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Isolation and identification of bacterial wilt causing *Ralstonia solanacearum* from groundnut (*Arachis hypogaea* L.)

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Abstract Bacterial wilt caused by *Ralstonia solanacearum* is one of the production constraints of groundnut. The wilt incidence, distribution, losses, symptoms and their epidemiology were investigated as well as the field survey to evaluate the severity and disease incidence in the regions of Andhra Pradesh and Karnataka. Result showed that 80 isolates were derived from wilted samples, and identified by the biochemical, pathogenicity and molecular characteristics. Among 80 isolates, sixty five isolates were categorized as Race 2 biovar III and the remaining 15 isolates belonged to biovars I and V based of sugars utilization. Pathogenicity assay suggested that out of 80 isolates, 25 isolates were highly pathogenic, 30 isolates showed 70-80% wilt incidence, 16 isolates showed 50% wilt, and 8 isolates did not cause any wilt symptoms. *R. solanacearum* is confirmed identification by molecular analysis.

Keywords: Groundnut, Bacterial wilt, *Ralstonia solanacearum*, Biovar, Pathogenicity

Introduction

Groundnut (*Arachis hypogaea* L.) is a legume crop grown nearly 24 million hectares globally with an overall yield of 36.45 million tons, and averaged production of 1520 kg/ha (FAOSTAT, 2011). The highest groundnut productions are cultivated in China, India, Nigeria, America and Myanmar. India is the second major producer of groundnuts worldwide. Being an oil seed crop, it is mainly used for oil production and the 13th most significant food crop in the world having 20-50% vegetable protein, 10-20% carbohydrates and 40-50% fat (Waliyar, 2006). In India, groundnuts are available in different varieties such as Kadiri-6, Kadiri-3, Kadiri-2, Kadiri-1812, Kuber, BG-2, BG-1, GUAG- 10, GAUG-1, PG-1, T-64, T-28, Chitra, Prakash, Chandra, Kaushal, Amber, etc. Bacterial wilt causes the damage of 15 to 55% crop losses annually

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(El-Argawy and Adss, 2016). Infested irrigation water, floor water and soil are the main sources of pathogen. Bacterial wilt infected by *R. solanacearum*, is one of the utmost dangerous plant infections, especially in groundnut (Maji and Chakrabartty, 2014; Caldwell *et al*., 2017). The symptoms begin with leaf drooping, followed by wilting of entire plant leading to plant death within few days of infection. Xylem infection due to bacterial colonization stops water and nutrition movement to the upper parts of the plant tissues leading to whole plant collapse (Islam *et al*., 2014). The diseased plants can also recover temporarily in the evenings, when temperature is cool but eventually dies due to permanent wilt. The roots and lower parts of the diseased plant stems have a browning of their vascular tissues. Sometimes, pathogen infected roots may cause rotting due to contamination from other bacterial species (Kurabachew and Ayana, 2017). *R. solanacearum* has a vast host range counting up to two hundred species in 50 families (Aliye *et al*., 2008; Elnaggar *et al.,* 2018). This pathogen is widespread possessing varied strains leading to socioeconomic affects (Narasimha Murthy and Srinivas, 2012). In India, the wilt influences a wide range of economically significant crops which includes groundnut, banana, tomato, potato, and eggplant (Anuratha *et al*., 1990). Bacterial wilt caused by *R. solanaceous* is also called southern bacterial blight, wilt or other local names pertaining to different nations (Kelman *et al*., 1954). In the present study, field survey was conducted from different groundnut growing areas, followed by isolation of bacterial wilt pathogen. Identification was carried out by biochemical assay, pathogenicity assay, and molecular phylogeny to distnguish different biovars based on sugars utilization.

Materials and methods

Field survey and sample collection

A field survey was carried out during July and August 2016 to monitor the bacterial wilt of groundnut plants in Andhra Pradesh and Karnataka. The survey was based on its occurrence and severity from regions of Tumkur (Pavagada) from Karnataka and Anantapur (Penukonda, Madakasira, Hindupur and Acharya N. G. Ranga Agricultural Research University, Extension center, Kadiri) from Andhra Pradesh. The infected plants were observed with characteristic wilt symptoms viz., droopy leaves, yellowing of leaves, entire plant wilted and vascular browning. At least 10 samples of the infected plant samples, and rhizosphere soil was collected in sterile polyester bag from each surveyed zone and samples were kept in the laboratory for the isolation of *R. solanacearum.*

Monitoring wilt incidence

Monitoring of Ralstonia wilt of groundnut was assessed by wilt symptoms from three sites of five grower's fields. Wilt frequency was calculated by using the following formula:

$$\% \text{ wilt incidence} = \frac{\text{Number of wilted plants in each field}}{\text{Total number of plants in each field}} \times 100$$

The incidence of wilt was based on the scale as follows: - 1=no symptom, 2 = upper younger leaves wilted, 3 =two leaves wilted, 4 = four or more leaves wilted and 5 = plant dies (Horita *et al*., 2001).

