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Research Article

PHENOTYPIC AND GENOTYPIC DIVERSITY OF *RHODOCOCCUS FASCIANS,* USING RAPD-PCR IN FARS PROVINCE

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ABSTRACT
Rhodococcus fascians is a gram positive plant pathogenic bacterium that stimulates gall formation in many monocot and dicot plants. In the present study, 27 isolates of *Rhodococcus fascians* were collected from Shiraz, Jahrom and Darab (Fars, Iran). The phenotypic and genotypic characteristics of these isolates, a standard strain (981011) and two isolates of petunia and geranium, from northern Iran were assessed using RAPD-PCR. Analysis of phenotypic characteristics using NTsys-pc software, all isolates showed 80% similarity. On the basis of our finding, petunia isolates were very similar and were placed in a distinct cluster. Nasturtium isolates with northern petunia isolates, were placed in a separate cluster and were separable from the other hosts. Standard isolate had the most and least similarity to the Shiraz's petunia isolate and Darab's geranium isolate, respectively. Results showed that the nasturtium and petunia isolates were homogeneous based on phenotypic characteristics and are separable from the other hosts; geranium strains, on the other hand, showed more diversity and were placed in different groups. The investigation of genotypic characteristics using six RAPD-PCR primers showed that all strains were highly homogeneous. Genotypic dendrogram showed all isolates could be separated from each other at the 90% similarity level. This held true, especially in the case of geranium and tobacco isolates and the Iran's northern strains. Analysis of RAPD-PCR fingerprints, showed that *R. fascians* isolates could be distinguished from each other, based on the isolated host and geographical area. Comparison of the results of phenotypic and genotypic analysis showed that there was no relationship between these two methods.

Keywords*: Rhodococcus fascians, Phenotypic and Genotypic Diversity*

INTRODUCTION
Rhodococcus fascians is the only phytopathogenic member of *Rhodococcus* spp. (Vreecke *et al.*, 2002). This gram positive bacterium is a soil-borne plant pathogenic bacteria and causes leafy gall in both angiosperms and gymnosperms (Goethals *et al.*, 2001). Diseased plants show different symptoms such as leaf deformation, witches' broom, flat limb and leafy gall. Bacterial host range is wide, includes pea, geranium, petunia, nasturtium, clove, carnation, chrysanthemum and tobacco (Lacey, 1939; Vereecke *et al.*, 2000; Manes *et al.*, 2001; Pataky, 1991).
In Iran, the first report of leafy gall was on Urmia's petunia (Amani, 1976). Thereafter, disease reported from geranium in Tehran province (Rahimian, 1993). In 1996, *R. fascians* was isolated and studied in petunia in Semnan province (Zarei and Rahimian, 1996). Najafipour and Taghavi investigated phenotypic characteristics and host range of 122 different isolates of *R. fascians* in Shiraz (Najafipour and Taghavi, 2001).
According to cases mentioned above, despite wide distribution of *R. fascians* in Iran, comprehensive information about genotypic characteristics of pathogen does not exist. The aim of this study was comparison of phenotypic and genotypic characteristics of *R. fascians*, using RAPD-PCR in Fars province (Iran).

MATERIALS AND METHODS
Isolation
During 2011 to 2012, different regions in Fars province were investigated, and ornamental plants showing leafy gall were collected and transferred to the laboratory. Samples were washed with tap water, renised twice with sterile distilled water, ground in a small amount of sterile distilled water. One loop of suspension was cultured on sucrose nutrient agar and incubated on 27ºC. Five to seven days after

culturing, different colonies appeared with orange, yellow and white pigmentations. Small orange and yellow, slightly mucoid or non-mucoid, gram positive and obligate aerobes colonies were selected, purified and stored at 4°C for complementary tests (Schaad *et al.,* 2001). Biochemical and physiological tests were performed according to standard methods (Schaad *et al.,* 2001; Suslow *et al.,* 1982; Fahy and Hayward, 1983).

Pathogenicity Test

Pea seeds were surface sterilized, put in indirect sunlight on a sterile, wet filter paper. 2 to 3 days later, the germinated seeds were placed for one week in bacterial suspension with a concentration of 10^7CFU (OD_{600}=1) (Klambt *et al.,* 1966). Sterile distilled water was used as control.

