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ISOLATION, PURIFICATION AND IDENTIFICATION OF *RALSTONIA SOLANACEARUM* (SMITH) FROM GROUNDNUT

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MA & MZ designed the study & wrote the paper
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ABSTRACT

Groundnut bacterial wilt (BW) diseases is caused by *Ralstonia solanacearum* that occur in Pakistan's hot and humid areas. It is difficult to manage the bacterial wilt, since the pathogen is soil borne and has extensive host range. Resistance against this pathogen must be developed through the biotechnological approaches. It represents the main limitation in the worldwide production of solanaceous crops. As a first step, infected groundnut samples were collected from crops disease research institute (CDRI), NARC, Pakistan. Isolations were performed on triphenyl tetrazolium salt (TZC) medium. Samples were used for isolation. Bacteria with similar characteristics were isolated from all positive samples producing fluid which after incubation for 48 hours at 28°C on TZC medium where either entirely white or white with red center. Growth of *Ralstonia solanacearum* was observed from seeds plated on the TZC medium. For conformation of bacterial wilting causing the pathogen *Ralstonia solanacearum* standard isolated colonies were selected and purified. Bacterial cultures were multiplied and preserve in sterilized distilled water on TZC media. Virulence of an isolate can be determined on the basis of colony colour on this particular media was developed to differentiate between the two colony types. Virulent colonies are usually large, elevated, fluidal and either entirely white or with a pale red center and avirulent colonies appear dark red. On the basis of the characteristic's pathogen were found positive for gram staining, KOH test, Catalase test. Isolates were identified as *Ralstonia solanacearum*. Out of 05 samples, 2 were found positive and 3 were negative.

1. INTRODUCTION

Groundnut (*Arachis hypogaea* L.), is a one of the most important essential oil seed crops that play an important role in the economy of agricultural crops worldwide (Narda *et al.*,) [11]; (FAO) [6]. It is a highly source of nutritious contents such as 50% edible oil, 25% proteins and 10-25% carbohydrates (Christensen *et al.*,) [4]; (Shah *et al.*,) [12]. It is a dietary source of vitamin E, K, P, Ca, Mg, Fe, Zn, thiamine (B1) and riboflavin (B2).

This crop is also used as animal diet in the form of fodder, seeds, straw and hay (Smith) [13]. Groundnut is sown in different rainfed areas of Pakistan (Chakwal, Attock, Jhelum, Rawalpindi, Karak, Swabi, and Sahngar) on an area of 252928 acres with an average yield of 114700 tones and 1121 kg per hectare (Ahmed and Rahim) [1]; (Khan *et al.*,) [9]; (Naeem-ud-din *et al.*,) [10].

Yield of groundnut per acre is still low because of low rains, low inputs by the farmer with unprecedented environmental conditions and unavailability of high yielding varieties. Groundnut is

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basically a stifling plant and needs a lengthy and hot growing period with optimum rainfall (500 mm) and optimum temperature of 25 to 30ºC (Weiss) [14]. A well-drained sandy loam soils (pH 6.0-6.5) best thrives for groundnut and light soil support as simple pegs diffusion, their growth and harvesting (Basu and Ghosh) [2]. *Ralstonia solanacerum* had a very wide host range which including, *Arachis hypogaea* (groundnut), *Heliconia* spp., *Lycopersicon esculentum* (tomato), *Musa paradisiacal* (banana and plantain), *Nicotiana tabacum* (tobacco), *Solanum melongena* (aubergine) and *Solanum tuberosum* (potato) (Bradbury) [3].

A number of biotic factors include (bacteria, viruses, fungi and nematodes) and abiotic factors (temperature,

2. MATERIALS AND METHODS

Samples collection

Research work for isolation, purification and determination of host range of bacterial wilt was carried out at Crop Diseases Research Institute (CDRI) in National Agriculture Research Centre Islamabad (NARC). Groundnut seeds samples were taken from CDRI, NARC.

