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INCIDENCE AND COLONISATION OF *RALSTONIA SOLANACEARUM* (SMITH) YABUUCHI *ET AL.* IN BRINJAL (*SOLANUM MELONGENA* L.) SEEDS IN RAJASTHAN, INDIA

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Total seventy five seed samples of brinjal (*Solanum melongena* L.) collected from major areas of Jaipur districts of Rajasthan revealed 10-100% incidence of *Ralstonia solanacearum* (RS) on semi-selective TZC Agar medium. For the study all the seed samples were categorised into asymptomatic, shrivelled discoloured and heavily discoloured seeds. Dry seed examination of these samples revealed asymptomatic (07.75-97.5%), shrivelled discoloured (04.50-67.50%) and heavily discoloured (03.25-34.75%) seeds. Two naturally infected seed samples of brinjal carrying 100% incidence of RS were selected for histopathological studies. The heavily infected seeds were with or without epidermal appendages and with water-soaked symptoms. On bisecting such seeds the embryo and endosperm showed necrosis and browning. The pathogen was found confined to the outer seed coat layer particularly at funiculus in the asymptomatic seeds. In shrivelled discoloured seeds of brinjal it was found in seed coat, space in between seed coat and endosperm. It colonised in all the seed components including embryo and endosperm in heavily discoloured seeds. The pathogen caused necrosis, formation of lytic cavities, reduction in cell contents and aggregation of the bacterial cells. The pathogen was found extra- as well as intra embryonal.

Keywords: Discoloured seeds; Histopathology; Incidence; *Ralstonia solanacearum.*

Introduction

Bacterial wilt of brinjal caused by *Ralstonia solanacearum* (Smith) (Yabuuchi *et al*., 1995) previously known as *Pseudomonas solanacearum*, has been reported from various part of world[1-5] including India[6,7]. The disease is widespread in tropical, subtropical and warm temperate brinjal growing regions of world. It has been reported from Switzerland[2], London[1] and Africa[4,5]. In India, the disease has been reported in all the major growing areas including Guwahati[7] and Pantnagar[6]. In the present study incidence of the pathogen in seed grown in Rajasthan state and transmission of seed-borne inoculums from seed to plant were studied. The pathogen has been reported to be seed-borne in brinjal[1,2,8]. The characteristic symptoms of the disease are wilting, stunting and yellowing of the foliage followed by collapse of the entire plant.

Materials and methods

Total seventy five seed samples of brinjal collected from major area of Jaipur district of Rajasthan were subjected to dry seed examination, standard blotter method[9] and TZC agar medium plate method[10]. In dry seed examination, seed samples were categorised into asymptomatic, shrivelled discolouredand heavily discoloured seeds. All the seed samples were incubated on TZC agar medium to record the per cent incidence of the pathogen in the seed samples. The isolates of the bacterium were subjected to confirmative tests for identification[11,12]. The degree of discolouration, sign, shape, size, outgrowths on seed surface was studied.

All the collected seed samples of brinjal were studied by dry seed examination, incubation on moistened blotters[9] and TZC agar plate method to find the incidence of *Ralstonia solanacearum* in brinjal seed samples. The culture were maintained on nutrient agar (NA) and pure colonies after 72 h of incubation at 30^{o}C, bacterial were subjected to various tests namely gram's staining, KOH solubility test, levan formation, oxidase test[13, 14], potato soft rot test, nitrate reductase test[15], arginine dihydrolysis, gelatin hydrolysis test, hypersensitivity test in tobacco and pathogenecity tests for the identification of the bacterial species. For all the tests 24-48 h old bacterial isolates in the forms of culture or suspension identified by various methods (as described above) were subjected to pathogenecity tests on the host plant and other plant species[11,12,15-17].

The pure bacterial culture from NA medium was diluted (10-4 to 10-8 dilution) and plated onto TZC agar medium[9] and incubated for 48 hours at 30^{o}C. Amongst a large number of colonies one showing irregular viscous appearance with pink centre and white border was selected and purified.

