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Molecular Diversity Within *Pseudomonas fluorescens* Strains Reflects Their Antagonistic Effect Differentiations to *Verticillium dahliae* on Cotton

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Abstract

Verticillium wilt disease caused by Verticilium dahliae Kleb. is a major problem on cotton growing areas in Turkey. In the present study efficient differentiation by molecular markers on fluorescent Pseudomonas strains with antagonistic properties have been evaluated and tested in order to determine a possible correlation between their genetic diversity and the antagonistic effects to V. dahliae. Therefore, twelve RAPD markers showing high polymorphism ratios within fluorescent P. strains have been selected. The selected RAPD primers resulted in discrimination on selected antagonistic strains, displaying significant correlation with their antagonistic effects on suppressing Verticilium dahliae. Therefore the fragments produced after priming with selected RAPD markers can be suggested as a marker in selecting the effective antagonistic fluorescent P. strains to V. dahliae.

Key words: Fluorescent *pseudomonas*, RAPD-PCR, rhizosphere, genetic diversity, molecular markers

Introduction

Verticillium wilt, caused by *Verticillium dahliae* Kleb. is one of the most important disease, resulting in great economic losses in many crops (TJAMOS & al. [1]). Turkey is the first country ranked in the top ten organic cotton producing countries in the world. A substantial increase in organic cotton production has been observed in the world and 12.507 metric tons of organic cotton production was recorded in 2007. Aydın province is also the second-largest organic cotton producer in Turkey. Therefore efficient control of the pathogen is required to minimize crop losses, which directly affects textile industry of the country. The use of biological control agents shows increasement and the control of soil borne pathogens by alternative methods becomes a prominent preoccupation since sustainable and organic agriculture are mainly considered.

The recent approaches to control Verticillium wilt disease is the rhizobacteria-mediated biological control and the method has also been successfully applied for controlling *V. dahliae* (BERG & al. [2]; TJAMOS & al. [3]; ÇUBUKÇU and BENLIOGLU [4]; ERDOĞAN and BENLIOĞLU [5]). Furthermore (RhizoStar®) strain, *Serratia plymutica* (HRO-C48) isolated from the rhizosphere of oilseed rape has been reported as an effective registered biopesticide resulting in effective control of Verticillium wilt on strawberry (KURZE & al. [6]).

Recently, Verticillium wilt symptoms delaying by root treatments of olive plants with selected *P.fluorescens* isolates has also been reported by MERCADO-BLANCO & al. [7]. Molecular techniques based on genomic DNA have extensively been used for analysis of genetic diversity. When the reproducibility can stablly be rendered with replications by the same primers, Random Amplified Polymorphic DNAs (RAPDs) is also confidentally used since it is a fast, cheap and easy method in mapping and characterization. One prominent advantage of the RAPD is that it can easily be applied and employed on antagonistic gram (-) and gram (+) bacteria (CIRVILLERI & al. [8]; BAYSAL & al. [9]).

The objective of this research was to determine a possible correlation between molecular diversity within *Pseudomonas fluorescens* strains and their antagonistic effect to *Verticillium dahliae* on cotton, by molecular markers.

Materials and Methods

Dual tests carried out *in vitro*: 59 bacterial isolates were tested for their ability to produce antifungal substances against *V. dahliae* using a dual-culture in vitro assay on PDA plates. Each plate was inoculated with 4 droplets of 10 µl bacterial suspension (at a 10^8 cfu/ml concentration) symmetrically placed on four sites at equal distances (2 cm) from the center of plate. After 24 h incubation at 24 ^oC, a single 5-mm-diameter mycelial disc was placed in the center. As a control, a disc of *V. dahliae* was grown on a PDA plate. The radius of each fungal colony was measured after a 10-day incubation at 24 ^oC in darkness, and the relative growth inhibition was expressed as a percentage [(treatment–control)/control x 100]. This experiment was conducted twice in three replicates.

