

Screening of antagonistic activity in different *Streptomyces* species against *Paecilomyces variotii* and verification of some of the physiological properties of the antagonists

Laleh Ansari^{1*}, Rezvan Vazirzadeh Sirchi¹, Gholam Hosein Sahidi Bonjar¹ and Seyed Amin Ayatollahi Moosavi²

¹Department of Plant Pathology, College of Agricultural Sciences, Bahonar University of Kerman, Iran.

²Department of Mycology, College of Medical Sciences, Bahonar University of Kerman, Iran

Corresponding author: Laleh Ansari

ABSTRACT: *Paecilomyces variotii* is a filamentous fungus which inhabits in soil, water, and functioning as biodegrading agent. In this study, biological control of *Paecilomyces variotii* was evaluated by two *Streptomyces* isolates. Eighty isolates were screened among Kerman soil samples. Among *Streptomyces* isolates with antifungal activity against *Paecilomyces variotii*, LA5 and LA27 isolates showed the most antagonistic effect on considered fungus in the study. After antibiogram test against *Paecilomyces variotii*, antagonistic activity of isolates was evaluated by measuring diameter of inhibition zone. Two isolates were identified as *Streptomyces* according to observation of scanning electron microscopy (SEM) and biological characters and about LA27 isolate, genotype properties was provided for identification of this isolate. Phylogenetic studies were conducted based on 16s rRNA sequence. Active strains were evaluated for Minimal Inhibitory Concentration (MIC). Both isolates could produce extracellular enzymes and antifungal activity of *Streptomyces* on pathogen mycelia was fungicidal type. Moreover, each one of obtained antagonists can examine in future studies for identification and chemical structure of antifungal material. If they can obtain necessary standards of environmental indices, they can be considered as biological control in campaign management with the pathogen.

Keywords: biological control, antifungal activity, genotype, inhibition zone, Phylogenetic studies

INTRODUCTION

Paecilomyces variotii is a filamentous fungus that inhabits in soil and water and functioning as biodegrading agent (Houbraken et al., 2008). *Paecilomyces* species are emerging pathogens that can cause serious infections in immunocompromised patients and sometimes in immunocompetent hosts (Polat et al., 2015). *Paecilomyces* cause different infections in human (Houbraken et al., 2010). One of the most common infections is endophthalmitis which is produced by *Paecilomyces variotii* (Ortoneda et al., 2004). One of causal agent of pistachio die-back in Kerman province is *Paecilomyces variotii* (Ghelichi et al., 2012). Economically pistachio die-back in Iran is one of the most important diseases affecting the pistachio trees (Ghelichi et al, 2012). Because of its pathogenicity and resistance to antifungal agents, *Paecilomyces* has clinical interest (Pastor and Guarro, 2006). *Streptomyces* are a group of Gram-positive bacteria which are mostly found in the soil; include some of the most common soil life. Because of their ability to produce several types of secondary metabolites, *Streptomyces* species are very important (Ceylan et al., 2008). New antifungal compounds discover based on natural screening of *Streptomyces*. By increasing

demand for new antibiotics through quick spread of antibiotic resistant pathogens which cause dangerous infections and regarding to use of chemical materials in agriculture and environmental pollution and their destructive effects on different kinds of non-target microorganisms, use of microbes according to biocontrol factors has been reported as replacement or supplement of chemical materials in the most of reports. The aim of this study was screening of *Streptomyces* of Kerman soil to check antagonistic activity of *Streptomyces* isolates against *Paecilomyces variotii*. Active strains which controled considered fungus in vitro, they can use for gene donorse developing resistant transgenic plant or soil modification in future. Of course, proper condition for antagonistic survival in soil should be studied, since soil environment is very complicated and a lot of factors affect on microorganisms.

MATERIALS AND METHODS

Used Culture medium for the organisms: *Paecilomyces variotii* was obtained from Kerman Agricultural Research Center. Isolated fungal pathogen of *Paecilomyces variotii* was grown on Potato Dextrose Agar (PDA) culture medium and was allowed to grow for 3 days at 25°C. In this study, Casein Glycerol (or starch) Agar (CGA) culture medium was used for actinomycetes isolation as was described by Kalantar Zadeh (2006).

