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Novel Methods for Investigating the Pathogenicity of *Ralstonia solanacearum* in Three- to Four-Leaf Stage Brinjal Seedlings

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Abstract: Bacterial wilt disease, caused by *Ralstonia solanacearum* (Smith), is a serious plant disease that is responsible for heavy losses to a large number of economic host plants it infects. Bacterial wilt of brinjal is a severe problem in Jharkhand state due to acidic soil. This study aimed to enhance our understanding of artificial inoculation methods in brinjal plants, focusing on their role in proving pathogenicity and virulence. Among all other methods, needle inoculation methods were found to be the most efficient. The needle inoculation method recorded significantly higher bacterial wilt incidence, recording 51.7%, 81.7%, and 81.7% at 7, 15, and 30 days after inoculation, respectively followed by soil inoculation method with wilt incidence of 63.3%, 73.3%, and 78.3 at 7, 15, and 30 days after inoculation (DAI). Wilt incidence recorded in the leaf clipping method of inoculation was comparatively less, i.e., 31.7%, 38.3%, and 48.3% at 7, 17, and 30 DAI. However, no wilt symptoms were recorded from the seed inoculation method at 7, 15, and 30 DAI. The needle inoculation method of artificial inoculation is seen to have a higher degree of wilting from 7 DAI to 30 DAI as compared to other artificial inoculation methods.

Keywords: artificial inoculation, bacterial wilt, brinjal, pathogenicity, *Ralstonia solanacearum*.

INTRODUCTION

Brinjal, or eggplant (*Solanum melongena* L.), is a significant solanaceous crop of the subtropics and tropics. The word "brinjal" is widespread in the Indian subcontinent and is derived from Arabic and Sanskrit, whereas the name "eggplant" has been obtained from the shape of the fruit of some types, which are white and resemble chicken eggs in shape. It is also called an aubergine (French term) in Europe. The brinjal is of significant importance in the warm parts of the Far East, being grown extensively in India, Bangladesh, Pakistan, China, and the Philippines. It is also popular in Egypt, France, Italy, and the United States. In India, it is one of the most frequent, popular, and major vegetable crops growing throughout the country, except at higher altitudes. It is a versatile crop suitable for a variety of agro-climatic areas and can be grown throughout the year. It is a perennial yet is produced economically as an annual crop. The fruits of brinjal are widely consumed in various culinary preparations and are a rich source of protective nutrients (Hedges and Lister, 2007). The land of the Jharkhand state is suitable for the cultivation of brinjal. Brinjal is now considered among the major vegetables grown in all seasons. It can be cultivated in all types of soil, like sandy soil, loamy soil, and

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the acidic soil of Jharkhand. In Jharkhand, the geographical area is 79.70 lakh ha, the cultivated area is 38 lakh ha, but the net sown area is 25-26 lakh ha. In vegetable production, the total area is 295.273 ha, and the total production is 4196.700 MT annual production. The brinjal cultivation area was 16.582 ha, with the productivity of 184.484 tonnes (Anon, 2023-24).

Bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi et al. (*Pseudomonas solanacearum* E.F. Smith) is the main constraint for its cultivation. In the late eighteenth century, bacterial wilt disease was identified on several solanaceous crops. It is a destructive bacterial disease distributed throughout the world, infecting more than 450 plant species of different botanical families (Kelman, 1953; Hayward, 1991), and limits production of many economically important crops such as brinjal, tomato, tobacco, potato, jute, banana, etc. (Kelman , 1954). The disease is especially relevant in many temperate zones where it causes economically substantial damage, particularly to brinjal, potato, and tomato crops. In some Asian nations, *Ralstonia solanacearum* may be responsible for an overall 75% decline in the brinjal crop. The bacterium may move between apparently healthy roots, and aerial transmission is feasible by rain splash. The bacteria can live for several years in some soil types and temperatures; however, viable cells disintegrate rapidly in some other environments. The wide host range enables the bacterium to survive within weed species, and it is commonly present in soil immediately after native vegetation has been cleared for crop development.

