

Isolation And Identification of Seed Borne *Ralstonia Solanacearum* from Tomato And Brinjal in Bangladesh

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Abstract: Altogether 11 seed samples each of tomato and brinjal of different varieties were collected from different sources viz. government, private and farmer's level of Bangladesh for evaluating seed borne nature of *Ralstonia solanacearum*. A seed borne bacteria *R. solanacearum* varied from 14.00 to 77.00% in tomato and 21.00 to 93.00% in brinjal seeds on Nutrient Agar. Total 165 bacterial isolates were collected from different seed samples of tomato and brinjal. The pathogenic isolates of all groups of bacteria produced pink or light red color colonies or colonies with characteristics red center and whitish margin on Triphenyl Tetrazolium Chloride (TTC) medium indicated bacterial isolates were virulent and might be *R. solanacearum* that later on confirmed by biochemical tests. Post-emergence death, seedling mortality and chlorosis/necrosis were observed by pathogenicity and hypersensitivity tests and the isolates were identified as *R. solanacearum*. After harvest, seed to plant to seed transmission of *R. solanacearum* was confirmed by using plate method, Liquid assay method, Cassette holder method, Tissue planting method, Seedling symptom and Growing on test. 16.00% harvested seeds of tomato and 26.00% harvested seeds of brinjal were found transmitted with *Ralstonia* on Nutrient agar. Growing on test resulted 14.00% and 8.00% wilted seedlings caused by *R. solanacearum* in tomato and brinjal were found affected respectively.

Keywords: Seed borne, *Ralstonia solanacearum*, tomato, brinjal and Bangladesh

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I. Introduction

Tomato (*Lycopersicon esculentum* Mill), a member of the family Solanaceae, is widely grown in almost all the countries of the world due to its adaptability to wide range of soil and climate. Tomato was grown in 30608.12 hectares of land of Bangladesh, the production being 4,13,610 metric tons in 2014-2015 [1]. Brinjal (*Solanum melongena* L.) belongs to the family Solanaceae and it is Bangladesh's third most important vegetable in terms of both yield and area cultivated which is only surpassed by potatoes and onions [2]. Total acreage of brinjal is 18479.35 hectares in Kharif and 30919.03 hectares in Rabi and production is 13,9,792 metric tons in Kharif and 3,10,354 metric tons in Rabi season [1]. The disease causes more than 19% yield reduction in brinjal in this country [3]. Over 200 diseases have been reported to affect the tomato plants in the world [4]. There are many factors responsible for the low yield of vegetable and among these seed borne pathogens play a vital role. Sowing diseased seeds can reduce germination, vigour and potential yield by transmitting pathogen from seed to plants. The most adverse effect of seed-borne pathogen is contaminating disease free areas. Thus seed-borne pathogens act as a primary source of inoculum for disease development. Its present a serious threat to seedling establishment, close association with seeds facilitates the long term survival, introduction into new areas and widespread dissemination of pathogens [5]. Bacterial wilt (*Ralstonia solanacearum*) is a very destructive disease of the solanaceous family, resulting in some cases complete loss of the crop [6, 7]. In crops such as tomato and eggplant, the pathogen is carried in seed [8, 9]. The seed-borne nature of this pathogen in chilli has been established by Umesha [10]. Seed transmission of the pathogen is of significance for seed trade and in quarantine inspection for seed exchange. In this study, isolation, identification and transmission of *Ralstonia solanacearum* from seed to plant to seed in tomato and brinjal seeds available in Bangladesh were studied.

II. Materials And Methods

The present investigation was carried out in the Seed Pathology Centre (SPC), Eco-friendly Plant Disease Management Laboratory and Molecular Plant Pathology Laboratory of the Bangladesh Agricultural University (BAU), Mymensingh.

2.1 Collection of seed samples

11 seed samples of tomato (Novelty hybrid, Digonta, Utsab, Udayan F1, Ratan, RomaVF -Lalteer, Roma VF- Metal, Marglove, Roma VF- Khrishan Agro, Roma VF- Pashapashi and Bina Tomato – 5) and 11

seed samples of brinjal (Chalanger eggplant F1, ACI beguni, Uttara, Kranti, Shingnath, Khatkhatia, Kata begun, Laffa, Zhumki, Kaikka nandina and Islampuri) were collected from different seed companies, Research Institute, seed agency and seed stores in Bangladesh.

