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# Assessment of Genetic Variation in *R. solanacearum*Strains from Marathwada Region by RAPD

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**ABSTRACT**

The genetic variation of 20 strains of *Ralstonia solanacearum* isolated from four host plants was assessed by Random Amplification of Polymorphic DNA (RAPD) fingerprinting method. Based on their genomic fingerprint, the strains divided into two distinct groups. One group consisting of Turmeric and Banana isolates, second group consisting of Tomato and Brinjal isolates. The cluster analysis based on Jaccard similarity coefficient separated the 20 strains into two major clusters. Cluster I comprised of strains isolated from host plant Banana and Turmeric shared genetic similarity of 69%. Cluster II comprised of strains isolated from host plant Tomato and Brinjal shared genetic similarity of 54%. The RAPD analysis also revealed that genetic variation showed by strains of *R. solanacearum* is independent of geographical location but the marker grouped the strains based on the host specificity and later on the races identified. The primer selected were capable of distinguishing the strains based on host specificity geographical distribution.

**Keywords**: *R. solanacearum*, RAPD, Biovar

## Introduction

Bacterial wilt caused by *Ralstonia solanacearum* (Yabuuchi et al., 1995) is one of the mostserious diseases of crops in tropics, subtropics and warm temperate regions of the world. It hasincited severe crop losses worldwide and the disease is now receiving global attention (Allen et al., 2005). It is emerging as an important threat to fruits and vegetables in the developing as wellas developed countries.*R. solanacearum* is a diverse species that differs in host range, geographical distribution, pathogenicity, epidemiological relationship and physiological properties. The pathogen is divided into five races based upon host range. This includes solanaceae family (race 1), Musa spp (race 2), in temperate regions it affects potato and tomato (race 3), ginger (race 4), andgroundnut and mulberry (race 5) (Elphinstone and Swanson, 2005). Further, divided into six biovars based on utilization of three disaccharides and three hexose alcohols (Kelman, 1954; Buddenhagan and Kelman, 1964; Hayward, 1994). It has been further separated into three majordivisions. Division I include biovars 2, 4, and 5 originating from Asia and Australia, division II includes all the members of the biovars I and II originating from South America (Cook et al., 1989) and division III contains strains emanated from Africa (Poussier et al., 2000). The most external symptoms are bacterial wilting, stunting and yellowing of the foliage, adventitious roots growing in the stems, and the observance of narrow dark stripes corresponding to the infected vascular bundles beneath the epidermis (Kelman, 1953; Smith, 1920). In India bacterial wilt disease caused by *R. solanacearum* has been reported mainly for solanaceous crops. The major hosts are - Solanum lycopersicum (tomato), Capsicum annum (Chilli and Bell pepper), Solanum tuberosum (potato), Solanum melongena (brinjal), Nicotiana tabacum (tobacco), Arachis hypogaea (groundnut), Musa paradisiaca (banana and plantain), Zingiber officinale (Ginger) (Hayward, 1994).

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Marathwada region (64818 sq. km) of Maharashtra state (307710 sq. km) consisting 54 of eight districts namely Aurangabad, Nanded, Latur, Parbhani, Hingoli, Jalna, Beed and Osmanabad. Marathwada lagged the rest of Maharashtra in economic prosperity, as the economy of this region is depending on agriculture. Major fruits and vegetables grown in this region are Banana, Mango, Oranges, Papaya, Grapes, Tomato, Brinjal, Turmeric, Onions, Garlic, Ginger and many leafy vegetables. Favored by conditions of humidity and temperature during monsoon season the crops are heavily attacked by bacterial wilt caused by *R. solanacearum* in Marathwada which causes major loss to farmers. No detailed studies related on characterization of the pathogen strains present in the Maharashtra state and in Marathwada region have been conducted, such as genetic variability and ecological distribution. Therefore, it is important to investigate the incidence of this disease caused by *R. solanacearum* in different districts in Marathwada region. This may provide basis for improved prediction and diagnosis together with formulation of management strategies preferably breeding for resistance against the disease. RAPD has a potential to detect the polymorphism in the entire genome compared with other techniques. Use of random primers for identification of individuals in differing populations is based on the theory that if any oligonucleotide is chosen at random, its distribution within the genome of differing individuals will vary (James et al, 2003). Keeping all these aspects in mind, the present study was conducted to isolate and characterize *R. solanacearum* strains at molecular level. RAPD technique has been used for assessing the genetic variations among 20 different *R. solanacearum* strains isolated from eight districts of Marathwada region in Maharashtra collected from different geographical location and from four different host plants.

