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DETECTION OF BACTERIAL WILT PATHOGEN IN GROUNDNUT CROPS GROWN IN RAJASTHAN

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ABSTRACT

Bacterial wilt of groundnut caused by *Ralstonia solanacearum* is an important disease characterized by hasty wilting following death of entire plant without showing any yellowing or spotting of leaves. The bacterial wilt is an extremely destructive soil borne disease of groundnut. It appeared as rapid and serious wilting symptoms in the host. The pathogen entered through different wounds and easily disseminated through infected biological material, soil, contaminated irrigation water, surface water and farm equipments and could survive for many years in association with alternating hosts. It is a broadly distributed and much diversified soil borne pathogen having an unusual broad host range with long-term survivable ability. In the present study, wilt incidence, distribution, losses, symptoms and their epidemiology were investigated and field surveys was done to evaluate the severity and disease incidence in the different regions of Rajasthan. Sixty bacterial isolates were derived from wilted samples, and identified by the biochemical and pathogenicity characterization. Among 60 isolates, forty five isolates were categorized as Race 2 biovar III and the remaining 15 isolates belonged to biovars I and V based on sugars utilization. Pathogenicity assay suggested that out of 60 isolates, 19 isolates were highly pathogenic, 24 isolates showed 70- 80% wilt incidence, 11 isolates showed 50% wilt, and 6 isolates were found non-pathogenic.

KEYWORDS: Bacterial wilt, Biovar, Groundnut, Pathogenicity, *Ralstonia solanacearum*, Severity.

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is a legume crop grown nearly 29.6 million hectares globally with an overall yield of 48.8 million tons, and average yield production on global level is 1.65 Mt ha^{-} in year 2019. India occupies first position in terms of area and second position in terms of production and China is the largest producer of groundnut in the world with 175.73 lakh tones in 2019 followed by India (67.28 lakh tones) (FAOSTAT. 2019). Being an oil seed crop, it is the 13th most important food crop in the world having 20-50% vegetable protein, 10-20% carbohydrates and 40- 50% fat (Waliyar, 2006). In India, groundnuts are obtainable 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 15 to 55% crop losses annually (El-Argawy and Adss, 2016).

The bacterial wilt caused by *Ralstonia solanacearum* is a destructive soil borne groundnut disease and dispersed through infested soil, water and infected seeds (Anitha et al. 2003). Direct plating of 4-week old leaf-twigs, leaf-bits and seed on Tetrazolium chloride agar (TZCA) medium is used to identify the wilt pathogen in groundnut (Prasada Rao et al 2000). Bacterial wilt caused by *Ralstonia solanacearum*, is one of the utmost hazardous plant infections, especially in groundnut (Maji and Chakrabartty, 2014; Caldwell *et al*., 2017). It penetrate into cortical tissue of host roots, colonized and exploded in numbers and cause a rapid deadly wilt of plant (Van Elsas et.al., 2001). Infection in young plants result in speedy wilting of stems and foliage, while leaves retain their green colour (Mehan et.al., 1994).

Wilt symptoms can be observed three weeks after planting. The symptoms begin with leaf drooping, followed by wilting of entire plant leading to plant death within few days of infection. Xylem infectivity 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 ultimately dies due to permanent wilt. Occasionally, pathogen infected

roots may cause rottingdue to contamination from other bacterial species (Kurabachew and Ayana, 2017).

Ralstonia solanacearum has a wide host assortment expanding over more than 200 species in 50 families (Aliye *et al*., 2008; Elnaggar *et al.,* 2018). This pathogen is widespread possessing diverse strains leading to socioeconomic affects (Narasimha Murthy and Srinivas, 2012). In India, the wilt influences a wide range of economically important crops which includes groundnut, banana, tomato, potato, and eggplant (Anuratha *et al*., 1990). Bacterial wilt caused by *Ralstonia solanacearum* is also called southern bacterial blight, wilt or other local names pertaining to diverse nations (Kelman 1954).