Isolation of Ralstonia solanacearum

The collected plant and soil samples were used for the isolation of *R. solanacearum*. 2, 3, 5 Triphenyl tetrazolium chloride (TZC) medium was used as specific medium for *R. solanacearum* isolation. The isolation of pathogen from infested soil was used by soil dilution method on TZC agar medium (Elphinstone *et al*., 1996). The infected plant segments (5mm-10mm) were surface sterilized with 1% NaOCl solution for roughly 2 min and rinsed with distilled water and blot dried. These surface sterilized plant segments were plated on TZC agar medium (Kelman *et al*., 1954). The plates were incubated for 24–48 h at 28 ℃, after this incubation, the pink centered colonies were selected for further studies. The selected bacterial isolates were subjected to morphological, physiological, cultural, molecular phylogenic identification. The pathogenicity assay was done for the confirmation of *R. solanacearum* (Vanitha *et al*., 2009). The isolated *R. solanacearum* strains were kept in steriled water at 25 ℃ in polypropylene tubes (Kelman and Person, 1961). For extensive period storage, the isolates were kept in glycerol stock at -80 ℃.

Identification of Ralstonia solanacearum

The identification of isolates was mainly based on the morphological, physiological, biochemical and molecular characteristics (Schaad *et al*., 1992). The isolated colonies were done by biochemical tests counting Gram's staining, catalase, motility, oxidase, production of fluorescence on King's B medium, starch hydrolysis, arginine dihydrolase, gelatin liquification, KOH solubility, H_2S production, Indole production, utilization of citrate and urease tests. The biovars was distinguished which depending on the utilization of disaccharides (maltose, lactose, sucrose, and trehalose) and oxidation of hexose alcohols (sorbitol and mannitol) (Table 1) (Rahman *et al*., 2010).

The biovar was determined in the mineral medium ($MgSO_4.7H_2O$-0.2g, agar-3.0g, KCl-0.2g, $NH_4H_2PO_4$-1.0g, peptone-1.0g and bromothymol blue 80 mg/1000ml distilled water) supplemented with 1% sugar, nearly 200 µl of sterilized broth was distributed into microtitre plate wells. It was added 20 µl of bacterial suspension containing 10^8 CFU/ml and incubated at 28 ℃-32 ℃ for 3 days. After incubation, the microtitre plate was observed for carbohydrate fermentation, and determined by color variation from blue to yellow (Rahman *et al*., 2010).

Table 1. The biovar characterization of *R. solanacearum* isolates

	Carbohydrate fermentation					
	1	**2**	**3**	**4**	**5**	**6**
Biovar	**Lactose**	**Maltose**	**Sucrose**	**Trehalose**	**Mannitol**	**Sorbitol**
I	-	-	-	-	-	-
II	+	+	+	+	-	-
III	+	+	+	+	+	+
IV	-	-	-	-	+	+
V	+	+	+	+	+	-

Note: += fermenters, - =non fermenters.

Pathogenicity of Ralstonia solanacearum isolates

The virulence of *R. solanacearum* isolates were assessed by bacterial wilt susceptible groundnut cultivars such as kadiri-6 and Harithandra. Twenty five days old healthy seedlings were subjected to pathogenicity assay. The bacterial inocula were prepared in sucrose peptone broth as per (Mitsuo *et al*., 2004), centrifuged at 12, 000 rpm for 10 min for pellet development and suspensions were prepared in distilled water to attain 10^8 CFU/ml which was confirmed by spectrophotometer (Ran *et al*., 2005).

Pathogenicity was conducted by root dip (Xue *et al*., 2009) and soil drenching methods (Williamson *et al*., 2002). The pathogenic interactions were recorded by wilt symptoms after 25 days inoculation. The isolates were grouped into 4 headed as highly pathogenic, moderately pathogenic, weakly pathogenic and non-pathogenic isolates mainly based on the variations in wilt symptoms. The uninoculated seedlings were used as control.