## Materials and Methods

### Collection of infected plant samples

The wilted plant samples were collected from four host plants namely Banana, Turmeric, Brinjal and Tomato from Nanded, Parbhani, Hingoli, Latur, Aurangabad, Beed, Jalna, Osmanabad districts of Marathwada region in Maharashtra. The infected leaves were collected after noting typical wilting. The collected samples were transferred in polyethylene bags they were labelled with location, date and sample type. A total 137 infected leaves and fruits samples of four plants were identified. Of these samples 53 samples were selected for isolation and further study. Thus20 strains in total and 5 strains each from four host plants were selected in this study.

### Isolation of bacteria

Bacterial strains used in this study were isolated on KZC media (Peptone 10gm; Hydrolyzed Casein - 1gm; Glucose - 0.5gm(Filter sterilized); Agar - 17gm; Distilled water- 1000ml; 3ml of TZC) (Hi-media chemicals, Mumbai India). Appearing of reddish fluidal colonies with red centre was noted. After incubation isolated colony was selected morphological and biochemical characterization (Kelman, 1954). Isolated colonies were streaked on TZC slants. Slants were again incubated at 28°C for 24-48 hours. After incubation when plenty growth was noted on slants they were kept in refrigerator forfurther use.

### Physiological and biochemical test

Twenty strains of *R. solanacearum* isolated from Banana, Turmeric, Brinjal, and Tomato from eight districts of Marathwada region in Maharashtra were characterized by using following tests. These test included fermentation, Catalase production, Oxidase production, Starch hydrolysis,additionally the tests such as Levan production, Arginine utilization, Urease productions, Tyrosine utilization Tween 80 hydrolysis, Gelatin liquification, Aesculin hydrolysis

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was also performed for species identification was carried out using the methods described in theLaboratory Manual for phytobacteriology (Goszczynska et al., 2000). Biovar characterization was carried out based on the ability of strains to oxidize three disaccharides, maltose, lactose, cellobiose and three hexose sugar alcohols, mannitol, sorbitol, dulcitol were prepared at 2% solution. Each medium was inoculated separately with loopful of48hrs old bacterial culture of each strain and the tubes were incubated at $28^0$C for 48-72hrs as described by (Hayward, 1964).

**Extraction of genomic DNA**

Genomic DNA of all the 20 strains of *R. solanacearum* was extracted by phenol chloroform method (Ausbel et al., 1994). The purity of the DNA was checked by measuring absorbance at 260nm/280nm by using EppendorfBiophotometer (Sambrook et al., 1989)

**Random primers and PCR Amplification**

Four primers of 10 decamer (OPA02, 5TGCCGAGCTG3, OPA07, 5GAAACGGGTG3, OPA09,5GGGTAACGCC3, and OPA19 5CAAACGTCGG3 of Operon series)(Merk Biosciences, Banglore India) were used in this study. The PCR amplification for RAPD analysis was performed according to the procedure described by James et al., (2003) with following modifications. All the amplification were carried out at final volume of 25 µl mixture consisting of 2.5µl 1x PCR buffer with 1.5 mM MgCl2, 0.2 mM of each dNTPmix (Merck Biosciences, Mumbai India), 0.6 u Taq DNA polymerase (Bangalore Genei Pvt Ltd Bangalore India), 10 pM primer and 50 ng of template DNA. The PCR amplification was performed using thermal cycler (Applied Biosystem 9700). The initial denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 1 min, annealing at 53.5°C for 1 min and extension at72°C for 2 min with final extension of 7 min at 72°C. All amplified DNA products wereresolved by agarose gel electrophoresis on (1.5% Agarose) in 1x TAE buffer at 120Volts for 1.5 hrs and stained with ethidium bromide. The gel was visualized on a UV transilluminator and the photo documented in Gel doc system (BioRad USA).

**Data analysis**

The gel images were scored using a binary scoring system, recording the presence and absence of bands as 1 and 0, respectively. The data were exported into a spreadsheet and formatted for the NTSYSpc (Rohlf, 1997) cluster analysis software. Cluster analysis was performed on thesimilarity matrix using the unweighted pair group method of arithmetic average (UPGMA) thedendrogram was analysed by similarity matrix based Jaccard Co-efficient.