MATERIALS AND METHODS

Isolation, identification, purification, and preservation

Isolation was done from the infected brinjal plant showing typical symptoms. Plants having initial symptoms were also directly plated on Triphenyl Tetrazolium Chloride (TZC) medium. The isolate was made from fresh samples. The infected plant tissue pieces were washed with tap water and surface sterilized with 0.1 percent mercuric chloride ($HgCl_2$) solution for 30 seconds, followed by five subsequent washes with sterilized distilled water in aseptic conditions. The sterilized pieces were then transferred aseptically under laminar airflow onto sterilized Petri plates containing 20 ml of TZC medium. The soil sample from the infected field was also plated on Selective Medium South Africa (SMSA) medium by serial dilution. The Petri plates were incubated in a B.O.D. incubator at 30 ± 2°C. Temperature for 36-48 hours. The bacterial colony developing from the infected tissues was subcultured aseptically on TZC-containing Petri plates. To confirm the isolates of *R. solanacearum*, the pathogenicity test was performed on a 15-day-old healthy brinjal plant by the needle inoculation method. Multiple colonies of *R. solanacearum* showing virulent, irregular, and creamy white with a pink color at the center were collected in a conical flask, and a prepared bacterial cell suspension (10^8 CFU/ml) was made. Bacterial suspensions were injected into the node of the plant with the help of a hypodermic syringe. Injected plants were observed daily and continued up to five days after the plant showed symptoms. The isolates of *R. solanacearum* were preserved at -20°C for a long time (6 months to 1 year) and at 4°C for a short time (2-3 months) in a refrigerator for subsequent biochemical studies.

Morphological characterization of *Ralstonia solanacearum* isolates

Triphenyl Tetrazolium Chloride Agar (TTC) contains 5 g of glucose, 10 g of peptone, 1 g of casamino acid, 5 ml of a 1% stock solution of Triphenyl Tetrazolium Chloride, and 17 g of agar per liter. (Singh et al., 2015). We looked at solid medium colonies of *Ralstonia solanacearum*, usually visible after 36-48 hours

of incubation at 30 ± 2°C. This medium was developed to differentiate between the two types of colonies: virulent colonies appear as white colonies with a pink center, and non-virulent colonies appear as dark red colonies.

Root inoculation methods

The pathogenicity of *R. solanacearum* in brinjal at the early seedling stage has been effectively studied in this paper, with the specifics of a simple and effective methodology offered. The approach is very simple and suitable for examining the bacterial wilt (*Ralstonia solanacearum*). Take a four-leaf stage seedling and wash it properly. The root of the Swarn Shree brinjal variety was sequentially immersed in 70% (v/v) ethanol for three minutes and 1% sodium hypochlorite solution for three minutes. After that, samples were aseptically rinsed with sterile distilled water (Anith *et al*., 2015). After washing the proper root, make a small cut in the tertiary or secondary root of the seedling. After that, put the liquid suspension of bacteria deep in it for 18 hours. The potting mix (loam soil to vermicompost in a 2:1 w/w ratio) was prepared and autoclaved for 15 minutes at 121°C, and 1% sodium hypochlorite was used to disinfect the plastic pots and keep them in the greenhouse.

Soil inoculation methods

To prove the pathogenicity of the bacteria by soil inoculation techniques, the inoculum of the bacteria was prepared as per the method given by Mohamed *et al.* (2014). The inoculum was prepared by streaking bacteria on triphenyl tetrazolium chloride (TZC) medium plates, and then from a pure culture of bacteria, inoculated TZC media plates were flooded with sterile distilled water to remove the bacteria and collected in a conical flask, and shaken for 2 hours at 160 rpm. The inoculum was mixed with sterilized soil in a 1:5 ratio. Each earthen pot was filled with 3 kg of sterilized soil, and the inoculum mixture was applied at 200 ml/kg soil. The next steps of soil inoculation were carried out by sowing surface-sterilized 3 seeds in each pot on the 6th day of soil inoculation. Three replications of each treatment were maintained. Uninoculated soil in an earthen pot served as a control. Observation on pre-emergence mortality was taken on the 7th day of sowing, while post-emergence mortality was observed on the 21st day of sowing. The infected seeds and seedlings were subjected to re-isolation to confirm Koch's postulates.