Screening of *Streptomyces*: The soil samples collected around the root zone of plants and each zone were mixed separately and air-dried at room temperature for ten days, then they were crushed and sieved. The *Streptomyces* Spp. was isolated from the soil samples by serial dilution method as described by Shahidi Bonjar (2005). The dilutions of 10⁻³ to 10⁻⁶ were plated on specific media CGA medium and plates were incubated at 27°C for 10 days. Then the pure colonies of *Streptomyces spp.* were sub cultured and stored at 4°C for further use. Antagonistic activity of actinomycete isolates against *Paecilomyces variotii* were screened by agar disk method as described by Shahidi Bonjar (2005).

Dual culture bioassay: A 3 days-old disk with 6 mm diameter from *Paecilomyces variotii* isolate was placed in the center of petri dishes PDA. Each actinomycete isolate was grown on CGA medium as pure culture and after incubation at 27°C for 10 days (Shahidi Bonjar et al., 2006), disks with 6 mm diameter were taken from 10 days-old cultures with appropriate growth, and were placed at 30 mm distance of fungus disk. Then, plates were incubated at 27°C, and selected based on the diameter of inhibition zone from growth of active isolates.

Scanning Electron Microscope (SEM): Active isolates were cultured for photography by electron microscopy on CGA culture medium. After 20 days, samples were placed in a SC7620 sputter coater for 2 min and a gold layer with 150 angstrom thickness was coated on samples. After sputter coating, samples were studied by CAMSCAN-MV 2300 scanning electron microscope, and 20 kVvoltage, then photographed by 6000-20000 X magnification.

DNA extraction and PCR amplification of LA27 of isolate: Preparation of DNA were performed as described by Rademaker and de Bruijn (1997) with slight modifications. Specifically, a loopful of each isolate was suspended in 500µL of buffer (0.15 M NaCl and 0.01 M EDTA, pH 8) in an Eppendorf tube, mixed by vortexing and centrifuged at 14000g for 15 min. The supernatant, including the viscous material, was discarded and the pellet was resuspended in 100µL of sterile distilled water. Ten µL of the suspension was transferred to another tube and 100µL of 0.05 M NaOH was added. The tube was incubated at 95°C for 15min, centrifuged at 14000g for 2min and 1µL of the supernatant was used per reaction as DNA sample. The 16Sr RNA of bacteria was amplified by PCR using universal primer pairs fd2 (5'-AGAGTTTGATCATGGCTCAG-3') and rp1 (5'-ACGGTTACCTTGTTACGACTT-3') (Weisburg et al., 1991). Each 50µL PCR reaction mixture contained 1µL lysed cell suspension along with 1X Taq buffer, 2mM MgCl₂, 2U of Taq DNA polymerase, 150 µM of each dNTP, 0.5 mM of each primer (fermentas) and distilled water. Amplification was performed by 33 cycles (my cycler™, Bio-Rad) of 1 min denaturation at 94°C, 1 min at 63°C for primer annealing, and 2 min at 72°C for primer extension, followed by the final step at 72°C for 10 min (Heuer et al., 1997). PCR products were electrophoresed (45min at 75 volts) on 1.2% agarose gels and stained with ethidium bromide (10 µg ml⁻¹) (Cook and Meyers, 2003) and visualized in UV transilluminator (UVtec, UK). PCR products was directly sequenced (Macrogen, South Korea). Retrieved sequences were edited by Seqman software (DNASTAR, USA) and aligned with BLAST in comparison with sequences from Gen Bank (Altschul et al., 1990).

Monitoring activity: 7 days-old cultures of *Streptomyces* disks were incubated separately in 250 ml of autoclaved CG medium. Flasks were placed in orbital shaker incubator at 30°C with 130 rpm shaking. After 3 days from incubation of flasks, sampling was performed for 20 days consecutive days. Antifungal activity was performed by well diffusion method (Kalantar Zadeh et al., 2006) against *Paecilomyces variotii*. In each petri dish, a well was considered as witness where was poured 150 µl CG medium.