DNA Preparation. Bacterial isolates were grown on nutrient agar medium at 25°C for 3 days. A loopful of colony from each strain was suspended in sterile distilled water to a concentration of 107 CFU (OD600=1). The suspensions were boiled for 10 min, cooled in the room temperature and used as DNA template (Yaish, 2006). DNA preparation was kept at -20°C for long preservation.

Detection of R. Fascians with Specific Primers

Two primers from *fas-1* gene [P_1: 5-GGGGATCCATATCGAACCGCCCT-3 and P_2: 5-GGGAATTCCGACGACGTATCCAGTGTGT-3] (Metabion Co., Germany) were selected for diagnostic PCR. These primers locate into the open reading frame of the *fas-1* gene and yield a 220-bp product (Stange *et al.,* 1996). The PCR reactions were performed in Bio-Rad I-cycler (USA) in 25 µl PCR mixture consist of 12.5 µl of PCR Master mix 2x, 1 µm of each primer and 2 µlit of DNA suspension. The PCR reaction was carried out for 1 cycle as a primary denaturation 94°C for 3 min, followed by 30 cycles as the following condition: 94°C for 1min, 65°C for 1 min and 72°C for 40 s and final extension was 72°C for 3 min (Stange *et al.,* 1996). The PCR products were electrophoresed on 1.5 % TBE agarose gel at 80V/cm for 1 h. The gels were stained with ethidium bromide, viewed and photographed under UV illumination (Bio Rad, USA).

RAPD-PCR

This test was performed using six random primers and a volume of 25µl (Chen *et al.,* 2007. Temperature cycles included one cycle of primary denaturation temperature of 94°C for 1 min, followed by 30 cycles of 94°C for 30 sec, 36°C for 90 sec, 72°C for 90 sec and a final extension of 72°C for 5 min. The sequences of primers are provided in Table1. Materials used in the RAPD-PCR reaction in 25µl volume included 0.2 µm from each dNTP, 1 µm of primer, 2.5 units of Taq DNA polymerase enzyme (Cinagen Co., Iran), 2.5 µl of 10x buffer, 2 µm of $Mgcl_2$, and 2µl of DNA suspension (Doyle, 1987). The PCR products of RAPD-PCR were electrophoresed on 1.5 % TBE agarose gel at 70V/cm for 100min. The gels were stained with ethidium bromide, viewed and photographed under UV illumination (Bio Rad, USA).

Analysis of Phenotypic and Genotypic Data

Using the Numerical Taxonomy and Multivariate Analysis System (NTsys-pc Version 2.02) software, the genetic distances of isolates were drawn. Genetic distance or similarity was determined among isolates based on molecular markers as the existence and/or non-existence of a band in the gel. Cluster analysis was performed based on the hierarchical technique; to investigate real distances among clusters, the unweighted pair-group method using the arithmetic average method (UPGMA) and simple matching (SM) of similarity coefficients were used (Rohlf, 2000). Phenotypic characteristics were defined as zero codes (for negative characteristics) and 1 (for positive characteristics). Genotype characteristics were defined based on RAPD-PCR as zero codes (for non–band) and 1 (for the existence of band) in this software. Based on the phenotypic and genotypic characteristics built on RAPD-PCR, the dendrogram related to the investigated strains was drawn by this software. The percentages of similarity among strains in groups were calculated based on the data obtained in this research (Rohlf, 2000).

RESULTS AND DISCSSION

Thirty strains of *R. fascians* were isolated from Shiraz, Jahrom and Darab in Fars province, Iran (Table 2). Characteristic of all strains were investigated on different media, such as SNA, YNA, YSB, SPA and GYCA. Most isolates were either dark or light orange in the culture media. Only one colony (981011) on

the SNA medium on the YNA, SPA and GYCA media appeared in yellow. Colonies of all isolates were nonmucoid on the YNA medium, but copious mucus was seen on the SNA medium, a little mucus was observed on the YSB and SPA media, and copious and shiny mucus was seen on the GYCA medium.
All isolates were positive in gram reaction and catalase, and negative in oxidase reaction, arginine dehydrolase, levan formation, starch hydrolysis and hypersensitive test on geranium. In addition, isolates were obligate aerobes and had the ability to produce H_2S from peptone (Table 3).