Needle Inoculation Methods

The ICAR-RCER, FSRCHPR, Ranchi, provided the seeds for the Swarn Shree brinjal cultivar. The surface of the seeds of the Swarn Shree brinjal variety was sequentially immersed in 70% (v/v) ethanol for three minutes and 1% sodium hypochlorite solution for three minutes. After that, seeds were aseptically rinsed with sterile distilled water (Anith *et al*., 2015). The potting mix (loam soil to vermicompost in a 2:1 w/w ratio) was prepared and autoclaved for two hours at 121 degrees Celsius, and 1% sodium hypochlorite was used to disinfect the plastic pots. In a greenhouse, brinjal seeds were sown in potting mix-filled containers weighing around three kilograms. Every day, the seedlings received irrigation as needed. Bacteria grow on TZC media *R. solanacearum* isolates for 48 hours at 30 ± 2°C, then TZC media plates were flooded with sterile distilled water to remove the bacteria and collected in a conical flask, and shaken for 2 hours at 160 rpm. Seedlings are transplanted into sterilized plastic pots. After a few days, the seedlings are healthy with the artificially inoculated *R. solanaceae* bacteria. Bacteria are inoculated into a node on the healthy plant with the help of a 1 ml syringe and inoculated with a 5-10 microliter suspension. 10–15 days after bacteria inoculation, wilt symptoms occur.

Seed inoculation method

Seed inoculation was conducted by the methodology given by Hanumanthappa *et*

al. (2013). Five plastic pots were filled with sterilized soil, which was autoclaved at 15 lbs for 15 minutes. The brinjal seeds were subjected to surface sterilization utilizing a 0.1% $HgCl_2$ solution for one minute, followed by five successive washings with sterile distilled water to eliminate the toxic effects of mercuric chloride. The surface-sterilized seeds were immersed for 16 hours in a 24-36-hour-old 1 x 10^9 cell/ml bacterial cell suspension of the pathogen. A total of five seeds were placed within each pot. The surface-sterilized seeds that were only immersed in sterilized distilled water were checked as the control. Observations regarding the nature of symptoms were continuously recorded. The infected seeds were subjected to re-isolation to substantiate the pathogenicity of the bacterium.

Leaf-clipping method

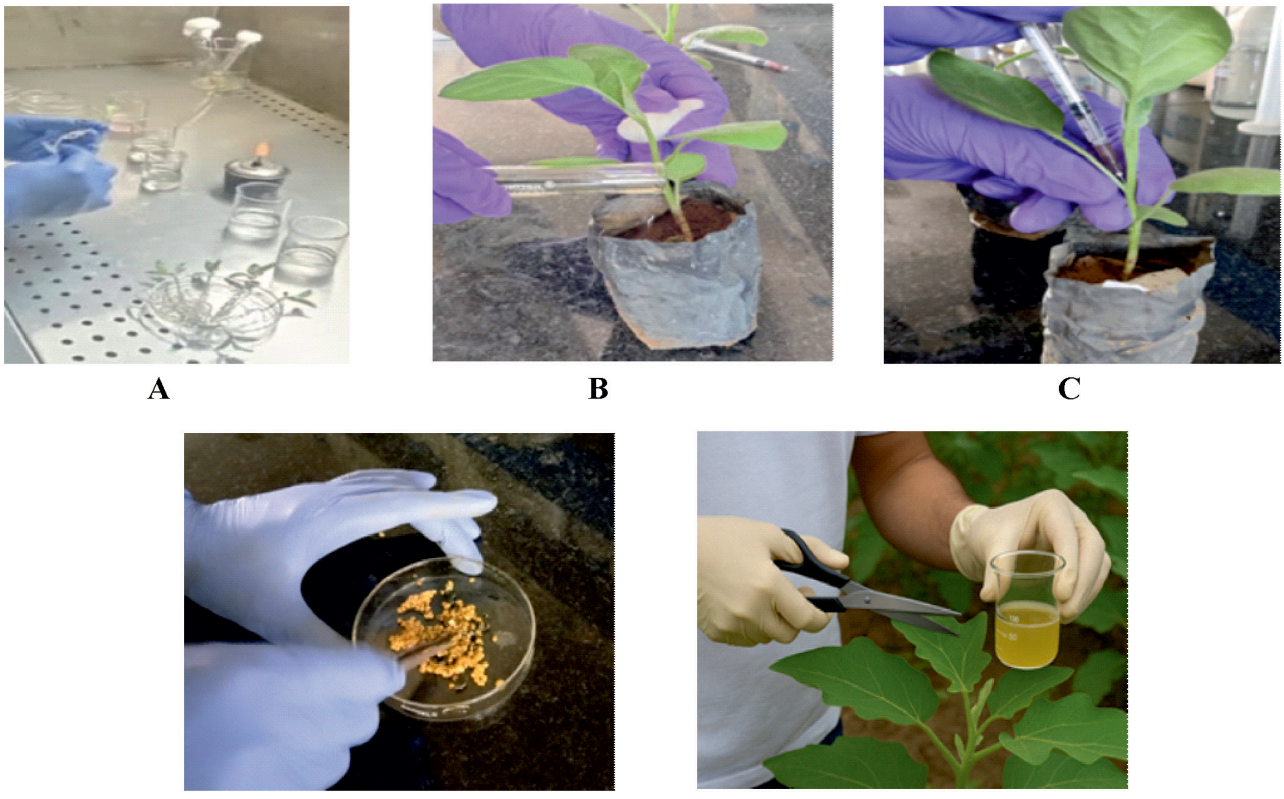
Sterile scissors are dipped into a bacterial liquid culture suspension of 1×10^8 CFU/ml. These scissors are then used to cut 5 to 6 leaves on brinjal plants that are 40 to 45 days old. This procedure is repeated with each plant to uniformly inoculate them and assess disease development.