**Result**

**Morphological studies**

The colonies of all the 20 isolates were spherical, fluidal with smooth margins, opaque, slimydull creamy white colonies with a red centre on KZC medium. Based on morphological studies itwas found that all the isolates were gram-negative, rod shaped, non-spore forming, and motileorganisms. They were named as RSMp(Pb) Parbhani, RSMp(A) Ardhapur, RSMp(Dk)Dongerkada, RSMp(H) Hingoli, RSMp(V) Vishnupuri, RSCl(Pu) Purna, RSCl(Pa) Pawadewadi,RSCl(V) Vishnupuri, RSCl(L)Latur, RSCl(N) Nivgha, RSLe(Au) Aurangabad, RSLe(Os)Osmanabad, RSLe(B) Beed, RSLe(U) Umerga, RSLe(J) Jalna, RSSm(Os) Osmanabad,RSSm(B) Beed, RSSm(Au) Aurangabad, RSSm(D) Daulatabad, and RSSm(P) Parli.Race 1 strains were isolated from two plants that included tomato and brinjal. Race 2 strains isolated from banana and race 4 isolated from turmeric.

**Biochemical variability**

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All the isolates were Catalase positive and Tyrosinase, Starch hydrolysis, Gelatin liquefaction, Aesculin hydrolysis were negative. Among the isolates RSCl(Pu), RSLe(Au), and RSSm(Os) were weakly Oxidase negative and remaining strains were showed Oxidase positive. Strains RSCl(V), RSSm(Au) showed Levan production negative and remaining strains were showed Levan positive. Strains RSMp(Dk), RSCl(L), RSSm(B), RSSm(P), and RSLe(J) were Arginine hydrolysis negative and remaining strains were showed Arginine hydrolysis positive. Strain RSMp(A), RSCl(N), and RSLe(U) were Urease negative and remaining strains were showedUrease positive. Strain RSMp (H), RSCl(Pa), RSCl(V), RSLe(Os), and RSSm(D) wereTween80 negative and remaining strains were showed Tween 80 positive (Table 1). Similarbiochemical tests were performed by (Shambhu et al., 2001) and (Chaudhry et al., 2011) forspecies identification.

Biovar determinations of the twenty strains tested five strains were from biovar 1 were failed to utilize disaccharidesmaltose, lactose, cellobiose and hexose sugar alcohols mannitol, sorbitol and dulcitol. Fifteenstrains were from biovar 3 were able to utilize disaccharides maltose, lactose, and cellobiose after 3 days incubation by changing the color of medium from red to yellow. The hexose sugar alcohols mannitol, sorbitol and dulcitol were used after 7 days of incubation. These propertiesshown by strains belonging to biovar 3 isolated from host plants Brinjal, Tomato, and Turmeric.This showed dominance of biovar 3 in Marathwada region. (Table 2)

**RAPD analysis of *R. solanacearum*:**

RAPD fingerprints of 20 strains were produced by primers OPA2 (Fig 1), OPA7 (Fig 2), OPA9 (Fig 3), OPA19 (Fig 4). Similarity coefficients were calculated based on the fingerprintsand a dendrogram was built by using UPGMA (Fig. 5). The banding pattern per primer also varied from minimum of 7 bands to maximum of 17 bands per isolated strains with molecularmarker range from 100bp to 3000bp. Information on the banding pattern for all the four primerswas used to find out genetic distance between the strains. This was based on simple matchingcoefficient genetic similarity to assess genetic relatedness among the 20 strains. Each strain thatdiffered by race or biovar represented a distinct fingerprint type (Fig 5). The strains tested wereseparated into two main groups; one with all race 1 strains, and the other with race 2 and race 4strains. Genetic similarity within race 1 strains was 54% and race 2 and race 4 was 69%,respectively, whereas average similarity between race 1, race 2 and race 4 was 26%. The two strains from race 1, from host plant Brinjal shared maximum genetic similarity of 65%,originated from different locations of Aurangabad district. The average genetic similarity of97%, and 97.8% was noted in the strains of race 1 isolated from host plant tomato, originatedfrom Aurangabad, Jalna and Osmanabad, and Beed.The three strains from race 2, shared maximum genetic similarity of 97% isolated from host plant Banana, originated from different locations of Nanded district. The two strains from race 4shared the maximum genetic similarity of 85% isolated from host plant Turmeric, originatedfrom different locations of Nanded district.

The dendrogram formed by using UPGMA from pooled data (Fig 5) clearly showed two majorclusters, I and II (Table 4). The RAPD analysis clearly distinguished the strains according to their hosts and biovars. Cluster I was further subdivided into two subclusters, subcluster Icomprised of strains of biovar 1 from host plant Banana, subcluster II comprised of strains of biovar 3 from host plant Turmeric. The cluster II was further subdivided into two subclusters,subcluster I comprised of strains of biovar 1 from the host plant Tomato. The subcluster IIcomprised of strains of biovar 1 from the host plant Brinjal. The four strains from host plant tomatowere found genetically related, though they are from different geographical locations.