Table 1: Sequences of random primers used in the RAPD-PCR

Primer	Sequence
S 301	5ó CTGGGCACGA 3ó
S 302	5ó TTCCGCCACC 3ó
S 304	5ó CCGCTACCGA 3ó
S 306	5ó ACGCCAGAGG 3ó
P 54	5ó CAGCACCCAC 3ó
P 86	5ó TGGACCGGTG 3ó

Table 2: Characteristics of *Rhodococcus fascians* used in this study

Bacterial code	Host	Region	Bacterial code	Host	Region
Sh2D	Pelargonium	Shiraz	A2	Petonia	Shiraz
Sh1	Pelargonium	Shiraz	Ash	Petonia	Shiraz
Sh2	Pelargonium	Shiraz	N1	Petonia	Jahrom
ShSh	Pelargonium	Shiraz	N2	Petonia	Jahrom
Shj	Pelargonium	Jahrom	Aj	Petonia	Jahrom
ShDarab	Pelargonium	Darab	ADarab	Petonia	Darab
Shshomal	Pelargonium	North of Iran	6O22	Petonia	North of Iran
A8	Petonia	Shiraz	Lj4	Nastatrium	Jahrom
AM3	Petonia	Shiraz	Lj5	Nastatrium	Jahrom
A16	Petonia	Shiraz	LSh1	Nastatrium	Shiraz
A6	Petonia	Shiraz	LSh2	Nastatrium	Shiraz
AK2	Petonia	Shiraz	Lj	Nastatrium	Jahrom
ACH8	Petonia	Shiraz	L Darab	Nastatrium	Darab
A3	Petonia	Shiraz	T5	Tobacco	Shiraz
AK1	Petonia	Shiraz	981011	Pea	Riverside- USA

In the litmus milk reaction, all isolates produced an alkali reaction. In the petunia and geranium isolates, a white precipitate was also seen. The tests of nitrate reduction to nitrite, gelatin hydrolysis, methyl red, Vogesproskauer, indole production, lecithinase, tyrosinase and the production of a reducing compound from sucrose were assessed negative in most isolates. All isolates were grown in 25°C, 28°C, and 30°Ctemperatures. Other results are recorded in.

Pathogenicity Test

The pathogenicity test was positive on pea seeds in all isolates. Symptoms produced on pea seeds included the growth of several seedlings from a seed.

Table 3: Phenotypic characteristics of *Rhodococcus fascians* isolated from different hosts

Test Name	Petunia	Pelargonium	Nastatrium	Bacterial isolate Tobacco	North's pelargonium	North's petonia	Standard isolate
Gram stain	+	+	+	+	+	+	+
KOH3%	+	+	+	+	+	+	+
catalase	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-
Potato rot	-	-	-	-	-	-	-
HR	-	-	-	-	-	-	-
H_2S from pepton	+	+(83.3)	+	+	+	+	+
Arginine dehydrolase	-	-	-	-	-	-	-
O/F	O	O	O	O	O	O	O
Gelatin hydrolysis	-(91.7%)	-	- (83.3%)	-	+	-	-
Levan production	-	-	-	-	-	-	-
Flourescent pigment on KB	-	-	-	-	-	-	-
Hydrolysis of starch	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	-
Litmus milk	K	K	K	K	K	K	K
Colour in YDC medium	Dark orange	Dark orange	Dark orange	Dark orange	Light orange	Dark orange	Dark yellow to orange
Simon citrat	+	+(83.3%)	+(83.3%)	+	+	+	+
Urease	+(91.7%)	+ (66.7%)	+ (83.3%)	+	+	+	+
Tyrosinase	-	-(80%)	-	-	-	-	-
Lipase (T80)	+	+ (83.3%)	+	+	-	+	+
Lecitinase	-	- (83.3%)	-	-	-	-	-
Ascoline hydrolysis	+	+ (80%)	-	-	+	-	+

Methyle red (MR/VP)	-	-(66.7%)	-	-	-	-	-
Growth at temprature (˚C)							
25	+	+	+	+	+	+	+
28	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+
Acetoein production	-(91.7%)	-(83.3%)	-	-	-	-	-
Endole production	-	-	-	-	-	-	-
Reducing substances from sucrose							
Acid production from:	- (91.7%)	-(66.7%)	-	-	-	-	-
Adonitol	-	-	-	-	+	-	-
Inositole	-	+	-	-	+	-	+
Arabinose	+	+	+	+	+	-	+
Trehalose	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	+	+	-
Salicine	-	-	-	-	+	+	-
Sucrose	±	±	+	±	+	+	±
Cellobiose	-	-	-	-	+	-	-
Dolcitol	-	-	-	-	-	-	-
galactose	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+
Fructose	+	-	-	+	+	+	+
Manitol	-	-	-	-	+	+	-
Melibiose	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	+