In the present study, field survey was conducted from different groundnut growing areas of Rajasthan, followed with isolation of bacterial pathogen. Identification was approved by biochemical assay and pathogenicity test to distnguish different biovars based on sugars utilization.

AIM OF THE STUDY

In the present study attempts were made to determine incidence and yield loss of bacterial wilt of groundnut in different growing areas of Rajasthan as well as to isolate and identify the bacterial pathogen.

MATERIALS AND METHODS

Field survey and sample collection

A survey was carried out to know the status of bacterial diseases in growing seasons of groundnut. The survey was based on its occurrence and severity from Bikaner and Dausa districts of Rajasthan. Samples have been collected at various intervals during july to October year of 2020. The infected plants were observed with characteristic wilt symptoms such as droopy leaves, entire plant wilted and vascular browning. All the samples have been collected of diseased plants from several sites within the field. At least ten samples of the infected plants 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.* Samples were packed in paper bags and labeled with the place and date of collection, and other valuable information such as host variety, growth stage, etc.

Monitoring wilt incidence

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

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

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 = entire plant dies (Horita *et al*., 2001).

Isolation of *Ralstonia solanacearum*

The composed plant and soil samples were used for the isolation of *Ralstonia solanacearum*. 2, 3, 5 Triphenyl tetrazolium chloride (TZC) medium was used as specific medium for isolation of *Ralstonia solanacearum.* The isolation of pathogen from infested soil was used through 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 by distilled water and blot dried. These surface sterilized plant segments were plated lying on TZC agar medium (Kelman 1954). The plates were incubated for 24–48 h at 28°C, following this incubation, the pink centered colonies were selected for further studies. The selected bacterial isolates were subjected to morphological, physiological and cultural identification.

The pathogenicity assay was completed for the confirmation of *Ralstonia solanacearum* (Vanitha *et al*., 2009). The isolated *Ralstonia solanacearum* strains were reserved in sterile water at 25°C in polypropylene tubes (Kelman and Person, 1961). For extensive period storage, the isolates were kept in glycerol stock on -80°C.

Identification of *Ralstonia solanacearum*

Ralstonia solanacearum is considered to be a "species complex" due to significant variation within the group (Fegan and Prior 2005). It identified from either symptomatic or asymptomatic plants and from water or soil samples by way of several microbiological and molecular methods (Weller *et al.* 2000). The identification of isolates was mainly based on the morphological, physiological and biochemical characteristics (Schaad 1992). Screening tests can make easy early detection of *Ralstonia solanacearum* in plants or contaminated soil and water samples, but they cannot be used to identify the race or biovar.

The isolated colonies were completed 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, polymerase chain reaction (PCR) with specific primers and pathogenicity tests using susceptible hosts, such as tomato seedlings (Elphinstone *et al.* 1996, Weller *et al.*, 2000). Isolation from symptomatic material can simply be performed using Kelman‟s tetrazolium chloride (TZC) medium. In the case of secondary infections, isolation of the pathogen on selective media was necessary.

Biovar test is a biochemical assay which can be recognized from a panel of disaccharides and sugar alcohols (Hayward (1994b). 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 dispersed into microtitre plate wells. It was added 20µl of bacterial suspension containing 10^8 CFU/ml and incubated at 28°C -32°C for three days. After incubation, the microtitre plate was observed for carbohydrate fermentation and determined by color disparity from blue to yellow (Rahman *et al*., 2010).

Table 1: The biovar characterization of *Ralstonia solanacearum* isolates.

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

Note: += fermenters and - =non fermenters

Pathogenicity of *Ralstonia solanacearum* isolates

The virulence of *Ralstonia solanacearum* isolates were assessed with bacterial wilt susceptible groundnut cultivars such as kadiri-6 and Harithandra. Twenty five days old healthy seedlings were subjected to pathogenicity test. The bacterial inocula were ready in sucrose peptone broth (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 with root dip (Xue *et al*., 2009) and soil drenching methods (Williamson *et al*., 2002). The pathogenic interactions were recorded through wilt symptoms after 25 days inoculation. The isolates were grouped into four headed as highly pathogenic, moderately pathogenic, weakly pathogenic and non-pathogenic isolates mostly based on the variations in wilt symptoms. The uninoculated seedlings were used like control.