Isolation of bacteria by direct Plating method

In direct method, seeds were surface sterilized with 2% sodium hypochlorite solution for 60 seconds followed by washing with 70% ethanol and 2-3 washings with sterilized water and dried on sterilized blotter paper. The seeds were placed on the Petri plates containing TZC medium and incubated at 28°C for 24-48hrs.

Preparation of Media

The following Basal medium 2, 3, 5 triphenyl tetrazoliuin chloride (TZC) were used for the isolation of *Ralstonia solanacearum*.

Dextrose	5.0g
Peptone	5.0g
Casamino acid (Difo)	0.5g
Agar	9.0g
Water (Distilled)	500ml

After autoclaving 5 ml of a 1% stock solution of 2, 3, 5-tripheny tetrazolium chloride was added in medium before pouring.

Plating and storing

Twenty ml of TZC medium was poured in each petri plate. Keep 1-2 days before use to permit surface drying (long storage may result in poor bacteria growth). On solid media colonies of *R. solanacearum* usually are visible after 48-72 hours of incubation at 28 ºC. This media was developed to differentiate between the two colony types. Virulent colonies appear white with pink centers and non-virulent colonies appear dark red.

***Purification of Ralstonia solanacearum*:**

After the growth of different bacteria on plates streaking method was used to separate the bacterial colonies from the composite culture of bacteria as shown in Figure 1.

Streaking

On the basis of their unique shape, colour, and size the desired single bacterial colony was choosen. The technique of Streaking was replicated many times to get the pure colonies.

- After the spirit lamp flame became red hot, the streaking loop was allowed to cool down.
- The sterilized streaking loop was used to capture bacterial colonies.
- Using a back-and-forth motion, the circle holding the bacterial colony was progressively streaked across a quarter of the petri dish.
- On the flame, loop was heated again and let it cool after the first streak.
- The same process was repeated again at an angle of 90^0 from the edge of the second stripe.

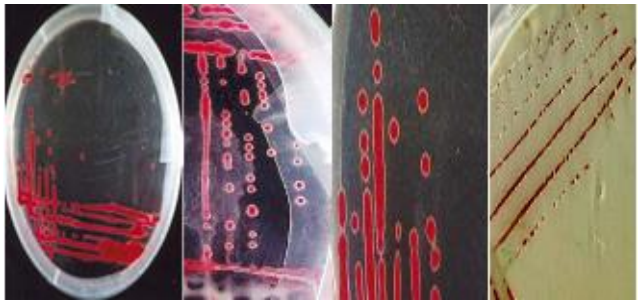

Figure-1. Isolation and confirmation of *Ralstonia solanacearum* from infected groundnut.

Biochemical Test:

Characterization of the pathogen:

Different biochemical tests were performed to characterize *Ralstonia solanacearum*.

a. Gram staining:

A loop full of the bacterium was spread on a glass slide and fixed by heating on a very low flame. Aqueous crystal violet solution (0.5%) was spread over the smear for 1 minute and then washed with running tape water for one minute. It was then flooded with iodine for one minute, rinsed in tape

water and decolorized with 95% ethanol for 15 seconds. After washing the specimen was counter-stained with safranin for approximately 30 seconds to 1 minute, washed with water, dried and observed microscopically at 100X (oil emulsion lens) using oil.

b. Potassium hydroxide test:
Bacteria were aseptically removed from Petri plates with a tooth pick or an inoculating wire loop, placed on glass slide in a drop of 3% KOH solution, stirred for 30 seconds and observed for the formation of slime threads.

c. Catalase oxidase test:
Young agar cultures (18-24 hrs) and 3% hydrogen peroxide (H_2O_2) were used to observe production of gas bubbles. A loop full of bacterial culture was mixed with a drop of H_2O_2 on a glass slide and observed for the production of gas bubbles with naked eye and under a dissecting magnification of 25X.