K, alkaline reaction; +, positive reaction; -, negative reaction; ±, weak positive reaction

***Phenotypic Analysis Using* NTsys-pc v.2-2**

The numerical analysis of phenotypic characteristics of 30 isolates of *R. fascians* using NTsys-pc v.2-2 showed that petunia isolates were significantly similar to each other and were placed in a group with 100% homology. Isolates with a higher than 96.5% similarity level showed the most similarity with the standard strain (981011). Moreover, nasturtium isolates and the northern petunia isolates were classified in a group and distinguished from other isolates at a 96% similarity level. Meanwhile, the northern geranium in a different group at a 92.5% similarity level and the Jahrom geranium with 92% similarity were separated from the other geranium and petunia isolates. The Jahrom nasturtium was distinguished from the other isolates at an 87% similarity level. The Shiraz geranium and the Jahrom petunia had a 90% similarity level with each other and were separated from the other isolates at an 84% level. Meanwhile, the Darab geranium was separated from the other isolates at an 80% similarity level.

Polymerase Chain Reaction

The Diagnostic PCR test was used to investigate 25 strains obtained from different hosts for the presence of the *fas-1* gene. Results showed that all isolates were able to amplify a part of DNA with an approximate size of 225 bp (Figure 2).

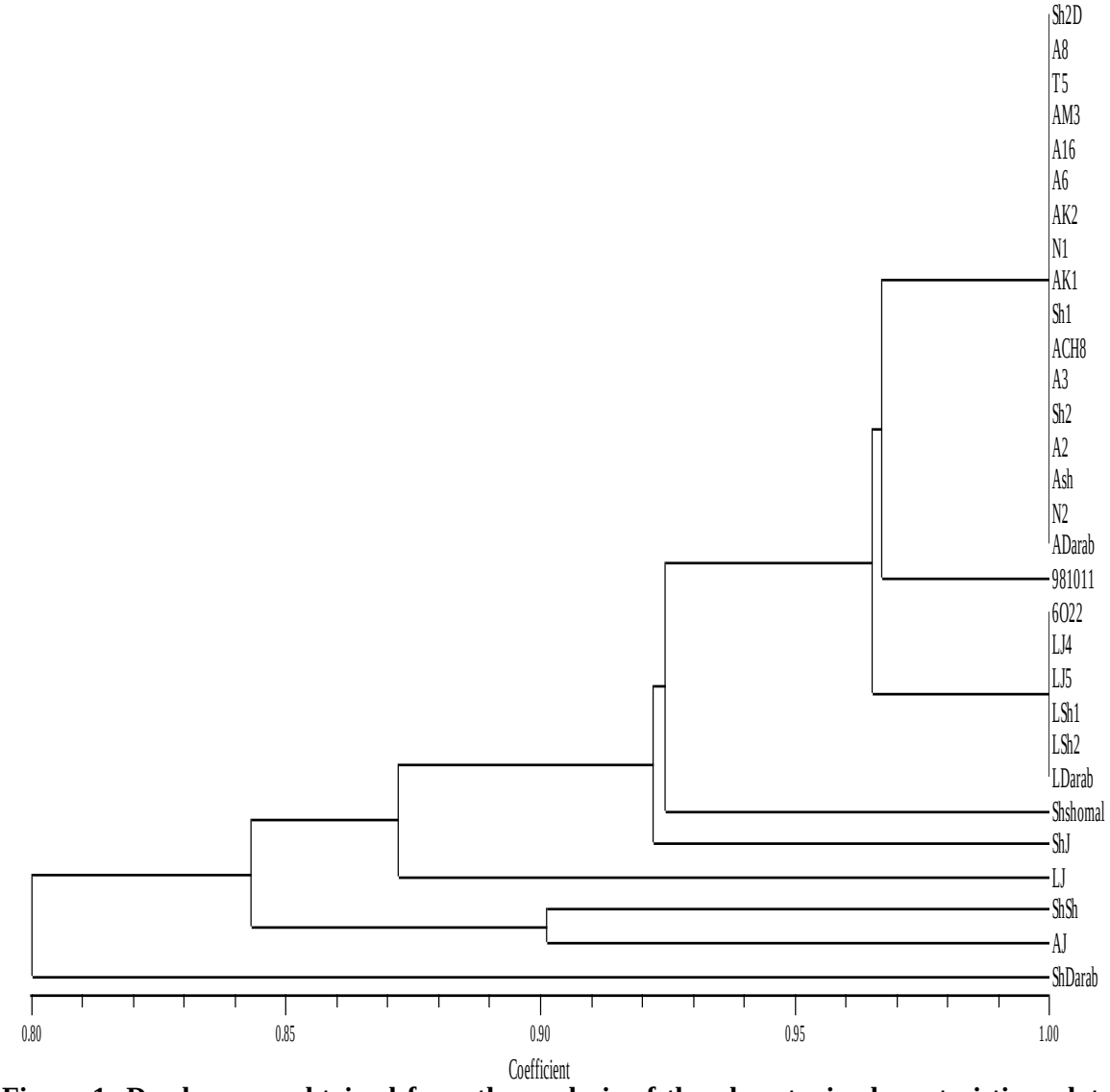

Figure 1: Dendrogram obtained from the analysis of the phenotypic characteristics related to 30 Rhodococcus fascians isolates

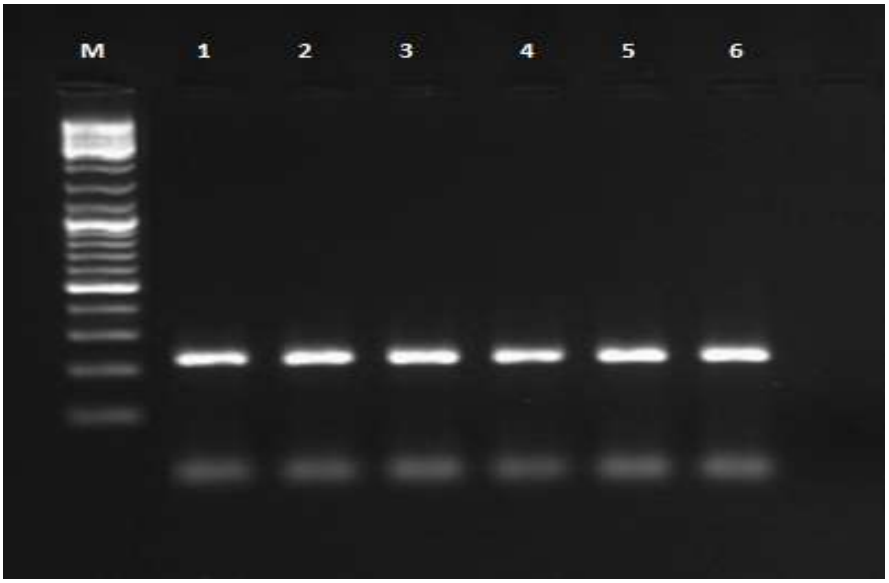

Figure 2: Electrophoresis gel of PCR product using primers of fas-1 gene. M: 100bp DNA molecular marker; 1-6, R. fascians strains

RAPD-PCR

In this test, results obtained from six random primers (Figure 3) were incorporated and analyzed (Figure 4). Results showed that isolates were separated from each other at an 81% similarity level. Most isolates from the three areas, Shiraz, Darab and Jahrom, were separated from the standard strain at an 87.5% level. The Jahrom isolates and the Darab geranium isolates were separated from the other isolates at a 92.5% similarity level. Meanwhile, the Jahrom petunia isolates and the Darab geraniums were classified with 100% similarity in a cluster and were placed in a group with the Jahrom nasturtium isolates. The last isolates showed similarity with the Jahrom geranium at a 95% level. Moreover, the petunia and northern geranium isolates showed similarity with each other at a 91% level and were placed in a group. One Shiraz geranium isolate was separated from all the above-listed isolates at an 81% level.