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 Bikaner and Dausa districts of Rajasthan (Figure 1 & 2). The maximum bacterial wilt incidence was recorded from Bikaner (35%) and the least bacterial wilt was recorded from Dausa (17%). Besides, those differences of wilt incidence could be attributed to *Ralstonia solanacearum* diversity and the variations in soil factors prominent in several places surveyed.

Figure 1: Bacterial wilt infected groundnut fields in Bikaner. Figure 2. Bacterial wilt infected groundnut fields in Dausa.

Isolation of *Ralstonia solanacearum*:

Later than 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 sixty isolates were isolated from the different infected groundnut plant and soil samples viz. 42 isolates were from Bikaner and 18 isolates were from Dausa (Table 2).

Table 2: Incidence and severity of bacterial wilt in selected groundnut growingareas of India.

Areas Surveyed Rajasthan	Number of isolates	Biovars detected	Wilt incidence (%)
Bikaner	42	I, III&V	35
Dausa	18	I, III&V	17

Identification of *Ralstonia solanacearum*

Virulent bacterial colonies were observed as white or cream coloured by a light pink centered irregular shape, highly fluidal and opaque on TZC media (Figure 2a), while avirulent colonies were round, deep red in shade. Microscopic observation was exposed that *Ralstonia solanacearum* isolates were Gram'snegative rods (Figure 2b), non-capsular and non-spore forming. All isolates were able to grow at 37°C, but they could not grow at above 40°C.

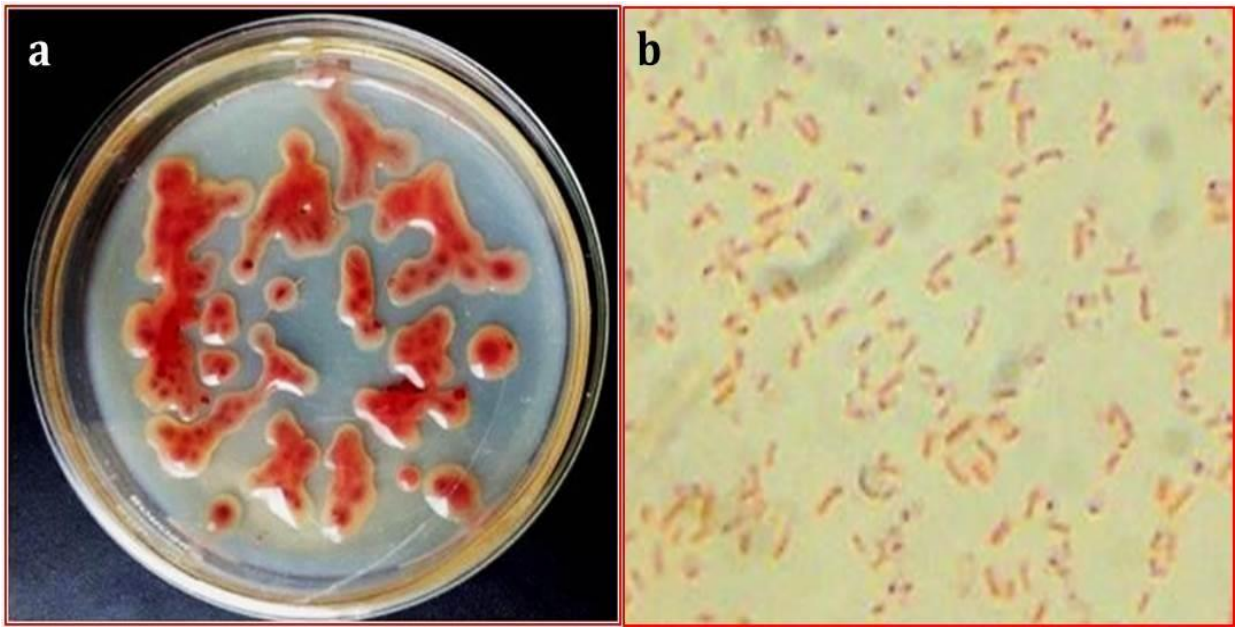

Figure 2: A. virulent colonies of *R. solanacearum* on TZC agar medium; B. Microscopic observation.