Two seed samples (ac nos SM017 and SM032) of brinjal naturally infected with pathogen and carrying 100% incidence as revealed on TZC agar medium were selected for histopathological studies. Serial microtomed and hand cut sections were used for study. For microtome sectioning, categorised seeds naturally infected with pathogen were soaked in sterilized distilled water at 60°C kept in hot air oven for 30 min. The selected seeds were fixed in 70% alcohol for 48 hrs in a vial, dehydrated through tertiary butyl alcohol (TBA) series, infiltered and embedded in paraffin wax. The embedded material was cut into blocks sectioned at 8-10 micron thickness, deparaffinised, stained with safranine and light green combination and mounted in DPX[18]. Microtome sections were studied under compound microscope (x20-1000). Some seeds were also hand cut using sterile blade.

Results

Infection of *Ralstonia solanacearum*- In dry seed examination, seed samples of brinjal were categorised into asymptomatic, shrivelled discoloured and heavily discoloured seeds (Fig. 1A). The discoloured seeds showed shrivelling, water-soaked, translucent areas and bacterial ooze forming crust like growth on the seed surface. The seed surface of asymptomatic seeds looking healthy, bright colour with typical texture but in discoloured seeds these properties of seed were lost (Fig. 1A). The heavily discoloured seeds on bisecting found the discoloured embryo and endosperm with necrosis and browning.

The virulent (colonies with pink or light red color or characteristic red center and whitish margin) and avirulent (smaller, off-white and non-fluidal colonies) strains of

R. solanacearum were identified in Triphenyl Tetrazolium Chloride (TTC) medium containing 0.005% TTC. Isolated colonies of *Ralstonia solanacearum* from

Table 1. Location study of *Ralstonia solanacearum* in different seeds components of brinjal in various categories in microtome sections

Seed categories	Seed components						
	Hilum	Seed Coat		Space in between endosperm and seed coat	Endo-sperm	Embryo	
		Outer layer of testa	Inner layer of testa			Embryonal axis	Cotyledons
Sample Ac. No. SM017							
1.Asymptomatic seeds	3	2	2	0	0	0	0
2.Shrivelled discolouredseeds	5	6	7	7	5	6	4
3.Heavily discoloured seeds	6	8	9	9	8	8	9
Sample Ac. No. SM011							
1.Asymptomatic seeds	2	2	3	1	0	0	0
2.Shrivelled discolouredseeds	3	7	8	6	6	5	6
3.Heavily discoloured seeds	8	9	8	8	9	7	8

Table-2: Area wise occurrence and incidence of bacterial species in brinjal in SBM and on semi-selective medium

Selected Area	Incidence of *Ralstonia solanacearum*		
	UT	PT	TZCA
East	32 (03-70)	28 (02-65)	32 (40-100)
West	15 (06-48)	12 (04-38)	15 (10-70)
South	13 (02-40)	10 (02-30)	13 (30-80)
North	15 (08-65)	12 (04-56)	15 (30-70)
Total	75 (02-70)	62 (02-65)	75 (10-100)