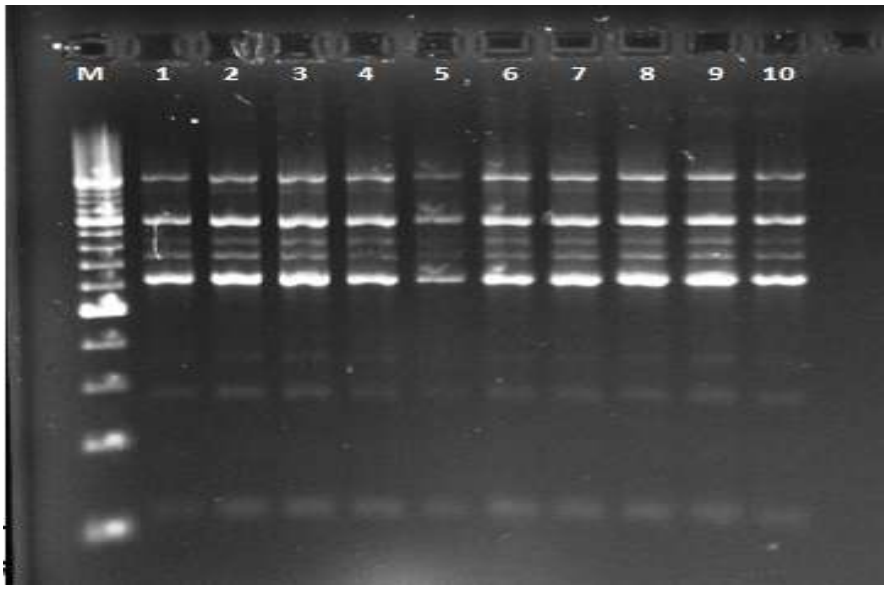

Figure 3: RAPD-PCR fingerprint patterns of *R. fascians* strains isolated from different hosts using P86 primer: M, 100bp DNA molecular marker; 1-10, *R. fascians* strains

