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Isolation, purification and identification of bacterial wilt pathogen from rhizosphere of *Solanum melongena* of Hadoti region

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Abstract
Solanum melongena L. (Brinjal), quite common and popular vegetable crop grown extensively during warm weather in India and is a major source of income for the small and marginal farmers as well. The major constraint in the production of brinjal is the bacterial wilt disease caused by *Ralstonia solanacearum*. Crop rotation with non-host plants has not been effective, since *R. solanacearum* has its disseminating and survival stages in the soil. The race and strain diversity of the pathogen has made breeding for resistant cultivars ineffective. That's why study was done to isolate wilt causing bacteria *Ralstonia solanacearum* from the infected roots of Brinjal plant of Hadoti region and characterize the isolated bacteria through various methods. Initial cultures were grown by streaking on nutrient agar medium. After, bacterial wilt pathogen *Ralstonia solanacearum* was identified; it was confirmed by PCR (Polymerase chain reaction) amplification. Isolate of *R. solanacearum* were characterized by various DNA Markers.

Keywords: DNA markers, crop rotation, cultivars, bacterial wilt disease, rhizosphere

1. Introduction
Plants contribute carbon to support the growth of bacteria in the rhizosphere, while bacteria are capable of supplying plants with nitrogen, solubilizing phosphorus and other nutrients which can limit plant growth [1]. Bacteria are also important in controlling soil-borne pathogens that can decrease the capacity of roots to absorb water and nutrients [2, 3]. Bacterial wilt is a widely spread soil born bacterial disease of *Solanum melongena* (Brinjal) plant which occurs throughout of the world [4]. *Ralstonia solanacearum* is the disease causing agent of bacterial wilt, brown rot or southern wilt in Brinjal, Tomato, Tobacco and in few of ornamental plants and also moko disease of Banana [5]. It is highly diversified bacteria that are considered as a species complex and heterogeneous group of related strains of *Ralstonia solanacearum* [6]. Due to variability of *Ralstonia solanacearum*, it is very difficult to control bacterial wilt due to its diversity [7].
Bacterial wilt caused by *R. solanacearum* has been described on a wide range of hosts in many regions of the world [8]. In the absence of susceptible crops, alternative weed hosts and non-host plants play important roles for the survival of *R. solanacearum* strains [9]. *R. solanacearum* is responsible for causing lethal wilting disease in more than 200 plant species [10], while more than 450 plant species were listed as host plants for *R. solanacearum* including many important and economic crops [20]. Host range of *R. solanacearum* includes several hundred species representing 44 families of plants and many newly recognized hosts. *R. solanacearum* biovar 3 has been described on some woody perennial hosts including cashew (*Anacardium occidentale*) and custard apple (*Annona* spp.) [12].
The reason for these highly different and heterogeneous bacterial pathogen is not obvious; nevertheless it is assumed that specific pathogenic strains for certain hosts may have evolved only in certain parts of the world and are not found elsewhere or these hosts may only be susceptible where a number of environmental factors such as temperature, rainfall, soil type, inoculums and other soil biological factors are conductive to disease expression coincide [20]. There is an extensive literature on the disease, particularly in terms of host range, geographic distribution, occurrence of various biovars, strains and the ability to survive in soil and in association with plants debris and weed hosts [13].

Material and Methods

Diseases plant and rhizospheric soil samples from wilt affected brinjal plants showing characteristic features of bacterial wilt were collected from infected fields from various districts of Hadoti region, Rajasthan. Villages in Kota district were Arjunpura, Girdharpura, Sinta, Nanta, Borkhera, Bhadana, Nayagaon and in Bundi villages were Mayja, Kaperen, Lesarda, Khatkar and Talwas while in Baran, villages were Anta,Mangrol and Khanpur and in Jhalawar villages were Suket, Kheravad and Sunel. The isolated samples were put at 4 ºC.

Root with spot symptoms were washed with sterilized distilled water, and then treated with 0.5% solution of hypochlorite (bleach) for 1-2 min to remove the contaminants from the outside, rinsed with distilled water, and cut into small pieces with a scalpel. These pieces were immersed in sterilized saline buffer and then vortex strongly. A tenfold-dilution series was prepared and 100 µl of each dilution & the undiluted extract was spread on nutrient agar (NA) with three replications of each dilution [14]. Cultures were incubated for 3 to 5 days at 30 °C. Discrete colonies were re-streaked on the media plates for pure culture isolation. One colony of the purified bacteria from each sample was selected and maintained on nutrient agar slants at 4 °C for further tests [15] (Fig 1).