2.2 Incidence and identification of seed borne bacteria

All seed samples of tomato and brinjal were studied on Nutrient Agar plate method, Liquid assay method and Triphenyl Tetrazolium Chloride (TTC) test [11] to find the incidence of *Ralstonia solanacearum* in seed samples as well as to isolate and characterize the bacterium of *R. solanacearum*. Typical bacterial colonies isolated from seeds on Nutrient Agar (NA) medium after 48 hrs of incubation at 27 °C were transferred on TTC medium to check the virulence and characterization of pathogens. The bacterium from nutrient agar plates were subjected to various tests namely Potato soft rot test, KOH solubility test, Levan test, Kovac's oxidase test [12], Catalase oxidase test, Sugar utilization test, Fluorescent pigment production test, Pathogenicity test and tobacco hypersensitivity test. For all the tests, 24-48 hrs old cultures [13] and bacterial suspensions [14] were used.

2.3 Pathogenicity test of isolated Ralstonia solanacearum

2.3.1 Seed dipping method

Surface sterilized seeds of a susceptible tomato and brinjal seed samples were soaked in a suspension (10^7 cells/ml) of 24 hrs bacterial culture growth on NA media (1:1) for 1 hour. The soaked seeds were then sown in sand culture in trays (100 Seeds/tray). Then the tray were placed in Net house and kept for at least 14 days for the appearance of symptom.

2.3.2 Root dipping method

Tomato and brinjal seedlings were grown on sterile sand and uprooted carefully at the 3 – 4 leaves stage and the root were cleaned thoroughly in running tap water. The tips of the roots were surgically removed to create an injury and the roots were then dipped for 1 hr in the culture suspension of bacteria (10^7 cells/ml). The seedlings were then transplanted to sterile soil in the pot. Five seedlings were transplanted in each pot and the seedlings were monitored routinely.

2.4 Hypersensitivity response (HR) test

To determine the pathogenic nature of the isolated *R. solanacearum* isolates, hypersensitive reaction was studied on tobacco (*Nicotiana rustica* var. MT 95) plants by infiltration of bacterial suspension into the intracellular space of the tobacco leaves [15]. Tobacco (30 days old) plants were used to inject bacterial suspension (10^7 cfu/ml) of each isolate. Bacterial suspension was injected into the intracellular space of the leaf with a hypodermal syringe. Hypersensitive response was observed daily and continued up to 5 days of infiltration till the appearance of the symptoms.

2.5 Testing of seed to plant to seed transmission of R. solanacearum collected from Tomato and Brinjal seed samples

Harvested seeds of naturally infected seed samples of tomato and brinjal (obtained from first year experiment) were tested using Nutrient agar plate method, Liquid assay method, Slide cassette holder method, Sectioning of diseased seedling, Tissue planting method and seedling symptom test (Pot experiment).

III. Results and Discussion

3.1 Incidence of bacteria (Nutrient Agar Plate Method)

Seed borne bacteria associated with tomato and brinjal seed samples of different varieties collected from different sources are presented in Table 1. In case of tomato seed samples, the highest bacterial infection by 77.00% was recorded in TS11 (Bina tomato-5) and the lowest (14.00%) was counted in TS6 (Roma VF). In case of brinjal seed samples, highest percent seeds infected with bacteria (93.00%) was found in BS10 (Kaikka nandina) followed by BS7 (Kata begun) (88.50%) and the lowest percent seeds infected with bacteria (21.00%) was found in BS1 (chalanger eggplant F1). Bacterial cell was grown on Nutrient Agar media (Fig. 1). Many scientists were followed NA plate method for detection of bacteria [16]. Researcher of Egypt reported that the prevalence of seed borne bacterial pathogens in imported tomato seed lots of different cultivars in Egypt [17]. He used liquid assay method for detection of the bacteria, and seed extracts were plated on different semi selective media. *Pseudomonas corrugate* and *xanthomonas campestris* pv. *Vesicatoria* were detected in 14.7% and 12.0% of the seed samples tested respectively. Another scientists [18] reported that any level of seed contamination contributed to initial infections and one infected seed in 10,000 was sufficient to cause bacterial disease epidemics in dry beans, while one percent seed infection in tomato transplants caused 100% *Clavibacter michiganensis* sub sp *michiganensis* infection [19].