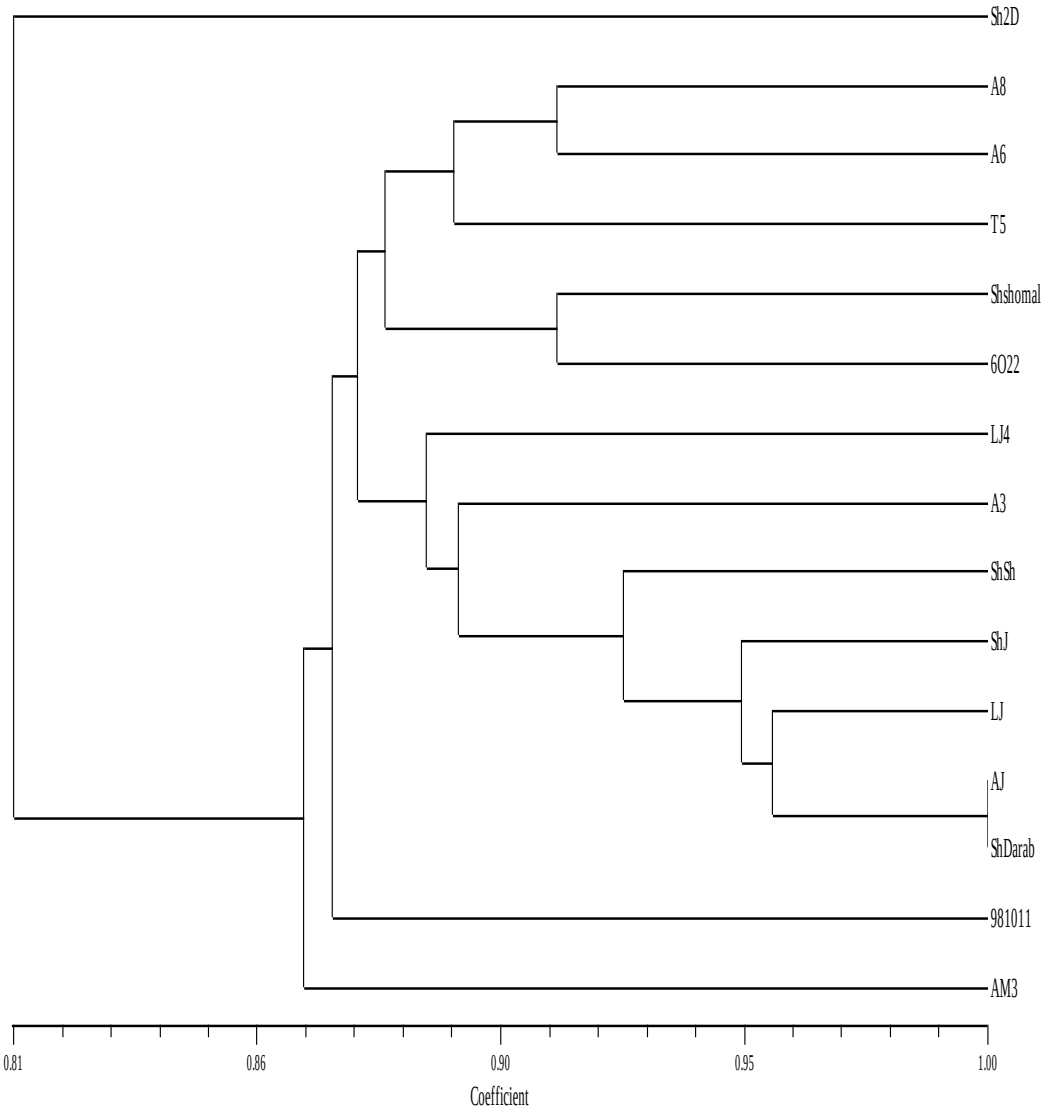

Figure 4: Dendrogram based on RAPD-PCR fingerprints of *Rhodococcus fascians* isolates from different hosts. Characteristics of each isolate were mentioned in table 1

Discussion

Our observations indicated that leafy gall disease is distributed in the Shiraz, Darab and Fasa, in Fars province. In most of the regions in which the petunia, geranium, and nasturtium plants were cultured, leafy gall disease was seen. Ornamental plants that were planted in warm, sunshiny regions of parks showed no symptoms, while in regions where plants were grown under suitable temperature and moisture conditions, the symptoms were severely increased.

In phenotypic characteristics, the northern geranium isolate had the ability to produce acid from adonitol, while all the other isolates lacked it. Geranium isolates of Fars province, the northern geraniums, and the standard isolate had the ability to produce acid from inositol, but the rest of the isolates lacked this ability. Moreover, in producing acid from salicin, the geranium and northern petunia isolates showed differences with all isolates. These results correspond with the findings of Najafipour and Taghavi (2001). The geraniums and nasturtiums of Fars province were distinct in that they had no ability to produce acid from fructose, and the geranium and northern petunia isolates were different from all other isolates in using mannitol. Furthermore, all isolates showed a difference from the standard strain in not using maltose. These results are in accordance with the findings of Najafipour and Taghavi (2001). Based on

observations in the present study, it was clear that the isolates from Fars province have the desired homogeneity, but they also showed differences with the northern isolates. In the pathogenicity test on peas, all isolates were found to be infectious.

In analysis of phenotypic data using NTsys–pc (v. 2.2), it became clear that all isolates were similar to each other at an 80% level. It should be noted that in phenotypic investigations, the least similarity rate is taken among isolates should be 80 percent (Swing and Heyward, 1990). The petunia isolates were significantly similar to each other and were placed in a distinct cluster, while the northern petunia isolates were separate from the other petunia isolates at a 96% level. Furthermore, the nasturtium isolates were placed with the northern petunia isolates in a distinct cluster and were separable from the other hosts. The standard strain had maximum and minimum similarity rates with the Shiraz petunia isolates and the Darab geranium isolates, respectively. Based