The whole experiment was carried out under controlled conditions in three replications. Percent wilt incidence was recorded after 7, 15, and 30 days of inoculation (DAI).

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

RESULTS AND DISCUSSION

Brinjal plants grown were evaluated against bacterial wilt disease under a net house in plastic pots, and data on percent wilt incidence were recorded to carry out pathogenicity and confirmation tests of different inoculation



(Figures A to E show different methods of artificial inoculation: A- Root inoculation, B- Soil inoculation, C-Needle inoculation, D- Seed inoculation, and E- Leaf-clipping)

methods. The infected plant was cut from the stem with vascular discoloration using a sterilized sharp knife or blade. Milky white strands containing bacteria and extracellular polysaccharides streamed out from the cut of the xylem vessel.

Data on different artificial inoculation methods given in Table 1 showed that the needle inoculation method was found to be the most effective among all other methods. The needle inoculation method recorded significantly higher bacterial wilt incidence of 51.7%, 81.7%, and 81.7% at 7, 15, and 30 days after inoculation, respectively followed by soil inoculation method with wilt incidence of 63.3%, 73.3%, and 78.3% at 7, 15, and 30 days after inoculation and root inoculation methods with wilt incidence of 63.3%, 70.0%, and 75.0% at 7, 15, and 30 days after inoculation (DAI). Wilt incidence recorded in the leaf clipping method of inoculation was comparatively less, i.e., 31.6%, 38.3%, and 48.3% at 7, 17, and 30 DAI. However, no wilting was recorded from the seed inoculation method at 7, 15, and 30 DAI, so this method is less effective than other methods of artificial inoculation. The needle inoculation method of artificial inoculation is seen to have a higher degree of wilting from 7 to 30 DAI as compared to other artificial inoculation methods, where the seed inoculation method was the least effective, even after 7-30 DAI.

Table 1: Wilt incidence (%) in different inoculation methods

Sl. No.	*Inoculation methods*	*Wilt incidence (%)*		
		7DAI	*15 DAI*	*30 DAI*
1.	Root Inoculation Method	63.3	70.0	75.0
2.	Soil Inoculation Method	63.3	73.3	78.3
3.	Needle Inoculation Method	51.7	81.7	81.7
4.	Seed Inoculation Method	0	0	0
5.	Leaf-Clipping Method	31.7	38.3	48.3

The results also revealed that all methods of artificial inoculation produced bacterial wilt symptoms with variable and non-uniform patterns, except the needle inoculation method, which was shown to be the most reliable and approachable and produced uniform vascular bacterial wilt disease development. The disease mainly affected the brinjal plant, showing wilting with necrosis, and drooping leaves as symptoms. The best method of inoculation for screening tomato, brinjal, and chilli is the soil drenching method, which is less cumbersome, reliable, and effective, as it exactly simulates the natural infection, as the bacterium is soil-borne and enters the plant through roots under field conditions (Kelman and Sequeira, 1965; Schmit, 1978; Vasse *et al*., 1995., Kesharwani and Lakpale, 2021).

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