Biovars and biochemical characterization

Among 60 isolates, 45 isolates were identified as biovar-III based on utilization disaccharides and hexose alcohols. 14 isolates were confirmed as a biovar-V; they utilized all disaccharides and one hexose alcohol mannitol. One isolate was identified as biovar-I there was no utilization of hexose alcohols and disaccharides. The results of biochemical tests for selected isolates are tabulated in the Table 3.

Table 3: Biochemical and physiological characterization of *R. solanacearum* from wilted groundnut plants collected from Rajasthan.

Isolates	Gram-staining	Mot-ility	Catal-asetest	Oxid-asetest	3%-KOH	Gelatin Lique-faction	Starch hydro-lysis	Arginine dihydro-Lase	H_2S Produ-ction	Indole Produ-ction	Citrate Utili-zation	Ure-ase	Fluore scenceon King's B
DAL02	--	+	+	+	+	--	--	--	+	--	+	--	--
DAL04	--	+	+	+	+	--	--	--	+	--	+	--	--
DAL07	--	+	+	+	+	--	--	--	+	--	+	--	--
DAL10	--	+	+	+	+	--	--	--	+	--	+	--	--
DAL13	--	+	+	+	+	--	--	--	+	--	+	--	--
DAL18	--	+	+	+	+	--	--	--	+	--	+	--	--
BKK21	--	+	+	+	+	--	--	--	+	--	+	--	--
BKK25	--	+	+	+	+	--	--	--	+	--	+	--	--
BKK28	--	+	+	+	+	--	--	--	+	--	+	--	--
BKL31	--	+	+	+	+	--	--	--	+	--	+	--	--
BKC36	--	+	+	+	+	--	--	--	+	--	+	--	--
BKC38	--	+	+	+	+	--	--	--	+	--	+	--	--
BKN43	--	+	+	+	+	--	--	--	+	--	+	--	--

BKN45	--	+	+	+	+	--	--	--	+	--	+	--	--
BKN48	--	+	+	+	+	--	--	--	+	--	+	--	--
BKP51	--	+	+	+	+	--	--	--	+	--	+	--	--
BKP54	--	+	+	+	+	--	--	--	+	--	+	--	--
BKP57	--	+	+	+	+	--	--	--	+	--	+	--	--
BKP60	--	+	+	+	+	--	--	--	+	--	+	--	--

Note: "+" =positive for the reaction, "--" =negative for the reaction. DA=Dausa, (L=Lalsot), BK=Bikaner, (K= Kolayat, L= Loonkaransar, C= Chhattargarh, N= Nokha, P=Pugal)

Pathogenicity assay of *Ralstonia solanacearum*

Pathogenicity was highlighted with the occurrence of wilt symptoms from inoculated seedlings after 25 days. The pathogenicity was also confirmed through reisolation and identification of the pathogen from infected seedlings. The results of pathogenicity assay was exhibited that the 19 isolates (DAL02, DAL04, DAL07, DAL10, DAL13, DAL18, BKK21, BKK25, BKK28, BKL31, BKC36, BKC38, BKN43, BKN45, BKN48, BKP51, BKP54, BKP57, BKP60) highly pathogenic and observed complete wilting of seedlings. The 24 isolates (DAL01, DAL03, DAL06, DAL09, DAL12, DAL15, DAL17, BKK22, BKK24, BKK27, BKL30, BKC33, BKC37, BKN40, BKN44, BKC46, BKC47, BKN49, BKN52, BKN53, BKP55, BKP56, BKP58 and BKP59) exhibited 70-80% wilt incidence, whereas 11 isolates (DAL05, DAL11, DAL14, BKK19, BKK23, BKK29, BKL34, BKC39, BKN41, BKN42 and BKP50) showed 50% of wilting; 6 isolates (DAL08, DAL16, BKN20, BKN26, BKP32 and BKP35) did not cause any wilt symptoms and seedlings remained healthy.

