against mycelial growth and sporulation of *B. allii*

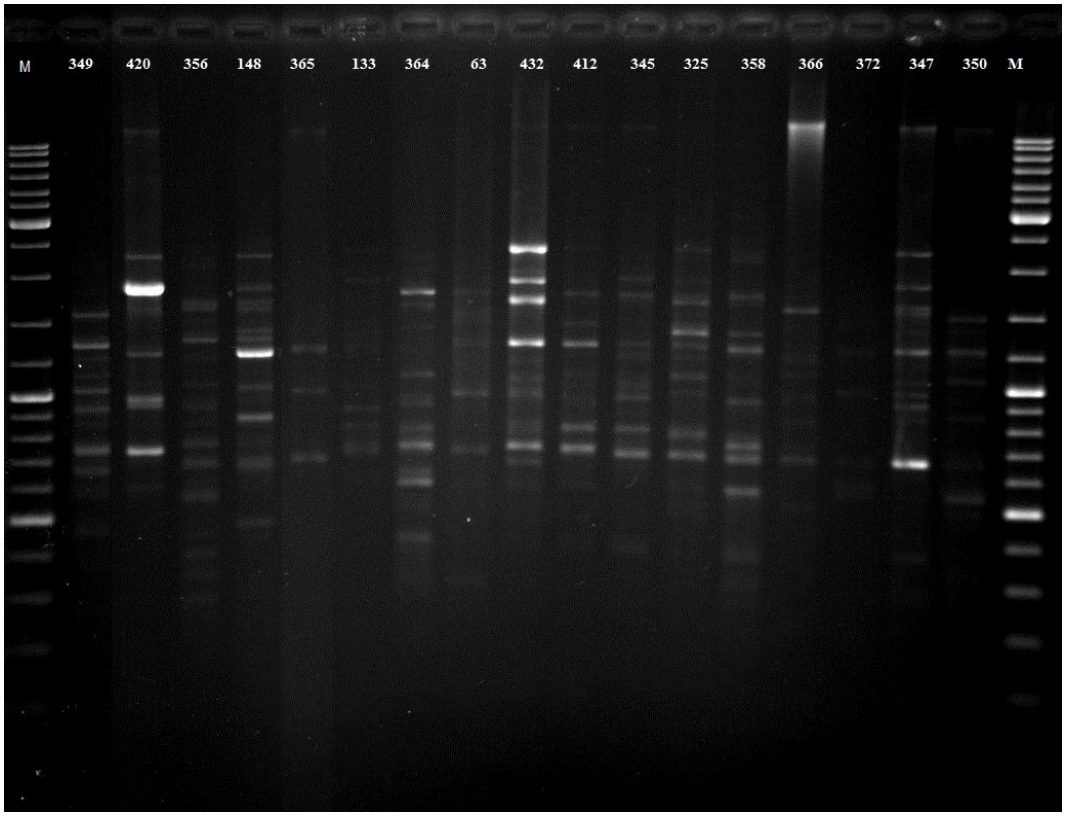

Figure 2. RAPD fingerprints profile of *Streptomyces* isolates on 1.2% agarose gel electophoresis amplified using primer 2.80.11. Lane M: 100bp DNA molecular weight marker, lanes 2-18 corresponds to the *Streptomyces* isolates: lane 2: 349, lane3: 420, lane 4: 356, lane 5: 148, lane 6: 365, lane7: 133, lane8: 364, lane 9:63, lane 10: 432, lane 11: 412, lane 12: 345, lane 13: 325, lane 14: 358, lane 15: 366, lane 16: 372, lane 17: 347, lane 18: 350.

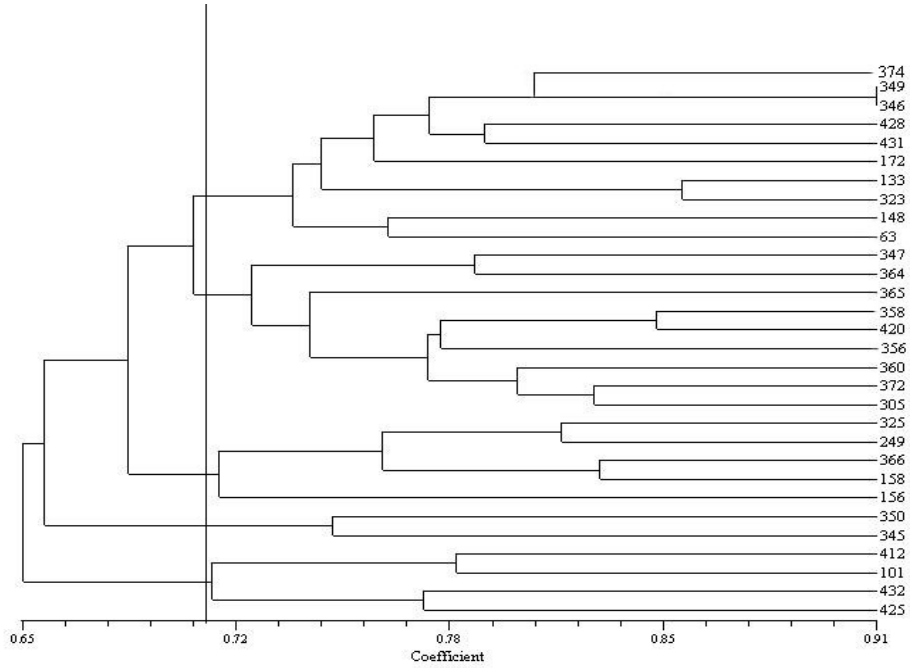

Figure 3. Dendrogram obtained from RAPD analysis using UPGMA.

Table 1. Oligonucleotide Primers Used for PCR Analysis

Primer	**Sequence** (5' to 3')	**Refrences**
70-34	GGACCGCTAG	Roberts & Crawford, 2000
1.80.5	ACCCCAGCCG	Martin *et al*., 2000
2.80.11	GCAGCAGCCG	Martin *et al*., 2000
4.80.35	CACCTGCCGC	Martin *et al*., 2000
4.80.37	CGCCAGGAGC	Martin *et al*., 2000

Table 2. Antifungal inhibitory effect of 30 isolates of *Actinomycetes* against *Botrytis allii*, the agent of onion gray mold base on El-Tarabily *et al*. method. (+++): strong inhibition, (++): moderate inhibition, (+): weak inhibition and (-): no inhibition zone (El-Tarabily *et al*., 2000)

Isolate	**Inhibition Zone**	**Isolate**	**Inhibition Zone**
148 UK	-	374 UK	-
133 UK	-	428 UK	-
323 UK	-	349 UK	-
431 UK	-	346 UK	-
63 UK	-	172 UK	-
350 UK	+++	347 UK	+++
347 UK	+++	364 UK	+++
366 UK	+	425 UK	+
249 UK	+	432 UK	+
158 UK	+	101 UK	+
156 UK	+	412 UK	+
372 UK	++	325 UK	+
305 UK	++	365 UK	++
356 UK	++	358 UK	++
360 UK	++	420 UK	++

DISCUSSION

In present study the resulting dendrogram divided the isolates with about 71% similarity into five major groups. Isolates which don't have any antagonistic

activity against *B. allii* separated into a cluster group and isolates with antagonistic activity classified into four groups. The results indicate that RAPD is an efficient method for discriminating and studying genetic diversity of *Streptomyces* isolates.

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