on phenotypic tests, the northern petunia isolates were found to be different from the Shiraz petunia isolates and the standard strain. This result is in accordance with the results of Najafipour and Taghavi (2001). They also stated that the northern petunia isolate differed from the Shiraz petunia isolate. Results of the current study showed that the nasturtium and petunia isolates were homogeneous based on phenotypic characteristics and were separable from other hosts. The geranium isolates showed more differentiation and were distributed in different groups. These results are in accordance with the results of Rahimian and Zarei, who showed that geranium isolates are remarkably varied in phenotypic characteristics. In summation, an analysis of the present phenotypic data showed that, based on the phenotypic tests of *R. fascians* that have been isolated from petunias and nasturtiums, they are classifiable in clear clusters. However, this is impossible for geranium isolates. In RAPD-PCR, the results obtained from six random primers were combined and analyzed. All isolates were similar to each other at an 81% similarity level. Despite much genetic similarity between the isolates, at a 90% similarity level, some isolates could be distinguished from the others. For instance, the geranium isolates at the mentioned similarity level, three geranium isolates showed more uniformity and grouped in a distinct cluster. These results showed that geranium isolates, despite more phenotypic diversity, have more genotypic uniformity than other hosts. In addition, the tobacco isolate (T5) were placed in a separate group. Due to there being only one tobacco isolate, certain assessment are impossible about the recent grouping. Two north's isolates had been separated from Fars's petunia and geranium isolates and were located in a separate group. Indeed, these isolates were slightly different from the Fars isolates. Genotypic analysis using RAPD-PCR showed that the petunia and nasturtium isolates were more varied than the geranium isolates; five petunia isolates were placed in four different groups, and two nasturtium isolates were also placed in two distinct groups. The standard strain that was isolated from the pea was located in a distinct group. These results show that, by using genetic analysis with RAPD-PCR primers, *R. fascians* strains can be separated from each other based on host and geographical region. A comparison of the results of phenotypic and genotypic analysis for *R. fascians* strains showed that no relationship between phenotypic characteristics and genetic fingerprints obtained from RAPD-PCR could be found.