Table 1: % infection of seed borne *R. solanacearum* of collected tomato and brinjal seed samples (Nutrient Agar plate method)

Tomato seed samples		Brinjal seed samples	
Variety	% seed infection	Variety	% seed infection
TS1 (Novelty hybrid)	41.00 d	BS1 (Chalanger eggplant F1)	21.00 h
TS (Digonta)	34.00 e	BS2 (ACI beguni)	72.50 e
TS3 (Utsab)	39.00 d	BS3 (Uttara)	84.00 c
TS4 (Udayan F1)	41.00 d	BS4 (Kranti)	48.00 g
TS5 (Ratan)	70.00 b	BS5 (Shingnath)	56.00 f
TS6 (RomaVF)	33.50 e	BS6 (Khatkhatia)	84.50 bc
TS7 (Roma VF)	56.00 c	BS7 (Kata begun)	88.50 b
TS8 (Marglove)	42.00 d	BS8 (Laffa BAU)	76.00 de
TS9 (Roma VF)	14.00 f	BS9 (Zhumki)	78.00 d
TS10 (Roma VF)	71.50 b	BS10 (Kaikka nandina)	93.00 a
TS11 (Bina tomato-5)	77.00 a	BS11 (Islampuri)	83.50 c

Values within the same column having common letter (s) do not differ significantly at 5% level of significance

3.2 Isolation and Identification of the bacterial isolates

A total of 165 isolates of bacteria were collected from the 11 seed samples of tomato and 11 seed samples of brinjal of different varieties collected from different sources. Isolation was done by liquid assay method. A total of 44 isolates of *Ralstonia solanacearum* were isolated from infected potato, tomato and wild species of potato [20]. The isolates obtained from the different seed samples of tomato and brinjal of different varieties were tested for potato soft rot test. Pectolytic activity in potato tubers has been recorded which is supported by different researches [21,22 and 23]. The isolates of all groups which gave positive reaction in potato soft rot test were tested for characterization. The pathogenic isolates of all groups of bacteria collected from different sources of tomato and brinjal seed samples of different varieties produced pink or light red color colonies or colonies with characteristics red center and whitish margin on TTC medium (Fig. 1). *Ralstonia solanacearum* produced fluidal colonies with pink or light red color on TTC media after 24 hours of inoculation [21,24,25 and 26]. These indicated that all of the groups of bacterial isolates were virulent and these were *Ralstonia solanacearum* causal organism of bacterial wilt of tomato and brinjal. Avirulent colony types of *Ralstonia solanacearum* could be easily differentiated by the pigmentation from the wild virulent types of *Ralstonia solanacearum* [11]. Bacteria with similar characteristics were isolated from all positive samples were found positive for KOH test, Kovac's oxidase test, Catalase test, Oxidation/fermentation of glucose and negative for Levan test and production of fluorescent pigment (Fig. 1). By these biochemical tests confirmed that isolated bacterial isolates were *R. solanacearum* (Table 2).

The isolates of *Ralstonia solanacearum* collected from the different seed samples of tomato and brinjal were tested for hypersensitive response in tobacco cv. MT 95. Slight localized chlorosis followed by necrosis and collapse of whole tissue was evident in HR positive isolates. The result showed that all the tested isolates were able to induce HR (death of local cell of tissues) between veins of tobacco leaves. Tobacco leaves infiltrated with bacterial suspension induced a distinct yellow zone at the spreading edge of the lesion (Fig. 1). Tobacco leaves infiltrated with sterile water were unaffected. It has been shown that many but not all pathogenic bacteria can induce hypersensitivity necrosis in leaves of tobacco or other non-host plants [15]. Since only the phytopathogenic but not the saprophytic bacteria have this property, the tobacco test is ideal for quick detection of the pathogenicity of the bacterium in question [15,27]. The reaction of tobacco leaves to all groups of *Ralstonia solanacearum* isolates under study was presented in Table 2.

Table 2: Biochemical test of *Ralstonia solanacearum* of different isolated groups of tomato and brinjal seed samples

Tests		Reaction	Inference
Colour test on TTC media		+	<i>Ralstonia solanacearum</i>
Kovac's oxidase test		+	<i>Ralstonia solanacearum</i>
Temperature sensitivity test (°C)	27°C	+	<i>Ralstonia solanacearum</i>
	37°C	-	<i>Ralstonia solanacearum</i>
	41°C	-	<i>Ralstonia solanacearum</i>
Levan test		-	<i>Ralstonia solanacearum</i>
Production of fluorescent pigment		-	<i>Ralstonia solanacearum</i>
Catalase test		+	<i>Ralstonia solanacearum</i>
HR test		+	<i>Ralstonia solanacearum</i>
Sugar fermentation test	Dextrose	+	<i>Ralstonia solanacearum</i>
	Sucrose	+	<i>Ralstonia solanacearum</i>
	Galactose	+	<i>Ralstonia solanacearum</i>
	Lactose	+	<i>Ralstonia solanacearum</i>