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## Discussion

Genetic variations of twenty strains of *R. solanacearum* have been studied by RAPD analysis.Knowledge of existence of biovar type and genetic variability in the pathogen is important forplant breeding and crop improvement programmes. We found that in Marathwada region biovar3 is more dominant than biovar 1. The strains of Tomato, Brinjal, and Turmeric were found positive for hexose alcohols. Similar findings were reported by (James et al 2003) for chilli, brinjal and tomato plants (Shambu et al 2001) for potato plant. Krishnappa et al (2011) reported of the 57 isolates 54 from biovar 3, (Lemessa et al 2010) reported of the 62 isolates 43 from biovar 2/race 3 and 19 from biovar 1/race 1 of host plants potato, tomato and pepper. (Jeong et al 2007) stated 440 strains from biovar 1, 3, 4 and 35 from biovar 2.

In the present study, dendrogram built clearly showed two major clusters, cluster I comprised of biovar 1/race 2 and biovar 3/race 4 strains. Cluster II comprised of all biovar 3/race1 strains suggesting that RAPD is a useful method for assessing the genetic relationships among biovarsand hosts of *R. solanacearum* isolates. Horita et al (2000) reported formation of two clusters,Cluster I included all biovar 3, 4 and 5 strains and Cluster II included biovar 1, 2 and N2 strains from rep-PCR fingerprinting. Kumar et al (2004) reported formation of three clusters, Cluster I and II comprised of ginger strains and Cluster III comprised of strains of potato on REP-PCR, ITS-PCR and RFLP-PCR fingerprinting. Lemessa et al (2010) recorded two groups, group 1 comprised of biovar 2 and group 2 comprised of biovar 1 strains on rep-PCR fingerprinting.

## Conclusion

This study confirmed that Banana, Turmeric, Tomato and Brinjal crops with symptoms ofbacterial wilt that were surveyed in Marathwada region were all infected with *R. solanacearum*strains belonging to biovar 1 and biovar 3. In Marathwada region, 15 strains (75 %) from recentlyidentified hosts are biovar 3 (For the first time). Our results also highlighted that biovar 3 is dominant in Marathwada region and have the potential to be compatible with more hosts. The molecular tools could cluster the most pathogenic isolates in a cluster at 100% similarity coefficient in conformity with their host origin and biovar.

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**Table 1: Biochemical characterization of *R.solanacearum* strains isolated from infected banana, turmeric, tomato and brinjal**

| Sr no | Strain | Location | Name of the biochemical test | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Catalase | Oxidase | Levan | Arginine | Urease | Tween80 | Tyroniase | Starch | Gelatin | Aesculin |
| 1 | RSMp5 | Ardhapur | + | + | + | + | - | + | - | - | - | - |
| 2 | RSMp9 | Dongerkada | + | + | + | + | + | + | - | - | - | - |
| 3 | RSMp13 | Hingoli | + | + | + | + | + | - | - | - | - | - |
| 4 | RSMp1 | Parbhani | + | + | + | + | + | + | - | - | - | - |
| 5 | RSMp17 | Vishnupuri | + | + | + | + | + | + | - | - | - | - |
| 6 | RSSm12 | Aurangabad | + | + | - | + | + | + | - | - | - | - |
| 7 | RSSm8 | Beed | + | + | + | - | + | + | - | - | - | - |
| 8 | RSSm16 | Daulatabad | + | + | + | + | + | - | - | - | - | - |
| 9 | RSSm4 | Osmanabad | + | Wn | + | + | + | + | - | - | - | - |
| 10 | RSSm20 | Parli | + | + | + | - | + | + | - | - | - | - |
| 11 | RSLe3 | Aurangabad | + | + | + | + | + | + | - | - | - | - |
| 12 | RSLe11 | Beed | + | + | + | + | + | + | - | - | - | - |
| 13 | RSLe19 | Jalna | + | + | + | - | + | + | - | - | - | - |
| 14 | RSLe7 | Osmanabad | + | Wn | + | + | + | - | - | - | - | - |
| 15 | RSLe15 | Umerga | + | + | + | + | - | + | - | - | - | - |
| 16 | RSCl14 | Latur | + | + | + | - | + | + | - | - | - | - |
| 17 | RSCl18 | Nivgha | + | + | + | + | - | + | - | - | - | - |
| 18 | RSCl6 | Pawadewadi | + | + | + | + | + | - | - | - | - | - |
| 19 | RSCl2 | Purna | + | Wn | + | + | + | + | - | - | - | - |
| 20 | RSCl10 | Vishnupuri | + | + | - | + | + | - | - | - | - | - |