Genomic DNA extraction from *P. flourescens* isolates: DNA was isolated and subjected to RAPD analysis. Genomic substraction was performed for tester strains. DNA was extracted from bacterial cultures grown in Nutrient Broth. After 24 h, 50 mL of culture was removed and centrifuged at 3000 × g for 5 min, after which the cells were washed in 0.85 % NaCl solution, re-centrifuged and resuspended in 2 mL of TE buffer (100 mM EDTA; 150 mM NaCl; 100 mM Tris-HCl, pH = 8.0) containing 4 mg mL-1 lysozyme. The suspension was incubated at 37°C for 45 min and 0.5 mL of 8.5% SDS was added, followed by incubation at 75°C for 30 min before the addition of 1.5 mL of potassium acetate (5 M, pH=5.2) and incubated for 20 min at 4°C. The DNA was extracted with chloroform: isoamylalcohol (24:1), precipitated with ice-cold isopropanol, washed with 70 % ethanol, briefly dried and re-suspended in 200 µL of TE buffer. The concentration of DNA was measured using a Biowave S2100 Diode Array spectrophotometer and stored at -20°C until further use.

RAPD-PCR analysis: PCR reactions were performed in 15 µl volume containing 20 ng DNA, 1,5 µl buffer, 2 mM MgCl2, 0.1 mM dNTPs, 0.6 U Taq polymerase (Biorun, Nantes, France), 3–10 µM primer. The primers resulting in clearly polymorphism have been selected. Primer and their sequences used in this study are presented in Table 1. PCR reaction condition for RAPD primers: 94°C for 3 min, 35 cycles of 94 °C for 1 min, 38 °C for 45 s, 72 °C for 1 min and 72 °C for 10 min. PCR products were separated on a 1.5 % agarose gel and visualized with ethidium bromide staining under ultraviolet light, and photographed with a Kodak Gel Logic 200 system.

Table 1. Primers and their sequences used in discrimination of *Pseudomonas flourescen*s isolates.

Primer name	Sequence (5'-3')
OP-A-01	CAGGCCCTTC
OP-A-09	GGGTAACGCC
OP-A-11	CAATCGCCGT
OP-E-02	GGTGCGGGAA
OP-E-04	GTGACATGCC
OP-F-06	GGGAATTCGG
OP-G-19	GTCAGGGCAA
OP-H-19	CTGACCAGCC
OP-T-04	CACAGAGGGA
OP-T-07	GGCAGGCTGT
OPM-20	AGGTCTTGGG

Scoring and Data Analysis: The RAPD fragments were scored as present (1) or absent (0) and the genetic relationship among populations was calculated using the similarity coefficient of Nei and Li [10]. A dendrogram was constructed using NTSYS (Numerical Taxonomy and Multivariate Analysis System) developed by ROHLF [11]. Amplified bands from each primer were scored as present (1) or absent (0). Only those bands showing consistently were considered; smeared and weak bands were excluded from the analysis. Dice's coefficient of similarity (Dij) was determined between each pair of strains. Dice's coefficient has been recommended for the evaluation of genetic similarities when using RAPD data (Lamboy, [12]). Genetic dissimilarity (GD) was calculated as GD = 1 - Dij. The estimates of similarity between strains were used for cluster analysis by the unweighted pair group method with arithmetic average (UPGMA; SNEATH and SOKAL [13]) using the NTSYS package version 2.02 for Windows (ROHLF [14]).

Results

The results indicated a strong correlation between the inhibitory effect of FP isolates and genetic variation. The origin of FP isolates and their inhibitory effect to *V. dahliae* (VD-11) is presented in Table 2. 15 FP isolates having different origins showed inhibitory effect by (>% 40) on *Verticillium dahliae.* The highest inhibition was 56.0 % obtained by FP5 (*Portulaca* sp. from Nazilli) and FP35 (*Convolvulus arvensis* from Söke) in dual cultures performed in *in vitro* conditions. Fluoresan Pseudomonas isolates which were collected from rhizosphere of *Portulaca* sp., *Convolvulus arvensis*, *Solanum nigrum*, that were the hosts of the pathogen, displayed high inhibitory effect to *V. dahliae* isolated from different cotton fields. The inhibitory effect of Fluoresan pseudomonas isolates collected from weeds were significantly higher than isolates collected from cotton rhizophere.