Preparation of crude extracts and Minimal Inhibitory Concentrations (MIC) determination: In monitoring phase, after obtaining the most time of fungicidal fungus activation in liquid culture condition, active isolates (LA27 and LA5) were harvested in determinate time, and spores and mycelia were included by filtration through two layers of these cloths. After filtration, extracts were dried under low pressure and 50°C temperature and they were kept in refrigerator. MIC is minimum concentration which is produced in the zone of inhibition from growth. 20 mg/ml of crude extract was prepared in DM solvent. Then, seven successive dilutions (20, 10, 5, 2.5, 1.25, 0.625 and 0.312 mg mL⁻¹) were prepared and The MIC was defined as the lowest concentration able to inhibit any visible fungal growth (Kariminik and Baniyasi, 2010).

Fungistatic and/or fungicidal properties: The bioassay was performed by agar disk method. After production of inhibition zone on PDA culture medium, some disks were taken from there and transferred to petri dishes contain PDA culture medium. Then the petris were incubated at 27°C for 7 days, and at the end of incubation, the growth of pathogens was investigated. The growth of fungus represents fungistatic activity and the lack of its growth represents fungicidal activity of isolate.

Enzymatic activities

Chitinase activity: The actively growing mycelial disks (4mm in size) from *Streptomyces* isolates (LA27, LA5) were placed in 9-cm petri dishes containing 0.4% colloidal chitin and 1.5% agar with pH 7.2 (Gomes et al., 2000) and the plates were incubated at 29°C. The data were recorded after 3, 5, 7 and 10 days of incubation with clear zones (>8 mm in diameter around each colony) around each colony and used as index for chitinase activity.

Protease production: The protease production activity of the isolates (LA27, LA5) was studied in minimum culture medium containing glucose (1gm), casein (3gm), CaCl₂ (2 gm) and 15gm agar. After 24 hrs, actively growing mycelial disks (4mm in size) from *Streptomyces* isolates (LA27, LA5) were transferred to the minimum culture medium and incubated for 3-4 days. The hydrolysis of casein and proteolytic property of the isolates were observed with formation transparent halo around disks (Dunne et al., 2000).

Amylase activity test: The amylase activity of the isolates (LA27, LA5) was studied on nutrient agar (NA) culture medium containing 0.2% starch solution. The actively growing mycelial disks (4mm in size) from *Streptomyces* isolates (LA27, LA5) were transferred to the nutrient agar (NA) and incubated for 3 days. After 3-4 days of incubation the plates were coloured by starch indicator (Iugols solution). The starch hydrolysis can be visualized around the place of bacterial colonies by formation of colourless halo zone.

Lipase production: The lipase activity of isolates (LA27, LA5) was studied on the medium provided with 10gm of Peptone, 5gm NaCl, 0.1gm CaCl₂ and 15gm Agar in 1000 ml distilled water. Then the media was autoclaved. After autoclaving 10 ml of Tween 80 was added to a medium before cooling. The actively growing mycelial disks (6mm in size) from *Streptomyces* isolates (LA27, LA5) were transferred to the above media and incubated for 5-7 days. After a few days, sedimentary halo zone around the place of bacterium growth represents tween hydrolysis (Jeffrey, 2008).

RESULTS AND DISCUSSION

Screening and dual culture bioassay: For the study of antifungal activity of actinomycete isolate, 80 isolates were screened. Among actinomycete isolates with antifungal activity against *P. variotii*, two isolates (LA27 and LA5) showed the most antagonistic effect on *P. variotii* in vitro in this research (Figure 1). Antagonistic activity of isolates against *P. variotii* was evaluated by measuring of halo diameter of growth inhibition.

Scanning Electron Microscope (SEM): Scanning electron micrograph of mycelia of LA27 is shown in Figure 2.