The findings of the pathogenicity test of seed dipping method and root dipping method are presented in Table 3. In seed dipping method mean germination 67.58% and 70.28%, mean seedling stand after 14 days 62.90% and 65.00%, mean seedling mortality 4.68% and 5.28% were found in tomato and brinjal seed samples of different varieties respectively. In root dipping method, seedlings showed browning and rotting of plumule and cotyledonary leaves within 3 days after inoculation and ultimately showed mortality (Fig. 1). Mean mortality was calculated by 58.18% and 23.64% from tomato and brinjal seed samples respectively.

Table 3: Pathogenicity test of different isolates of *Ralstonia solanacearum* collected from different seed samples of tomato and brinjal (Seed dipping and Root dipping method)

Crop	Seed dipping method			Root dipping method
	Germination (%)	Seedling stand at 14 days (%)	Mortality (%)	Mortality (%)
Tomato	67.58	62.90	4.68	58.18
Brinjal	70.28	65.00	5.28	23.64
T value	NS	NS	NS	17.645

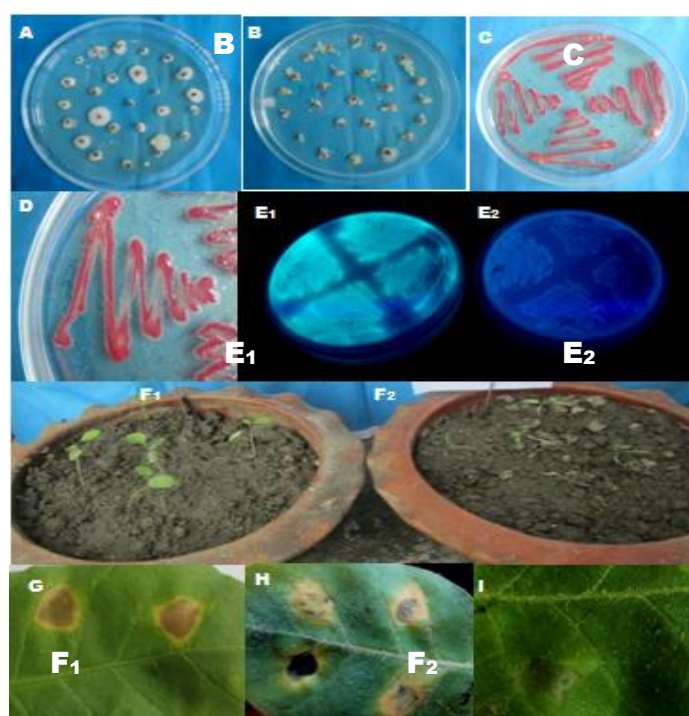


Fig. 1: Bacterial growth around the seeds (A: Tomato and B: Brinjal) on Nutrient Agar medium, Virulent colonies of bacterial isolates collected from different seed samples of tomato and brinjal on TTC media(C-D), Production of fluorescent pigment under UV light in the plate inoculated with collected bacterial isolates for 48 hrs at 28-30°C (E₁: green diffusible fluorescent pigment was evident in fluorescent strains and E₂: brown diffusible pigments which are the characteristic of *Ralstonia solanacearum* strains), Root dipping method for pathogenicity test of seedlings (F₁: Control and F₂: Inoculated), HR test: Tobacco leaves (*Nicotiana rustica* var. MT 95)) inoculated with *Ralstonia solanacearum* (G and H) and Control- Infiltrated with sterile water (I)

3.3 Seed to plant to seed transmission of *Ralstonia solanacearum*

3.3.1 Nutrient agar plate method

Seed borne nature of *R. solanacearum* was studied on Nutrient agar plate method where bacterial oozing around the seed on nutrient agar was found. Harvested seeds of tomato were found 16.00% seed infection and 26.00% found in harvested seeds of brinjal (Fig. 2). Transferred bacterial cell produced pink or light red colour colonies or colonies with characteristics red center and whitish margin on TTC medium (Fig. 4).