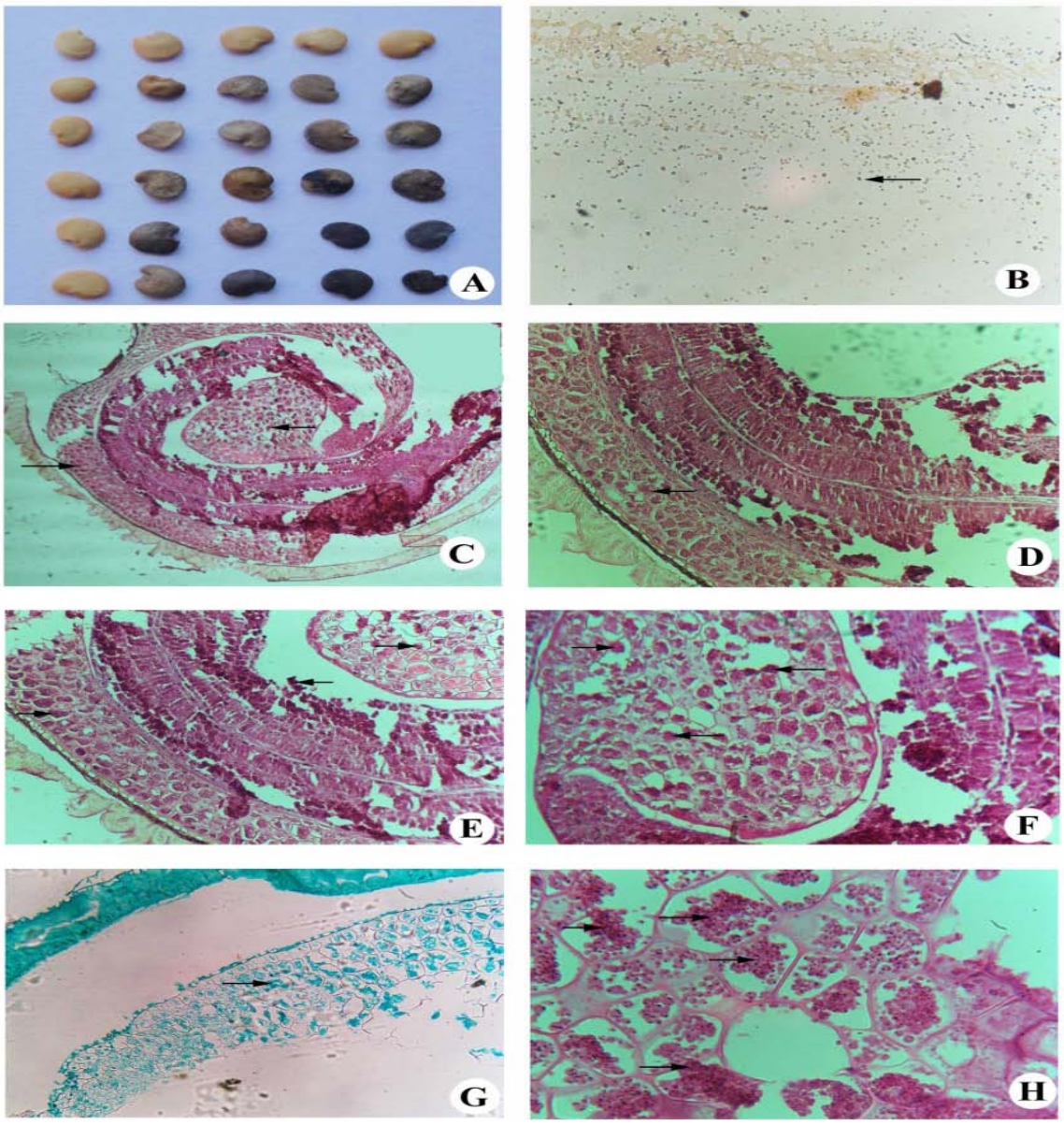
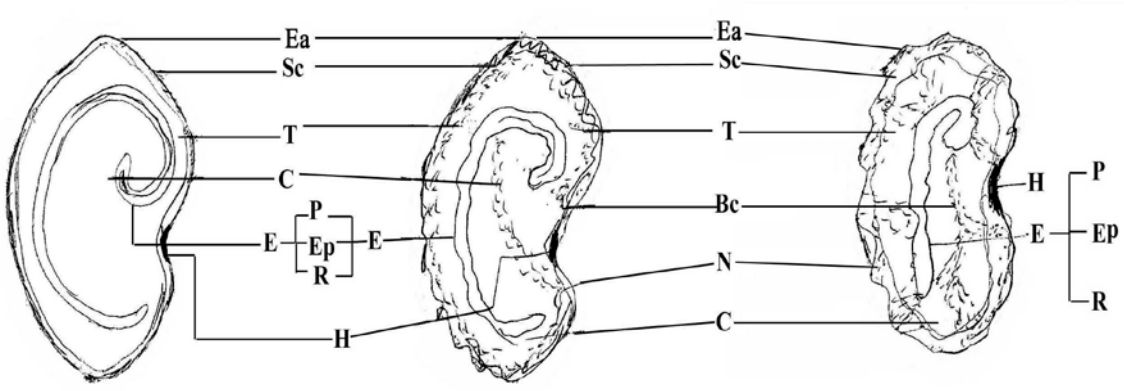