Table 2. Inhibitory effect of *Pseudomonas flourescens* isolates to *V.dahliae* (VD-11) in dual culture tests.

Isolates	Origin	Region	*In vitro* inhibition (%)
FP 1	*Chenopodium album*	Nazilli	48.5
FP 5	*Portulaca* sp.	Nazilli	56.0
FP 11	*Portulaca* sp.	Söke	50.0
FP 12	*Gossypium hirsutum* (cv BA 119)	Söke	43.9
FP 15	*Solanum nigrum*	Çine	53.0
FP 18	*Gossypium hirsutum* (cv Carmen)	Çine	45.0
FP 21	*Solanum nigrum*	Koçarlı	53.0
FP 22	*Xanthium strumarium*	Koçarlı	48.5
FP 23	*Portulaca* sp.	Koçarlı	43.9
FP 25	*Chenopodium album*	Aydın Merkez	43.9
FP 29	*Portulaca* sp.	Aydın Merkez	53.0
FP 30	*Gossypium hirsutum* (cv Carmen)	Aydın Merkez	43.9
FP 35	*Convolvulus arvensis*	Söke	56.0
FP 39	*Sinapis* sp.	Çine	50.0
FP 53	*Gossypium hirsutum* (cv Giza 45)	Nazilli	40.1
Control			00.0

The genetic relationship among all RAPD patterns of FP isolates based on the combination of data obtained with the selected primers is represented in the dendrogram shown in Figure 1. The isolates displaying high inhibition to pathogen were classified and discriminated by selected RAPD markers and Rohlf similarity index was calculated.

The RAPD profiles were compared by numerical methods and the dendrogram revealed genetic diversity (Fig. 1). Cluster analysis resulted in two main cluster groups designated 1 and 2. Most of the strains were exclusively grouped under subclusters, 3, 4, 5, and cluster 6 (Fig. 2). Cluster analysis also distinguished FP strains belonging to antagonistic group under subcluster 3-6. On the whole, grouping of isolates based on RAPD profiles was partially correlated with strain origin. The first cluster consisted of two sub- groups, one including 11, 14 and 15 FP isolates and a second sub-group including 3, 4, 5, 1 and 2 FP isolates respectively. The second group was clustered with two sub-groups. The first sub-group included 6, 7 and 8 FP isolates and the second sub-group contained 9, 10, 12 and 13 FP isolates respectively.

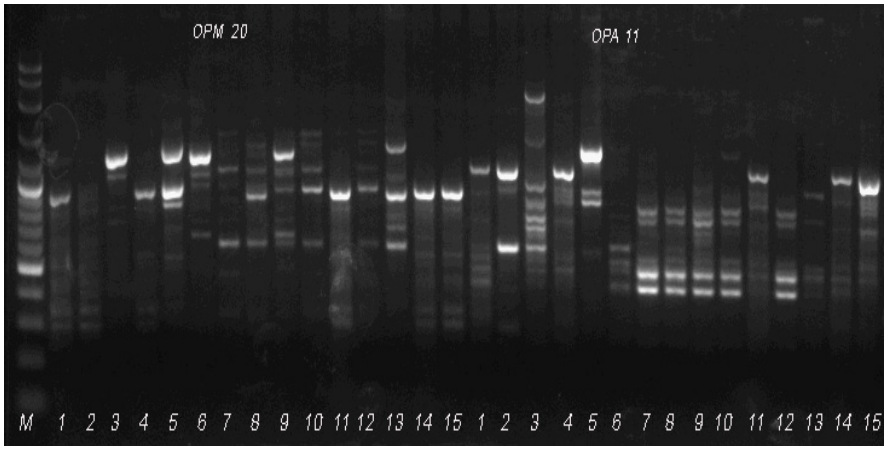

Figure 1. RAPD profiles amplified from DNA of FP (1-15) isolates analyzed using both primer OPM 20 and OPA 11 M: Gen Ruler Fermantas 100-1000 bp.