3. RESULTS AND DISCUSSION

Isolation
A typical of *Ralstonia solanacearum* growth was observed from seeds plated on TZC medium. Typical isolated colonies were picked and purified for confirmation of bacterial wilt causing pathogen *Ralstonia solanacearum*. Bacterial cultures were multiplied on TZC media and stored in sterilize distilled water. Virulence of an isolate can be determined on the basis of colony colour on this particular media. Virulent wild type colonies are usually large, elevated, fluidal and either entirely white or with a pale red center; avirulent mutant colonies were butyrous, deep-red often with a bluish border (French *et al.,)* [7].

Microscopic observation
Gram staining
Bacteria retaining reddish pink colony color showed that these are gram negative (G- ve), while gram-positive (G+ ve) bacteria stained violet blue. All 5 isolates were gram –ve.

Biochemical test:
KOH test:
All isolates found gram-negative by gram staining gave a positive KOH loop test. Slime threads were formed when fresh bacterial cultures (24-48hrs old) were mixed with 3% KOH solution. Formation of slime threads or loop is positive test and is indication of being gram-negative because gram negative bacteria have relatively fragile cell walls which are bounded by an outer membrane (Fig 2).

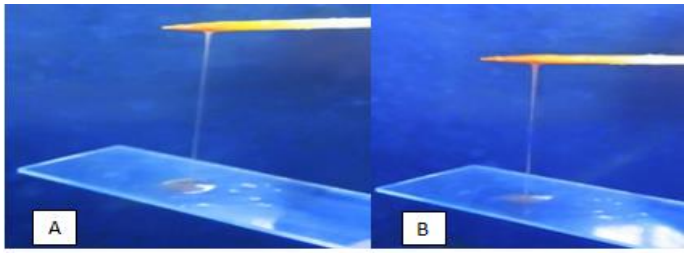

Figure-2. *Ralstonia solanacearum* are looping by KOH test: Fig A and B

Catalase oxidase test

All aerobic bacteria produce gas bubbles when these are mixed with a drop 10 % of H_2O_2 on glass slide. Production of gas bubbles gives a clue for presence of aerobic and facultative anaerobic bacteria. In my studies all the isolates tested produced gas bubbles during these tests (Plate), indicating that these might be *Ralstonia solanacearum.* Catalase is a hemi enzyme capable of decomposing hydrogen peroxide to water (H_2O) and oxygen (O_2) gas (Fig 3).

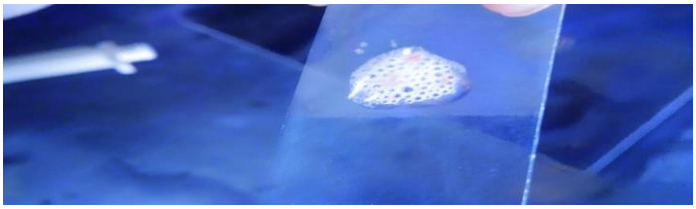

Figure-3. *Ralstonia solanacerum* producing bubbles during catalase test:

Table-1. Gram staining, Catalase oxidase test and Potassium hydroxide test for confirmation of *Ralstonia solanacearum*.

Sr. No	Isolate	Gram staining	Catalase test	KOH test
1.	S 1	-	+	+
2.	S 2	-	+	+
3.	S 3	-	+	+
4.	S 4	-	+	+
5.	S 5	-	+	+

4. CONCLUSION

Ralstonia solanacearum growth was observed from seeds plated on triphenyl tetrazoliuin chloride (TZC) medium. Typical isolated colonies were picked and purified for confirmation of bacterial wilt causing pathogen. Virulence of an isolate can be determined on the basis of colony colour on this particular media. Virulent wild type colonies are usually large, elevated, fluidal and either entirely white or with a

pale red center; avirulent mutant colonies were butyrous, deep-red often with a bluish border. On the basis of the characteristic's pathogen were found positive for gram staining, KOH test, Catalase test. Isolates were identified as Ralstonia solanacearum. Out of 05 samples, 2 were found positive and 3 were negative.

5. CONFLICT OF INTEREST

All authors have declared that there is no conflict of interests regarding the publication of this article.

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