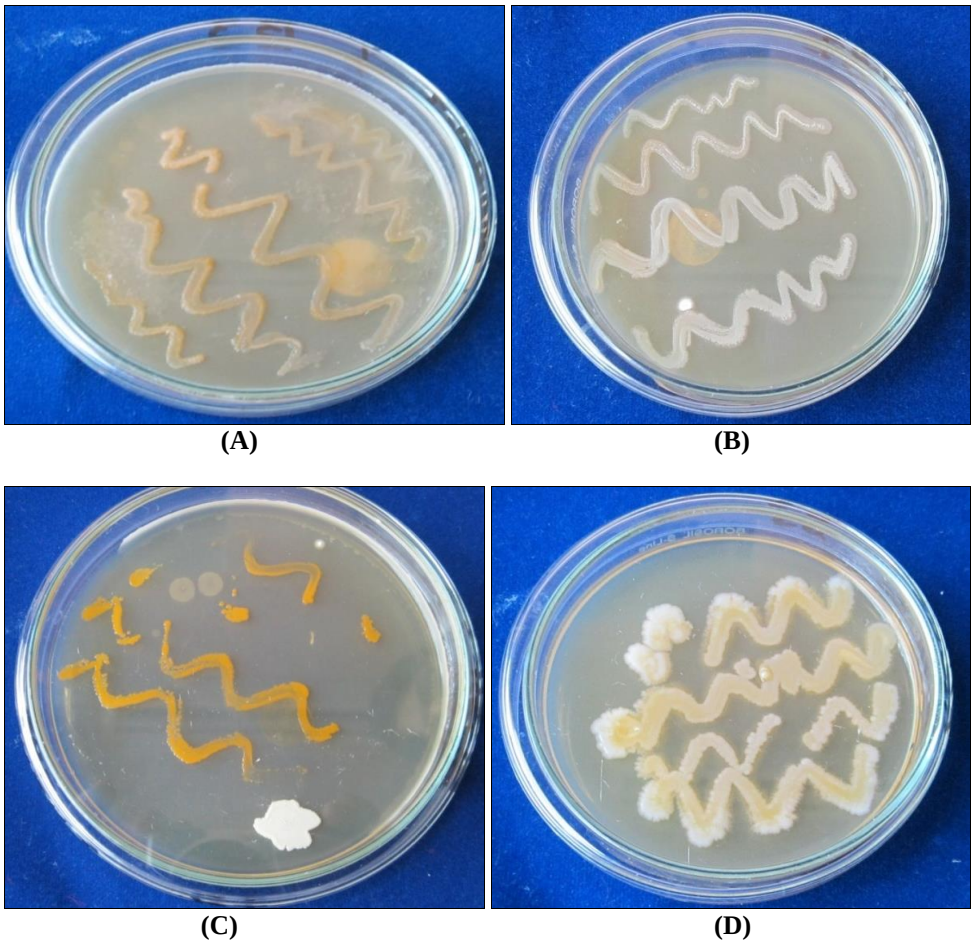

Fig 1: Culture of *R. solanacearum* on Agar plate (A) From Mangrol (B) From Khanpur (C) K. Patan (D) Lesarda

Fig 2: Cultural appearance of *Ralstonia solanacearum* on TZC media

Identification of virulent

The virulent colonies of *Ralstonia solanacearum* distinct with pink colour and characteristic red in center with whitish margin (Fig. 2) were streaked on TZC medium recognize as *Ralstonia solanacearum* [16].

Characterization of the Pathogen

Various biochemical test performed to characterize the *Ralstonia Solanacearum.* The formation of slime thread is an indication of Gram-negative. Isolates also appeared rod-shaped under the microscopic observation following simple staining mprocedure (Table 1).

Table 1: Primer sequence for characterization of *R. solanacearum.*

Universal Primer	Sequence of the primer	
OLI1	5'GGGGGTAGCTTGCTACCTGCC3'	~140 bp
Y1	5'CCCACTGCTGCCTCCCGTAGGAGT3')	~290 bp

Gram staining

Take the 14 loop full of the bacterial culture and spread on a slide fixed by the heating on a very low flame. After that aqueous crystal violet solution (0.5%) was spread on the smear for 30 seconds and then properly washed through water for 60 seconds [17]. Isolated strains showed yellowish creamish colour (Fig 1) gram staining test confirmed that *Ralstonia solanacearum* is gram negative in nature.