Fig. 1. Histopathology of brinjal seeds naturally infected with *Ralstonia solanacearum* **A:** Seeds categories in dry seed examination showing the degree of discolouration. Note the asymptomatic, shrivelled discoloured and heavily discoloured seeds (from left to right respectively), **Fig. B:** The cell of pathogen in Gram's staining X1000, **Fig. C:** Cross section of whole seed contains pathogen in different part of seed. Note lysis, depletion and necrosis of host cell X250. **Fig. D:** Part of L.S. of heavily infected seeds showing depletion, lysis, necrosis and cavities in (↗) endosperm, cotyledons and in testa. Note the clumps of pathogen in testa X250. **Fig. E:** Part of L.S. of asymptomatic seeds showing the cells of pathogen in endosperm, cotyledons and seed coat (outer and inner layer) X250. **Fig. F:** L.S. of shrivelled discoloured seed showing the bacterial cells of pathogen in embryo and endosperm. X1000. **Fig. G:** Part of L.S. of heavily discoloured seed showing bacterial cell and disintegration of host cells of cotyledons. Note the lysogenous cavities in host tissue X1000, **Fig. H:** Part of L.S. of heavily discoloured seed showing bacterial cells and depletion of host cells of testa (outer and inner layer) X250.

seeds of brinjal was incubated on TZC medium. After 48 hours of incubation at 30°C a viscous colony with pink centre and white border developed on the plate was selected as *R. solanacearum*. A single virulent colony of *R. solanacearum* isolates were used for pathogenicity test. The bacterial isolates were gram's negative, KOH solubility test positive, non-pigmented non levan forming from sucrose; gelatin hydrolysis weak: starch and aesculin not hydrolysed; nitrate reduced by nearly all strains, many produce gas (denitrify); no growth at 40°C; oxidase positive and arginine dihydrolase negative.

Two seed samples of carrying 100% natural infection of pathogen on TZC agar (semi-selective medium) in ac numbers SM017 and SM032 were categorised and used for histopathological studies respectively. (Fig. A).

The symptomatic discolored seeds showed translucent, water soaked spots on seed coat. On bisecting of such heavily infected seeds, deformed endosperm with black embryo were yielded. In asymptomatic seeds bacterial cells was confined to hilum, outer layer of seed coat and inner seed coat in both the samples. The bacterial cells were also colonised in between inner layer of seed coat and endosperm in ac. no. SM032. No bacterial cells were observed in endosperm and embryo.



I. Asymptomatic seed **II. Shrivelled discoloured seed** **III. Heavily discoloured seed**

(Bc = Bacterial cells, C = Cotyledons, E = Embryo, Ep = Epicotyl, Ea = Epidermal appendages, H = Hilum (funiculus), R= Redicle, N = Necrosis, Sc = Seed coat, E = Embryo, P = Plumule, T = Testa)

Fig. 2. Semi diagrammatic presentation of *Ralstonia solanacearum* in naturally infected seeds of brinjal.

In moderately discolored seeds, the bacterium was observed at hilum, outer layer of seed coat and inner layer of seed coat but not up to embryo. The bacterial cells were also observed in the space in between seed coat and endosperm cuticle in 7 and 6 seeds in ac nos SM017 and SM032 respectively. In the endosperm the depletion of cell contents, enlargement of host cells and bacterial clumps were observed in both the samples (Table 1).