Molecular phylogenic identification of Ralstonia solanacearum

The identification of selected isolates were also confirmed by molecular method based on 16s rRNA sequencing. The obtained sequences were done by NCBI BLAST search, and top hit sequences were multiple aligned to construct the phylogenetic tree by using Neighbor Joining (NJ) study with 1000 bootstrap replications based on the algorithm, and sequences were submitted to NCBI GenBank database for accession numbers.

Results

Assessment of bacterial wilt incidence

The groundnut field survey was conducted from main groundnut plantation areas to monitor bacterial wilt of groundnut in accordance with its occurrence and severity from Andhra Pradesh and Karnataka (Figure 1). The maximum bacterial wilt incidence was recorded from Andhra Pradesh (35%) and the least bacterial wilt was recorded from Karnataka (17%). Furthermore, those differences of wilt incidence could be attributed to *R. solanacearum* diversity and the variations in soil factors prominent in several places surveyed.

Figure 1. Bacterial wilt infected groundnut fields (Anantapur district Patharlapalli village)

Isolation of R. solanacearum

After incubation, the bacterial colonies on TZC media was observed as cream color or off-white color with pink centered colonies and these are selected for further studies. Of the eighty isolates were isolated from the different infected groundnut plant and soil samples viz. 65 isolates were from Andhra Pradesh and 15 isolates were from Karnataka (Table 2).

Table 2. Incidence and severity of bacterial wilt in selected groundnut growing areas of India

Areas Surveyed	Number of isolates	Biovars detected	Wilt incidence (%)
Andhra Pradesh	65	I, III&V	35
Karnataka	15	I, III&V	17

Identification of R. solanacearum

Virulent bacterial colonies were observed as white or cream coloured with a light pink centered irregular shape, highly fluidal and opaque on TZC media (Figure 2a), whereas avirulent colonies were round, deep red in shade. Microscopic observation was shown that *R. solanacearum* isolates were Gram's negative rods (Figure 2b), non-capsular and non-spore forming. All isolates were able to grow at 37 ℃, however they could not grow at 40 ℃.They were motile which was showed by hanging drop method.

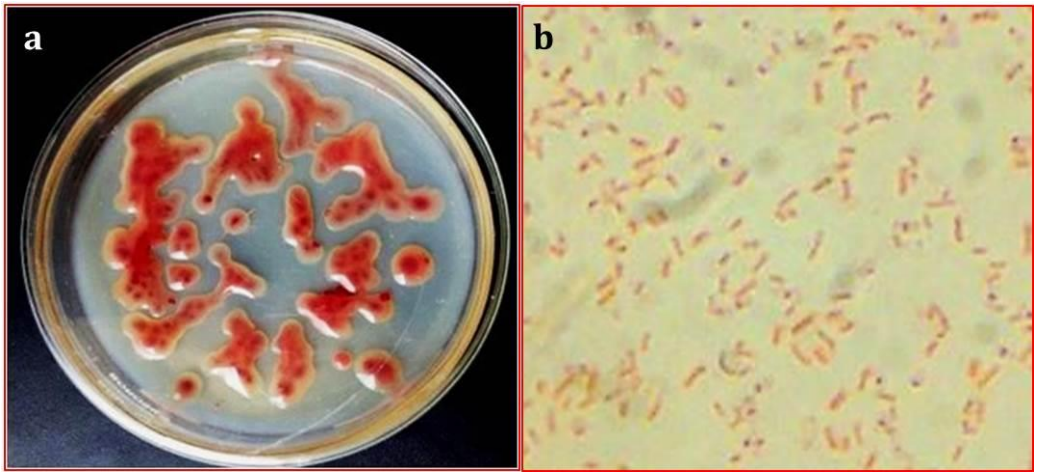

Figure 2. a. Pink centered virulent colonies of *R. solanacearum* on TZC agar medium; **b.** Microscopic observation

Biovars and biochemical characterization

Among 80 isolates, 65 isolates were confirmed as biovar-III based on utilization disaccharides and hexose alcohols. 14 isolates were identified as a biovar-V; they utilized all disaccharides and one hexose alcohol mannitol. One isolate was showed biovar-I as there was no utilization of hexose alcohols and disaccharides (Figure 3). The results of biochemical tests for selected isolates were tabulated in the Table 3.