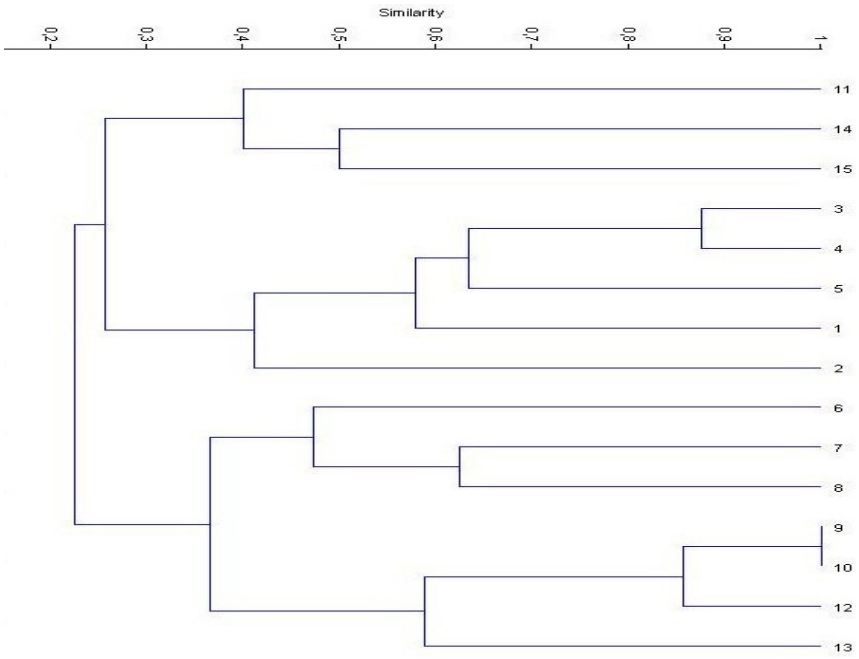

Figure 2. Dendrogram showing the genetic relationships among 15 *P. flourescent* isolates based on RAPD analysis.

The highest similarity index was between isolates 9 and 10, whose origins were Koçarlı and Aydın provinces. The isolates collected from weeds (common purslane and nettleleaf goosefoot) showed the similar effect on pathogen inhibition. Although origins of the

isolates collected from broad-leaf weeds showed differences, they had similar effect in dual culture tests. FP isolates 2 and 3 have similar origins and they have been isolated from the common purslane rhizosphere in Nazilli and Söke. Inhibition ratio of isolate 2 and 3 was above 50 % in dual culture tests. Although isolates 7 and 8 have showed similarity which is more than 60 %. However, they were isolated from different weeds. It is clear that although the isolate seems different in view of their host plants, the isolates showing similar inhibition on pathogen growth presented genetic similarity. The high genetic similarity of isolates collected from the same weed species is an expected result.

There was partially clear-cut relationship between clustering in the RAPD dendrogram and geographic origin, host genotype of the tested isolates, with a few exceptions. The similarity percent of each group oscillated between 40 and 87 %. The RAPD results analysis showed great genetic diversity among the FP isolates.

Discussion

This study clearly presents a high correlation between genetic similarity and inhibitory effect of the isolates as biological control agents. Therefore the study can be suggested as an effective tool for discrimination of isolates to be tested against *V. dahliae* Kleb. On the other hand, to rapidly selection of effective antagonistic isolates, once the fragments produced with amplification of selected RAPD primers are considered, these fragments can be sequenced and converted to SCAR markers. In another study, 29 fluorescent *Pseudomonas* tested to *P. ultimum* showed genetic diversity that was confirmed with their fatty acid profiles and RFLP analysis of the ribosomal DNA operon (ribotyping) (ELLIS & al. [15]). *P. fluorescens* strains were also classified by PCR–RAPD analysis, since biochemical methods limits their differentiation (RAMESH KUMAR & al. [16]).