In heavily discolored seeds, aggregation of bacterial cells and clumps were observed in hilum, outer layer of seed coat, inner layer of seed coat and endosperm. The inner layer of testa and endosperm cuticle disintegrated at several places in the two samples and the bacteria

aggregated around broken endosperm cuticle and in endosperm. In endosperm, the depletion of cell contents lysogenous cavities due to disintegration of cells and aggregation of bacterial (Fig. G) cells were observed (Fig. H). In embryo, the embryonal axis and cotyledons had the bacterial clumps in the two samples ac nos SM017 and SM032 respectively. Bacterial cells aggregated in space in between seed coat, endosperm and cotyledons (Fig. I) was also observed.

Discussions

Infection of RS in seeds of brinjal affected seed quality adversely causing discolourations, shrivelling, shedding of epidermal appendages and water- soaked symptoms. It was mentioned that *X. campestris* occurred on seed coat surface but those causing vascular or systemic infection are frequently found in the seed coat and other tissues of seed[8]. Such symptoms caused by *Xanthomonas campestris* in cow pea[18], by *X. c.* pv. *campestris* in rape, mustard[19]and pegion pea[20,21] and by *Ralstonia solanacearum* and *Xanthomonas axonopodis* pv. *vesicatoria* in tomato[22,23] have also been reported. The bacteria have been reported to be present below the seed coat in cabbage[24], rape, mustard[19] and pegion pea [20,21].

In the present study, seeds with discolourations were found associated with pathogen. Similar observations have been reported in other crops like cowpea, mustard, sunflower, pigeon pea, chilli, tomato etc. In cowpea, shrivelled seeds showed brown discolorations of seeds in bean halo disease caused by *Pseudomonas phaseolicola*[8]. Discoloured seeds with water-soaked translucent areas on seed surface due to *P. syringae* have been reported in sunflower[25]. Brown, pinkish discolourations by *Xanthomonas campestris* pv. *campestris* in mustard[19] and *X. cajani* pv. *cajani* in pigeon pea[20] and *Ralstonia solanacearum* in tomato[22] have been reported.

In this study, the bacterium was found associated with the epidermal appendages and also at hilar region as like tomato due to *Ralstonia solanacearum*[22]. It was reported that this may be due to gas exchange, water transport that is through the funiculus during the development of seed[26]. Formation of cells or clumps of bacterial cells near hilar region suggested the penetration of pathogen through funiculus as also suggested[27]. *Xanthomonas campestris* pv. *phaseoli* caused common and fuscous blight in *Phaselous* spp. and *Dolichos lablab* confined to be harboured both within the seed and on the seed coat[28].

X. c. pv. *malvacearum* located internally and externally on the seed. Internally in seed, it was located in chalaza, micropylar end of the seed coat and in the embryo[29,30]. *X. c.* pv. *glycines* and *X. oryzae* pv. *oryzae* were found located externally and internally up to endosperm[31-33]. *Pseudomonas syringae* pv. *phseolicola* in severly infected seeds of bean (*Phaseolus* sp.) are found associated in the hilum region of seed, surface of cotyledons and embryo[34].

In the present study, large number of cells or clumps was observed at funiculus (hilum) and it suggested being the site of penetration of bacteria in the seed. Earlier studies have been demonstrated that the bacterial pathogens may penetrate through micropyle, funiculus[35], through wounds[36], through stomata[37,38] and by mechanical injuries as wind or by sand[39].

Thus, the pathogen was found to be extra- as well as intra embryonal in seeds of brinjal. It was confined to outer layer of seed coat and funiculus in asymptomatic seeds and seed coat, in inner layer of testa,

endosperm and embryo in shriveled and heavily discoloured seeds. The bacterial cells were found in abundance at funiculus suggesting the possible mode of invasion and infection in seed through this area leading to systemic infection as earlier reported.

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