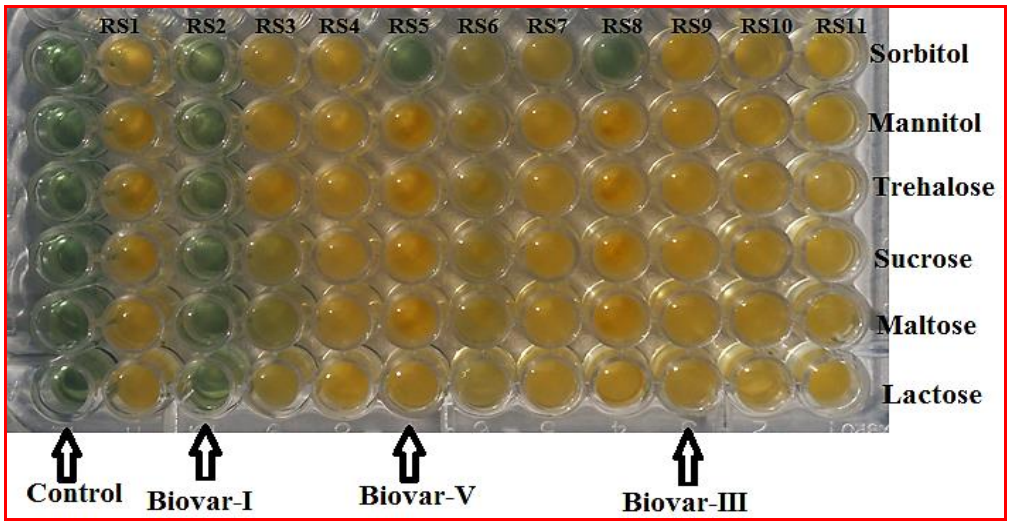

Figure 3. Biovar characterization of *R. solanacearum* based on utilization of sugars

Pathogenicity assay of R. solanacearum

Pathogenicity was highlighted with the occurrence of wilt symptoms from inoculated seedlings after 25 days (Figure 4). The pathogenicity was also confirmed through reisolation and identification of the pathogen from infected seedlings. The results of pathogenicity assay was exhibited that the 25 isolates (KAP01, KAP06, KAP08, APK10, KAP14, KAP17, KAP19, APH25, APH26, APH28, APA36, APA37, APM39, APM42, APM53, APP63, APP66, APP69, APP70, APP71, APP73, APP74, APP77, APP78, APP80) highly pathogenic and observed complete wilting of seedlings. The 30 isolates (KAP02, KAP07, KAP16, KAP20, APA33, APA67, APA69, APM44, APP65, APM40, APM41, APM43, APM45, APM47, APM51, APM55, APM57, APM59, APM60, APP60, APP65, APP67, APP68, KAP07, KAP16, KAP20, APP60, APK15, APK74, APK76) exhibited 70-80% wilt incidence, whereas 17

isolates (KAP05, KAP21, KAP22, KAP24, KAT55, KAS40, APA31, APA32, APA38, APA68, APA70, APK79, APK80, APM54, APH23, APH28, APP82) showed 50% of wilting; 8 isolates (APA34, APM58, APM61, APP61, APP75, APP76, APH21, APK11) did not cause any wilt symptoms and seedlings remained healthy (Figure 5).

Figure 4. Disease symptomatic variations on 1-5 scale for ground nut plants studied under greenhouse conditions. 1: No symptoms. 2: Slight chlorosis, 3: Moderate chlorosis, 4: Severe chlorosis, 5: Death of the plant