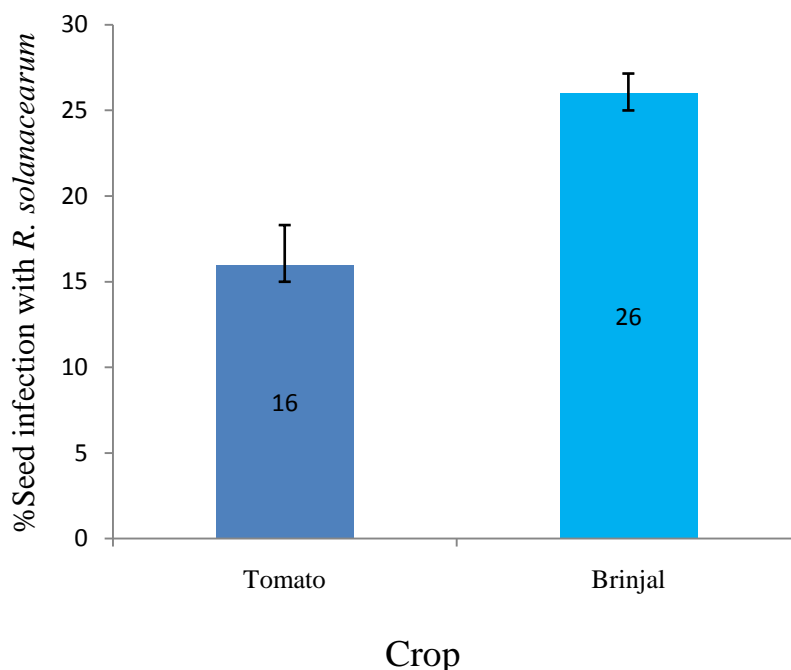


Fig. 2: Seed borne nature of *Ralstonia solanacearum* (% seeds infected with *R. solanacearum*) of tomato and brinjal seed samples (Nutrient Agar plate method)

3.3.2 Liquid assay method

Harvested seeds of tomato and brinjal were tested in Liquid assay method for studying transmission of *Ralstonia solanacearum* from seed to plant to seed. Isolated bacteria were able to produce pink or light red colour colonies or colonies with characteristics red center and whitish margin on TTC medium (Fig. 4). *R. solanacearum* was also isolated from infected plant material and seeds of Chilli [10].

3.3.3 Slide cassette holder method

Transmission of *R. solanacearum* from seed to seedlings of tomato and brinjal was found in slide cassette holder method. Harvested seeds of tomato and brinjal resulted different percent germination categories (normal seedlings, abnormal seedlings, diseased seedlings and dead seed) were counted in cassette holder method. Diseased seedling by 5.00% was found in harvested seeds of tomato and 2.00% was found in harvested seeds of brinjal (Fig. 3).

3.3.4 Test of seed borne nature of *R. solanacearum* causing wilt of tomato and brinjal following seedling symptom test (Pot experiment)

In this test, germination (86.00%) and wilted seedlings (14.00%) were recorded from harvested seeds of tomato as well as 72.00% germination and 8.00% wilted seedlings was recorded from harvested seeds of brinjal (Fig. 3). The growth of *R. solanacearum* from tissue of infected seedlings on TTC media is shown in Fig. 4. Plants raised from naturally infected seeds under greenhouse conditions also showed wilt symptoms [28].

3.3.5 Sectioning from diseased seedling

Stem of diseased seedlings were sectioned and prepared slide on glycerine and observed under compound microscope showed that the bacterial cells come out from the plant cell. This bacterial cell was able to produce pink or light red colour colonies or colonies with characteristics red center and whitish margin when streaked on TTC medium (Fig. 3).

3.3.6 Tissue planting method

The plant tissue of diseased seedlings produced bacterial colony of *R. solanacearum* on TTC (Fig. 3). Bacteria grew around the pieces of plant tissues on TTC, while bacterial colony was not found from the tissues of healthy seedlings.