KOH (Potassium hydroxide) test

The isolate was further subjected to KOH test to confirm the identification of Gram-negative bacterial pathogen. The KOH test is a widly used method for identification between gram positive and gram negative bacteria. For that a drop of KOH was used on slide by using a Pasteur pipette. After that single colony of the plant pathogen was removed from the agar medium using a sterilize wire loop, Bacteria were mixed into KOH [18]. The formation of slime thread is an indication of Gram-negative. Isolate also appeared rod-shaped under the microscopic observation following simple staining procedure (Table 2).

Table 2: Cultural Characterization, Inoculum Density of *Ralstonia solanacearum.*

Field	Bacterial Streaming	Plating test	KOH test	Shape	Pathogenicity Test
Baran, Mangrol	+	Pink	+	Rod	+
Jhalawar, Khanpur	+	Pink	+	Rod	+
Bundi, K. patan	+	Pink	+	Rod	+
Bundi, Leserda	+	Pink	+	Rod	+

Streaming test

A presumptive test was carried out on infected Brinjal plant for diagnose the presence of *Ralstonia solanacearum.* Take root of infected brinjal plant and which were put in test tube having water, four isolates showing positive result during streaming test (Table 2).

Result and Discussion

Molecular characterization of pathogen of bacterial wilt

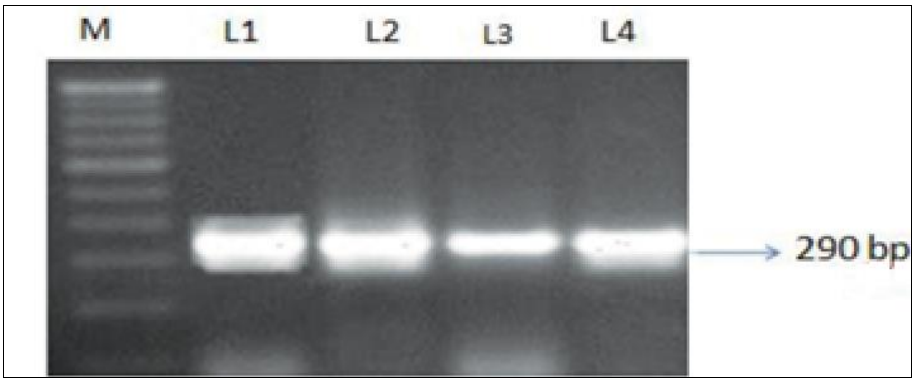

Fig 3: PCR amplified DNA from four isolates of *R. solanacearum* using the primer Y1

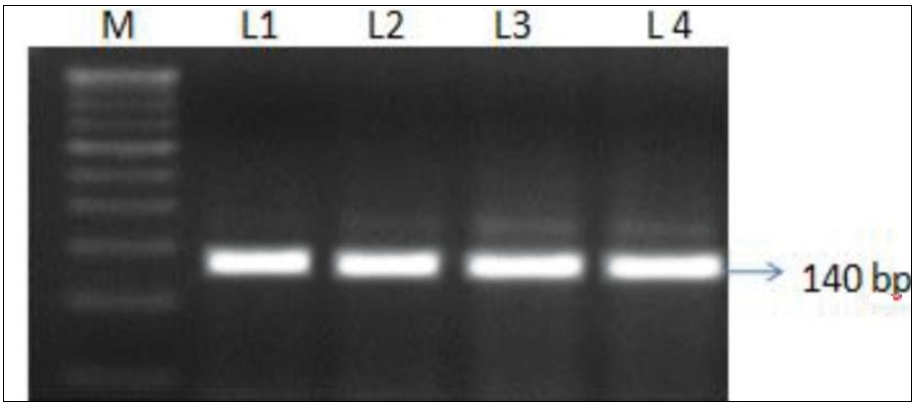

Fig 4: PCR amplified DNA from four isolates of *R. solanacearum* using the primer OL1

The bacterial wilt pathogen *Ralstonia solanacearum* was identified and confirmed by PCR (Polymerase chain reaction) amplification [15]. Isolate of *R. solanacearum* were characterized by various DNA Markers (Table 2). The

genomic DNA was subjected to PCR, the PCR amplification resulted (Fig 3) in a ~140 base pair (bp) product for (OLL1) and 290 bp product for Y1 from various isolates (Fig 4). These result confirmed that all bacterial isolates were *R. solanacearum*.

Author Contributions

Pooja Sharma designed and drafted the work. Neerja Shrivastava contributed in the final revision of the manuscript.

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Conflict of Interest

The authors declare that the research have no conflict of interest.

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