Molecular identification of LA27 isolate: The colonial morphology of the antagonist LA27 was grey colour, observably flat. It was spore forming bacilli (Figure 2). Based on the results, LA27 was preliminarily identified as *Streptomyces* species. Phylogenetic tree was constructed by neighbor-joining method (Figure 3), in which LA27 clustered with the isolates of *Streptomyces lividans*. BLASTn analysis showed that the 16S rRNA of LA27 shared 100% identity with those of *Streptomyces lividans* KC462527 based on the results, LA27 was classified as *S. lividans*.

Monitoring activity: In the study of incubation period time versus activity of actinomycetes after 20 days, the most concentration that produced a visible zone of inhibition was regarded at 8 and 10 days in strains of LA5 and LA27 respectively. Pertinent details of the monitoring are shown in Figure 4.

Fungicidal and/or fungistatic: Two isolates noted as negative for growth of pathogen that indicated the fungicidal activity (data not shown).

MIC: Minimal Inhibitory Concentrations (MIC) of the crude extracts of active strains was determined as 0.625 mg mL⁻¹ against *P. variotii* (Figure 5).

Enzymatic activities: After setting actinomycetes in chitin agar culture medium, clear zone was appeared around actinomycetes in the eighth day, which indicates the analysis of colloidal chitin and the result of chitinolytic property in each two isolates. In protease test, proteolytic activity and appearance of halo of inhibition from growth around growing actinomycetes indicate the ability of isolate in casein analysis. In the case of lipase and amylase activities, both isolates, can analysis the starch and tween hydrolysis. The results are shown in Figure 6.

Soil actinomycetes are gram-positive bacteria which showed a wide range of antifungal activity against pathogenic fungi (Jorjandi et al., 2009). Since actinomycetes have effective histories in the control of plant disease and produce more than half of antibacterial compounds of world, they are introduced as powerful and hopeful instruments in the case of biological control. In this study, we try to separate isolates of terrestrial actinomycetes and examine and screen them at the point of view of their antagonistic effects on *Paecilomyces variotii*. The antagonistic *Streptomyces* is related to the action of hydrolytic enzymes such as chitinase, β -1, 3-glucanase, chitosanase, and protease (Wang et al., 2002). Our findings showed that only 2 isolates (LA27 and LA5) from 80 actinomycete isolates, have antifungal compounds against *Paecilomyces variotii*. In this study, antagonist strain LA27 based on morphological property and partial nucleotide sequence of 16s rRNA was identified as *S. lividans*. Two selected isolates (LA27 and LA5) were studied in submerged culture tests. The curves of effective substance production for the isolates during 20 days of successively antibiogram tests showed that maximum inhibitions are on the eighth and tenth days after incubation. The trend of effective antifungal substance production curve for LA5 and LA27 isolates, shows that effective antibiotic substance of the isolates produce gradually, and will decrease gradually after reaching to maximum inhibition concentration.

Minimal inhibition concentration (MIC) for different isolates (LA27 and LA5) was 0.625 mg/ml that from antagonistic point of view have good effect on disease control. We can mention to determination of effective producer genes for active isolates actinomycete as long-term goals of this study. In the case that effective substances don't have any risk at biological point of view, or their transfer to human be harmless, it is necessary to determine effective genes in antagonistic property of the biocontrol factors and use for produce resistant transgenic plants to *Paecilomyces variotii* in agriculture for produce antifungal drug in pharmacy industry. Since *Paecilomyces variotii* is the agent of die-back in pistachio trees and also it depends on many types of human infection, so we can take advantage of the results of this study in agriculture and medicine.

In sum, this study was the first steps to reach to above aims and we hope that other researchers will be effective in progress of long-term goals of this study.



Figure 1: *In vitro* Agar disk bioassay of two *Streptomyces* isolates (top and bottom plugs) against *Paecilomyces variotii* (center plug) indicating antifungal inhibition. Left plug is control (plain agar plug).