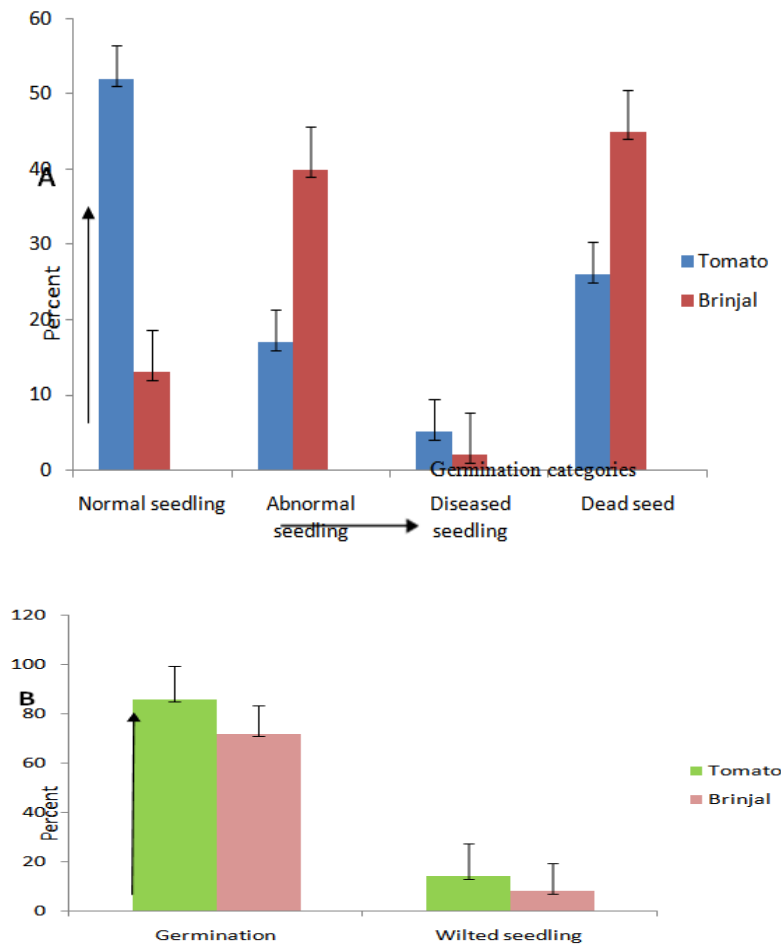


Fig. 3: Test of seed borne nature of *R. solanacearum* causing wilt of tomato and brinjal following seedling symptom test: Cassette holder method (A) and Pot experiment (B)

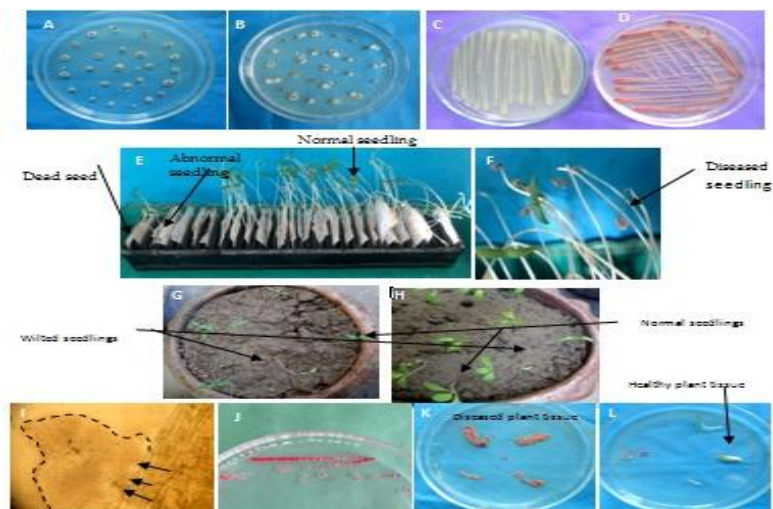


Fig. 4: Seed borne bacteria grew on harvested tomato seeds (A) and harvested brinjal seeds (B); Isolated bacteria of *Ralstonia solanacearum* on Nutrient Agar produced off-white coloured bacterial conoly (C) and on TTC medium produced colonies with characteristics red center and whitish margin (D); Seed borne nature of *R. solanacearum* causing wilt of tomato and brinjal following seedling symptom test (E-F: Cassette holder method), Seedlings showing wilting symptom of tomato (G) and brinjal (H); Outing of bacterial cells of *R. solanacearum* observed under compound microscope and growth on TTC media (I-J), Bacterial cell of *R. solanacearum* growth from tissue of diseased seedlings on TTC media (K) and no growth from healthy plant tissue(L)

IV. Conclusion

Seed borne bacteria of collected seed samples varied from 33.50 to 77.00% in tomato and 21.00 to 93.00% in brinjal on NA. A total of 165 isolates of bacteria were collected from seed samples of different varieties of tomato and brinjal. Biochemical tests confirmed that isolated bacterial isolates were *R. solanacearum*. In addition, seed to plant to seed transmission of *R. solanacearum* collected from different seed samples of tomato and brinjal seeds of different varieties collected from different sources was established.

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