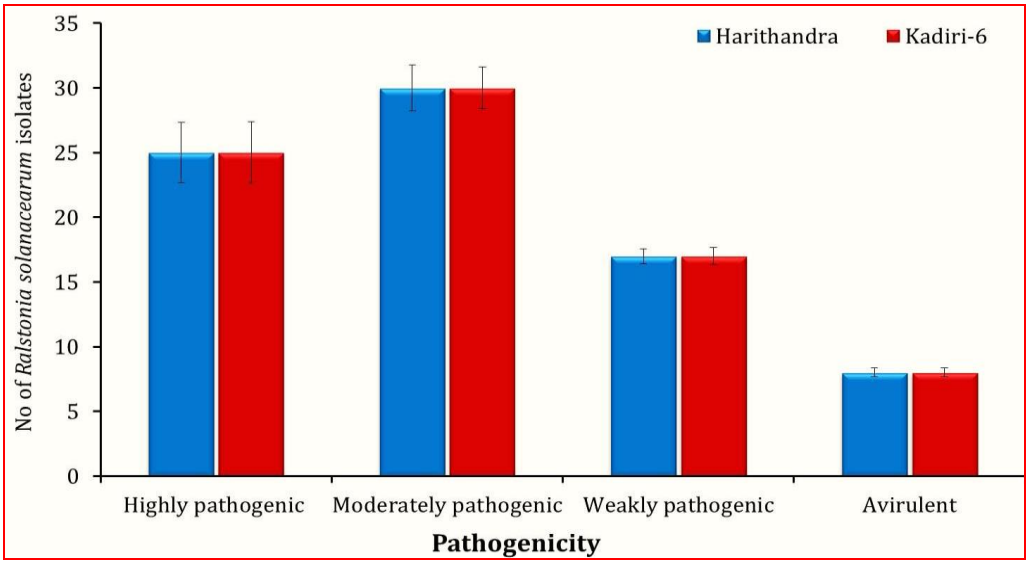

Figure 5. Grouping of *R. solanacearum* isolates based on pathogenicity variation

Molecular phylogenic identification of selected R. solanacearum

The identification of isolated *R. solanacearum* isolates were confirmed by molecular study. The BLAST analysis of their molecular sequences ensured 95%-98% identity to *R. solanacearum* strains. Amongst 80 isolates, two highly virulent isolates were identified as *R. solanacearum* with gen bank accession numbers APK76-MF973211 and KAK51-MF973210. The phylogenetic tree shown that the highly virulent strains belonged to closely related group of *R. solanacearum* (Figure 6).

Figure 6. Phylogenetic relationships of *R. solanacearum* isolates inferred by Neighbor-Joining (NJ) bootstrap tree analysis of 16s rRNA sequences

Table 3. Biochemical and physiological characterization of *R. solanacearum* from wilted groundnut plants collected from Andhra Pradesh and Karnataka

Isolates	Gram staining	Motility	Catalase test	Oxidase test	3% KOH	Gelatin liquefaction	Starch hydrolysis	Arginine dihydrolase	H_2S Production	Indole Production	Citrate Utilization	Urease	fluorescence on King's B
KAP01	--	+	+	+	+	--	--	--	+	--	+	--	--
KAP06	--	+	+	+	+	--	--	--	+	--	+	--	--
KAP08	--	+	+	+	+	--	--	--	+	--	+	--	--
APK10	--	+	+	+	+	--	--	--	+	--	+	--	--
KAP14	--	+	+	+	+	--	--	--	+	--	+	--	--
KAP17	--	+	+	+	+	--	--	--	+	--	+	--	--
KAP19	--	+	+	+	+	--	--	--	+	--	+	--	--
APH25	--	+	+	+	+	--	--	--	+	--	+	--	--
APH26	--	+	+	+	+	--	--	--	+	--	+	--	--
APH28	--	+	+	+	+	--	--	--	+	--	+	--	--
APA36	--	+	+	+	+	--	--	--	+	--	+	--	--
APA37	--	+	+	+	+	--	--	--	+	--	+	--	--
APM39	--	+	+	+	+	--	--	--	+	--	+	--	--
APM42	--	+	+	+	+	--	--	--	+	--	+	--	--
APM53	--	+	+	+	+	--	--	--	+	--	+	--	--
APP63	--	+	+	+	+	--	--	--	+	--	+	--	--
APP66	--	+	+	+	+	--	--	--	+	--	+	--	--
APP69	--	+	+	+	+	--	--	--	+	--	+	--	--
APP70	--	+	+	+	+	--	--	--	+	--	+	--	--
APP71	--	+	+	+	+	--	--	--	+	--	+	--	--
APP73	--	+	+	+	+	--	--	--	+	--	+	--	--
APP74	--	+	+	+	+	--	--	--	+	--	+	--	--
APP77	--	+	+	+	+	--	--	--	+	--	+	--	--
APP78	--	+	+	+	+	--	--	--	+	--	+	--	--
APP80	--	+	+	+	+	--	--	--	+	--	+	--	--

Note: "+" =positive for the reaction, "--" =negative for the reaction. KA=Karnataka, (P=Pavagada), AP=Andhra Pradesh, (K=Kadiri, H=Hindupur, A=Anantapur, M=Madakasira, P=Penukonda)