+; Positive reaction, - ; Negative reaction, Wn; Weakly negative

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**Table 2: Biovar characterization of *R.solanacearum* strains isolated from infected banana, turmeric, tomato and brinjal**

| Strain | Location | Disaccharide reaction | | | Hexose alcohol reactions | | | Race |
|---|---|---|---|---|---|---|---|---|
| | | Maltose | Lactose | Cellobiose | Mannitol | Sorbitol | Dulcitol | |
| RSMp5 | Ardhapur | - | - | - | - | - | - | II |
| RSMp9 | Dongerkada | - | - | - | - | - | - | II |
| RSMp13 | Hingoli | - | - | - | - | - | - | II |
| RSMp1 | Parbhani | - | - | - | - | - | - | II |
| RSMp17 | Vishnupuri | - | - | - | - | - | - | II |
| RSSm12 | Aurangabad | + | + | + | + | + | + | I |
| RSSm8 | Beed | + | + | + | + | + | + | I |
| RSSm16 | Daulatabad | + | + | + | + | + | + | I |
| RSSm4 | Osmanabad | + | + | + | + | + | + | I |
| RSSm20 | Parli | + | + | + | + | + | + | I |
| RSLe3 | Aurangabad | + | + | + | + | + | + | I |
| RSLe11 | Beed | + | + | + | + | + | + | I |
| RSLe19 | Jalna | + | + | + | + | + | + | I |
| RSLe7 | Osmanabad | + | + | + | + | + | + | I |
| RSLe15 | Umerga | + | + | + | + | + | + | I |
| RSCl14 | Latur | + | + | + | + | + | + | IV |
| RSCl18 | Nivgha | + | + | + | + | + | + | IV |
| RSCl6 | Pawadewadi | + | + | + | + | + | + | IV |
| RSCl2 | Purna | + | + | + | + | + | + | IV |
| RSCl10 | Vishnupuri | + | + | + | + | + | + | IV |

+; Positive reaction, (Color of medium changes from red to yellow), - ; Negative reaction (color of medium was not changed).

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**Table 3. Strains of *Ralstonia solanacearum* used in this study and classifications established according to race, biovar and host.**

| Sr no | Code no | Strain Host | Geographical origin | Race | Biovar | Source |
|---|---|---|---|---|---|---|
| 1 | RSMp5 | Banana | Ardhapur | 2 | I | leaf |
| 2 | RSMp9 | Banana | Dongerkada | 2 | I | leaf |
| 3 | RSMp13 | Banana | Hingoli | 2 | I | leaf |
| 4 | RSMp1 | Banana | Parbhani | 2 | I | leaf |
| 5 | RSMp17 | Banana | Vishnupuri | 2 | I | leaf |
| 6 | RSSm12 | Brinjal | Aurangabad | 1 | III | leaf |
| 7 | RSSm8 | Brinjal | Beed | 1 | III | leaf |
| 8 | RSSm16 | Brinjal | Daulatabad | 1 | III | leaf |
| 9 | RSSm4 | Brinjal | Osmanabad | 1 | III | leaf |
| 10 | RSSm20 | Brinjal | Parli | 1 | III | leaf |
| 11 | RSLe3 | Tomato | Aurangabad | 1 | III | Fruit |
| 12 | RSLe11 | Tomato | Beed | 1 | III | Fruit |
| 13 | RSLe19 | Tomato | Jalna | 1 | III | Fruit |
| 14 | RSLe7 | Tomato | Osmanabad | 1 | III | Fruit |
| 15 | RSLe15 | Tomato | Umerga | 1 | III | Fruit |
| 16 | RSCl14 | Turmeric | Latur | 4 | III | leaf |
| 17 | RSCl18 | Turmeric | Nivgha | 4 | III | leaf |
| 18 | RSCl6 | Turmeric | Pawadewadi | 4 | III | leaf |
| 19 | RSCl2 | Turmeric | Purna | 4 | III | leaf |
| 20 | RSCl10 | Turmeric | Vishnupuri | 4 | III | leaf |

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**Table 4. Grouping of strains of *Ralstonia solanacearum* on the basis of Random amplification of polymorphic DNA (RAPD) analysis**

| Sr no | RAPD group | No. of strains | Geographical origin | Race | Biovar | Original host |
|---|---|---|---|---|---|---|
| 1 | I | 5 | Pb,Dk,H,V,A | II | I | Banana |
| 2 | II | 5 | Pu,Pa,V,L,N | IV | III | Turmeric |
| 3 | III | 5 | B,U,J,Au,Os | I | III | Tomato |
| 4 | IV | 5 | Au,Os,D,P,B | I | III | Brinjal |

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