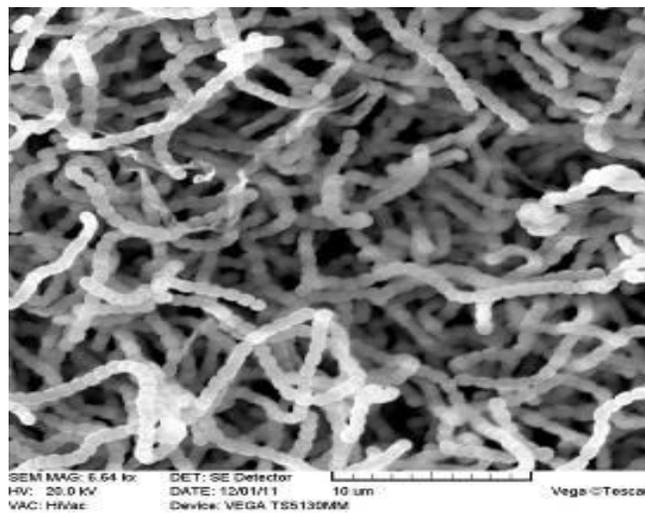


Figure 2: Scanning electron micrograph of mycelia of *Streptomyces* isolate LA27.

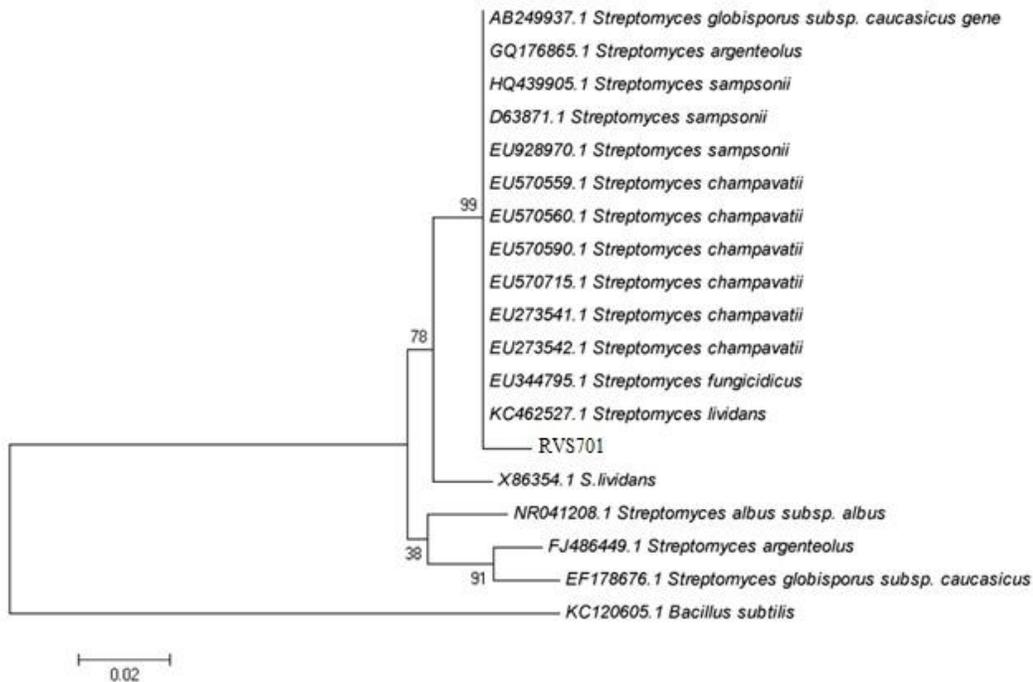


Figure 3: Phylogenetic tree of the antagonist LA27 (were shown RVS701 isolate in phylogenetic tree). Phylogenetic tree of the *Streptomyces* LA27 was constructed by the neighbor-joining method based on the partial nucleotide sequences of 16S rRNA from bacteria related to *Streptomyces* species. The sequences of 16S rRNA from *Bacillus subtilis* KC120605 was used as out group. The scales indicate the evolutionary distance of the nucleotide substitutions per site.