The cotton is a high value crop grown in the western and south parts of Turkey. *P. fluorescent* are gram (-) bacteria epiphytically colonizing the foliage and rhizophere of the plants. A strain of *P. fluorescens* displaying inhibition on *Rhizoctonia solani, T. basicola*, *Alternaria sp.* and *Verticillium dahliae* growth has been asociated with antibiotic (pyrrolnitrin) production (HOWELL and STIPANOVIC [17]). Therefore *P. fluorescens* and its pyrrolnitrin has been tested in infested soil with *R. solani* by pre-plant treatment of seeds, this treatment resulting in cotton seedling survival, which was increased from 30 to 79%. *P. aeruginosa*, *P. putida*, *P. fluorescens* ve *P. syringae* are common and particularly colonize on young roots and rootlet of the plants. *P. fluorescens* suppresses soil borne pathogens by competion (nutrient and place), antibiosis (2,4,5-chlorophenoxy asetic acid, pyrrolnitrin ve pyoluteorin) and results in induced systemic resistance on plants (BUYSENS & al. [18]; ZHANG & al. [19]; ZEHNDER & al. [20]; VIDYASEKHARAN & al. [21]; WALSH & al. [22]) with PGPR effects which positively stimulate plant growth (ENEBAK & al. [23]; WELLER [24]; GÖRE and ALTIN [25]).

The production of antimicrobial agents in soil is influenced by several environmental factors such as soil chemistry and microbial population. For example, presence of Zn^{2+} in soil increases the production of Phenazine (PCA)16 and low temperature (12°C) influences production of 2,4-diacetyl phloroglucinol (DAPG) by *Pseudomonas* sp. (NOWAK-THOMPSON & al. [26]). *P. fluorescenst* has competion with other microorganisms, inhibiting penetration of pathogen microorganism through the root surface. Moreover, the pathogen fungi spores have suffered from F^{+3} defficieny and their germination was inhibited (BORA & al. [27]).

Environmental *P. aeruginosa* isolates have been considered as potential biological control agents or inducers of systemic acquired resistance and also some strains have been

reported as plant-growth promoting rhizobacteria (ANJAIAH & al. [28]; BANO and MUSARRAT [29]). Population dynamics of antibiotic-producing *Pseudomonas fluorescens* are believed to play a significant role in soil suppressiveness (MCSPADDEN GARDENER & al. [30]).

A similar study on antagonistic *P. fluorescens* bacteria used to control of Verticilium wilt of strawberry in *in vitro* conditions has been reported (BERG and BALLIN [31]). In another study carried out on inhibitory effect of *P. fluorescens* on *Pythium carolinianum* on corn and cotton fields the control possibilities of the disease by the antagonistic isolates have also been informed (ABDELZAHER and ELNAGHY [32]). *P. fluorescens* isolates were tested to *V. dahliae* Kleb. and some of the prominent isolates showing inhibitory effect on dual culture tests have resulted in succesfully control of the disease in cotton fields (TEHRANI & al. [33]). The results indicated a clearly reflection of plant species or the habitat,from which the bacteria have been isolated. The bacteria strains varies according to habitat and origin of the host plants, even if all bacteria isolate belong P. *fluorescens*. The findings shows that the molecular markers renders possible the discrimination of the isolate origins based on their plant host.

Conclusion

RAPDs were a useful marker system for determination of molecular diversity within *Pseudomonas fluorescent* strains. The genetic cluster formed within *P. fluorescens* isolates can also be correlated well with their inhibitory effect on pathogen fungi and may be associated with their antibiotic production. The further studies in which characterization of antibiotic compounds produced by selected isolates and quantitative analysis on them are also necessary. Our studies will be maintained in this concept in the future.

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