REFERENCES

Amani B (1976). Occurrence of new bacterial disease in Iran. *Iranian Journal of Plant Pathology* **12** 27 (Abstr.).

Chen YF, Yin YN, Zhang XM and Guo JH (2007). Curtobacterium flaccumfascians pv. Beticola, A new pathovar pathogens in sugar beet. *Plant Disease* **91** 677-684.

Fahy PC and Hayward AC (1983). Media and methods for isolation and diagnostic tests. In: *Plant Bacterial Diseases, a Diagnostic Guide*, edited by Fahy PC and Persley CJ (Academic Press, Sydney, Australia) 337-378.

Goethals K, Vereecke D, Jaziri M, Van Montagu M and Holsters M (2001). Leafy gall formation by *Rhodococcus fascians*. *Annual Review Of Phytopathology Journal* **39** 27- 52.

Klambt D, Thies G and Skoog F (1966). Isolation of cytokinins from *Corynebacterium fascians*. *Proceedings of the National Academy of Sciences* USA **56** 52-59.

Lacey MS (1939). Studies in bacteriosis. XXIV. Studies on a bacterium associated with leafy galls, fasciations and cauliflower disease of various plants. Part III. Further isolation, inoculation experiments and cultural studies. *Annals of Applied Biology* **26**(2) 262-78.

Maes T, Vereecke D and Ritsema T (2001). The att locus of *Rhodococcus fascians* strain D188 is essential for full virulence on tobacco through the production of an autoregulatory compound. *Molecular Microbiology* **42**(1) 13-28.

Najafi Pour G and Taghavi SM (2001). Determination of phenotypic characteristics and host range of *Rhodococcus fascians* strains, the causal agent of fasciation on ornamental plants, in Shiraz. *Iranian Journal of Plant Pathology* **37** 35-37.

Pataky NR (1991). Fasciation or leafy gall. Department of Crop Science, University of Illinois at Urbana - Champaign, Report on Plant Disease 2.

Rahimian H (1993). Isolation of *Rhodococcus fascians* from germanium in Tehran province. In *Proceeding of 11th Iranian Plant Protection Congress Rasht, Iran* 245.

Rohlf FJ (2000). NTSYS-pc: Numerical taxonomy and multivariate analysis system, version 2.1. Exeter Software: Setauket, New York. 11733- 2870.

Schaad NW, Jones JB and Chun W (2001*).* *Laboratory Guide for Identification of Plant Pathogenic Bacteria* 2nd. edition (APS Press) 164.

Stange RR, Jeffares RR, Young C, Scott DB, Easom RJ and Jameson PE (1996). PCR amplification of the *fas-1* gene for the detection of virulent strains of *Rhodococcus fascians. Plant Pathology* **45** 407-417.

Suslow TV, Schroth MN and Isaka M (1982). Application of rapid method for Gram differentiation of plant pathogenic saporphytic bacteria without staining. *Phytopathology* **72** 917-918.

Vereecke D, Burssens S, Simo´n-Mateo C, Inze´ D, Van Montagu M, Goethals K and Jaziri M (2000). The *Rhodococcus fascians*-plant interaction: morphological traits and biotechnological applications. *Planta* **210** 241–251.

Vereecke D, Cornelis K and Temmerman W (2002). Chromosomal locus that affects pathigenicity of *Rhodococcus fascians*. *Journal of Bacteriology* **184** 1112-20.

Yaish MWF (2006). Genetic mapping of quantitative resistance to race 5 of *Pseudomonas syringae* pv. *phaseolicola in* common bean. *Euphytica* **152** 397-404.

Zarei A and Rahimian H (1996). Characteristics of *Rhodococcous fascians* isolated from petunia in semnan. *Iranian Journal of Plant Pathology* **32** 125 (Abstr.)