Discussion

Bacterial diseases in groundnut lead to drastic reduction of their yield and hence account for a detailed study. In the recent years, the disease has widely spread across noteworthy areas of groundnut farms Andhra Pradesh and numerous complaints from the farmers regarding the improper control of the suggested management practices have been unheard (Wang and Liang, 2014). The survey and surveillance from the indication for any actual plant health caters upon early discovery of the wilt incidence and timely adoption and application of disease preventive methods. In the present study showed that predominant of wilt incidence from different groundnut growing parts of Karnataka and Andhra Pradesh. Higher wilt incidence in Andhra Pradesh proposes a recurring problem in the groundnut cultivating regions and least efforts made to isolate the pathogen, identify and characterize them unless signs appeared. The present study clearly demonstrates that bacterial wilt disease prevails in all the surveyed regions of the state with varied degree of wilt occurrence and severity. This different grade of wilt occurrence and severity is anticipated because of the prevailing agro climatic conditions and the nature of the host cultivar. Hence, this work recommends that the periodic field survey is essential to know the development of bacterial wilt in groundnut plants. *R. solanacearum* was clearly identified by direct plating and spread plate approaches. TZC medium proved useful in the finding of *R. solanacearum* from groundnut and soil samples (Rahman *et al*., 2010). Difference of wilt frequency and disease severity were particularly expressed in groundnut because of the great diversity of plants distress from *R. solanacearum*, genotype and phenotype of this pathogen, its enormous geographical dispersal and the varied ecological situations favorable to bacterial wilt (Champoiseau et al., 2008; Ahmed and Kerstin, 2011).

In this study, the cultural characteristics of virulent *R. solanacearum* isolates from Andhra Pradesh and Karnataka look like these isolates from different areas of the world (Seleim *et al*., 2014). The *R. solanacearum* isolates formed fluidal colonies with pink centered on TZC medium that is main cultural characteristic of the pathogen (She *et al*., 2017).Virulent colonies were actually elevated, large, fluidal and entirely white with a pale pink center, whereas avirulent colonies were seen to be butyrous, small, deep red often with a bluish border (Jangir *et al*., 2018). The *R. solanacearum* isolates from various host plants were Gram-negative and rod-shaped bacteria (Afroz *et al*., 2011). Microscopic study clearly indicated that the isolates were rod-shaped and Gram-negative (Wang *et al*., 2017; Ibrahim *et al*., 2019).

The development of slime threads or loop was positive, because the Gram-negative bacteria possess extremely fragile cell walls that are well surrounded by an outer membrane. Production of gasoline bubbles indicates both aerobic and facultative anaerobic bacteria (Rahman *et al*., 2010). The isolates producing gasoline bubbles were demonstrated through catalase test (Lual, 2017). The isolates also exhibited the oxidase positive (Singh, 2014). Of the 80 *R. solanacearum* isolates, 65 isolates oxidized sugars with the characteristic colour change of the medium to yellow from green confirmed as biovar-III. Fourteen isolates were identified as a biovar-V; they utilized all disaccharides and one hexose alcohol mannitol and one isolate was showed as biovar-I. The utilization of many sugars by isolates was in accordance to of the results obtained by Hayward (Hayward, 1964).

The pathogenicity assay under greenhouse conditions showed that 25 bacterial isolates were highly virulent and infected groundnut seedlings with impressive wilt symptoms and had diverse infection in Kadiri-6 and Harithandra cultivars. It also showed that the inoculation attempts were possible when concentration reached 10^8 CFU/ml in the bacterial suspension (van der Wolf and De Boer, 2007; Baichoo and Jaufeerally, 2017).

Therefore, on the basis of physiological, biochemical, morphological, pathogenicity assays and molecular study, all the isolates were identified as *R. solanacearum*. Among eighty isolates, pathogenicity assay confirmed only two highly pathogenic isolates and these were selected for molecular identification. These two isolates were identified by 16S rRNA gene sequences and showed 95-98% similarity with the *R. solanacearum* strains from GenBank database.

In conclusion, the present research was suggested that the bacterial wilt of groundnut altered its occurrence, distribution and incurred losses in production of different varieties of groundnut. Isolation and identification of isolated *R. solanacearum* was conducted successfully from the groundnut samples and were later characterized by morphological, biochemical, pathogenicity and molecular approaches.

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