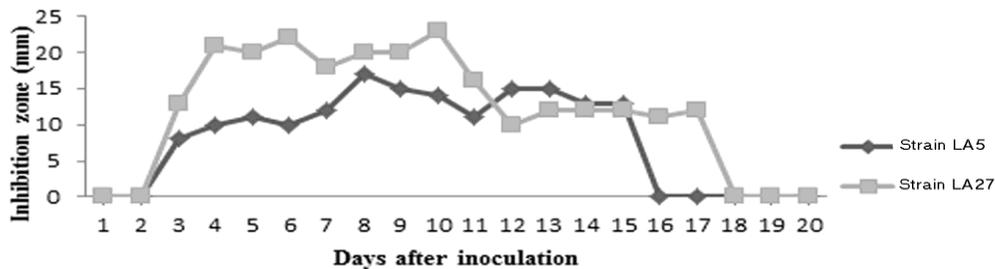


Figure 4: Activity versus post seeding time in submerged media culture of strains: LA27 and LA5 Against *P. variotii* monitored by well diffusion method.

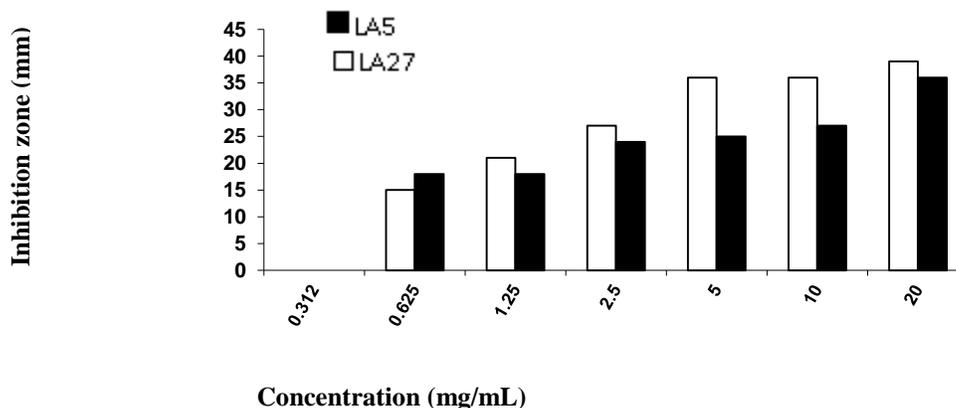


Figure 5: Dose response of *Streptomyces* strain LA27 and LA5 crude extract against *Paecilomyces variotii* isolate determined by agar diffusion method at 26°C for 7-10 days of inoculation.

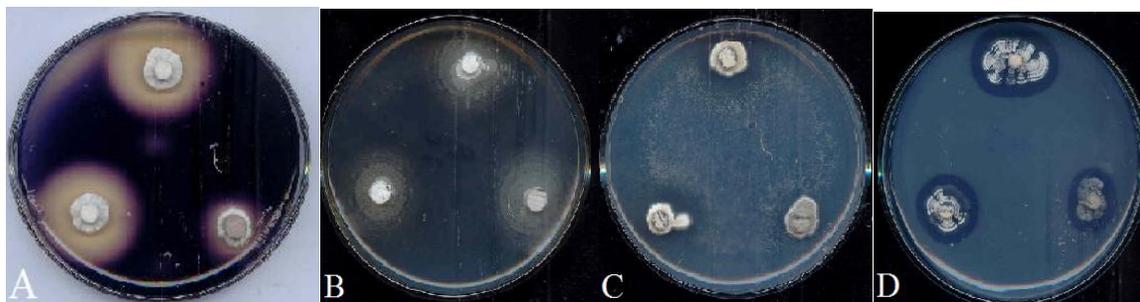


Figure 6: Clockwise from top strains of LA27, LA5 and LA27 (A). Inhibition zones are indicative of amylase activity of two *streptomyces* isolates (B) Inhibition zones are indicative of using tween as a lipid source and lipase activity (C) Inhibition zones are indicative of proteolytic activity of two *streptomyces* isolates (D) Inhibition zones are indicative of chitinolytic activity of two *streptomyces* isolates.

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