Conditions:- 1: No symptoms, 2: Slight chlorosis, 3: Moderate chlorosis, 4: Severe chlorosis, 5: Death of the plant

DISCUSSION

Bacterial diseases in groundnut lead to drastic decrease of their yield and therefore account for a detailed study. In the recent years, the disease has widely spread across notable areas of groundnut farms 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 implementation and application of disease preventive methods.

The present study showed that predominance of wilt incidence from different groundnut growing parts of Rajasthan. Higher wilt incidence in Rajasthan proposes a recurring problem in the groundnut cultivating regions and least efforts made to isolate the pathogen, identify and characterize them unless signs appeared. It also showed that the inoculation attempts were probable when concentration reached 10^8 CFU/ml in the bacterial suspension (Van der Wolf and De Boer, 2007; Baichoo and Jaufeerally, 2017). TZC medium proved helpful in the finding of *Ralstonia solanacearum* from groundnut and soil samples (Rahman *et al*., 2010).

Difference of wilt incidence and disease severity were predominantly expressed in groundnut because of the great diversity of plants suffering from *Ralstonia solanacearum*, genotype and phenotype of this pathogen, its enormous geographical dispersal and the diverse ecological situations favorable to bacterial wilt (Ahmed and Kerstin, 2011). The present study clearly demonstrates that bacterial wilt disease prevails in all the surveyed regions of the state with different degree of wilt incidence and severity. This different grade of wilt incidence and severity is predictable because of the prevailing agro climatic conditions and the nature of the host cultivar. Therefore, this work recommends that the periodic field survey is necessary to know the development of bacterial wilt in groundnut plants. *Ralstonia solanacearum* was clearly recognized by direct plating and spread plate approaches.

The *Ralstonia solanacearum* isolates created fluidal colonies with pink centered on TZC medium that is most important cultural characteristic of the pathogen (She *et al*., 2017). Virulent colonies were actually elevated, large, fluidal and completely white with a pale pink center, while avirulent colonies were seen to be butyrous, small, deep red often with a bluish border (Jangir *et al*., 2018). The *Ralstonia solanacearum* isolates from different host plants were Gram-negative and rod-shaped bacteria (Afroz *et al*., 2011). Microscopic study evidently 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 have extremely fragile cell walls that are well surrounded with an outer membrane. The consumption of many sugars by isolates was in accordance of the results obtained by Hayward (Hayward, 1964). Production of gasoline bubbles indicates equally aerobic and facultative anaerobic bacteria (Rahman *et al*., 2010). The isolates producing gasoline bubbles were demonstrated during catalase test (Lual, 2017). The isolates too be exhibited the oxidase positive (Singh, 2014).

Among the 60 *Ralstonia solanacearum* isolates, 45 isolates oxidized sugars with the characteristic colour

change of the medium to yellow from green confirmed as biovar-III. Fourteen isolates were recognized as a biovar-V; they utilized all disaccharides and one hexose alcohol mannitol and one isolate was showed as biovar-I. The pathogenicity test under greenhouse conditions showed that 19 bacterial isolates were highly virulent and infected groundnut seedlings with impressive wilt symptoms. Therefore, on the basis of physiological, biochemical, morphological and pathogenicity test, all the isolates were recognized as *Ralstonia solanacearum*. Among sixty isolates, pathogenicity test confirmed only two highly pathogenic isolates.

CONCLUSION

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

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