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Phylotype Analysis of *Ralstonia Solanacearum* Causing Bacterial wilt in Eggplants in Orissa in India

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ABSTRACT

Eggplant is prone to attack by several pests including bacteria, fungi, nematodes and insects. In this study, we have analyzed phylotype of bacterial wilt (*Ralstonia solanacearum)* infection in eggplant plants collected from Bhubaneswar (Orissa) in India. Bacterial wilt symptomatic five plant samples were collected from brinjal field in Bhubaneswar in 2016. The samples were macerated in sterile distilled water and grown on Kelman's triphenyltetrazolium chloride (TZC) agar media. Total genomic DNA of the bacterium were extracted and subjected to PCR amplification using the *R. solanacearum* specific universal primer pair 759/760. An expected single 280 bp fragment amplified in all the samples confirmed the identity of these as Ralstonia. To reconfirmed isolate of bacterium, the amplicon was sequenced in sequencer. In NCBI blast, the nucleotide sequence was 100% similar with *Ralstonia solanacearum* strain RS-lpxC-DOB-1 (AB910593) and the sequence was submitted in NCBI database under Acc. No. KY393266. To determined phylotype of strain used specific multiplex PCR with phylotype specific primers (Nmult: 21F1/2, Nmult: 22InF, Nmult: 23AF, Nmult: 22RR) revealed that all the five infected samples belonged to phylotype I as a 144-bp amplicon were observed in agarose gel. On the basis of above finding concluded that the bacterial wilt infected eggplant collected from Bhubaneswar was *Ralostonia solanacearum*, Phylotype I.

KEYWORDS: *Eggplant, bacterial wilt, Phylotype*

INTRODUCTION

Brinjal or eggplant (Solanum melongena L.) is an important solanaceous crop of sub-tropics and tropics region. India produces 12.5 million ton in a year and stand second position in the total world production of eggplant (*Solanum melongena*) after China (FAOSTAT, 2017). In India, it is one of the most common, popular and principal vegetable crops grown throughout the country except higher altitudes. Major eggplant growing states and its percentage production share in India are West Bengal (24%), Orissa (16%), Gujarat (11%), Madhya Pradesh (9.2%) and Bihar (9%) (National Horticulture Board, India, 2015-16).

The eggplant production limits up to 86% because of bacterial wilt disease (BW) caused by *Ralostonia solanacearum* in India (Sabita et al., 2000). The *Ralstonia solacearum* ranks among the most devastating pathogen infecting solanaceous crops viz, tomato, potato, brinjal etc. (FAO). The host of bacterium is more than four hundred plant species and spread across the world (Patil et 2017). This soil born bacterium has been classified into five races and six biovars based on host and trophic traits respectively (He et al., 1983). More recently, based on geographical origin of the strains of the bacterium, it is divided in four phylotypes: Phylotype I (Asia), Phylotype II (America), Phylotype III (Africa) and Phylotype IV (Indodenia) (Fegan and Prior 2005). In India, All the three phylotype (I, II, IV) are reported to infect potato crops (Patil et al 2017) but Phylotype I is reported to infect solanaceous crops including eggplants from different states of India but not from Orissa (Ramesh et al., 2014). In this study, we are reporting phylotype of Ralstonia solacearum infecting eggplants in Orissa.

Material and methods

Bacterial strains, media and growth condition

Five samples were isolated from bacterial wilt affected brinjal plants collected from three different brinjal growing regions of Bhubaneswar of Orissa in India during Kharif 2016 season. Stem pieces (8-10 cm long) of wilted brinjal plants were collected from each field washed thoroughly; air dried and brought to the laboratory for further studies. The samples were then surface disinfected with 70% ethanol, peeled, sub sampled and macerated in sterile distilled water (Fig. 1A). Macerates were streaked on Kelman's triphenyltetrazolium chloride (TZC) agar medium (Peptone, 10 g; glucose, 5 g; Casamino acid, 1 g; agar, 17 g; TZC, 50 mg L^{-1}; pH 6.5) (Kelman, 1954). Plates were incubated at 28°C for up to 72 h. Bacterial colonies developing the typical

irregular mucoid colonies were again streaked onto fresh TZC medium for further purification. Well separated typical wild type R. solanacerarum colonies were further transferred to medium modified by exclusion of TZC for multiplication of inoculums (Fig 1B).

DNA extraction from bacterial strains

The bacterial cultures were grown on nutrient broth and incubated at 28±1°C with 200 rpm for 48 h. The bacterial cells were harvested in form of pellet in tube by centrifugation for 3 min at 13,000 rpm. Supernatant was discarded and the pellet was resuspended into 300 µl of lysis buffer (40mM Tris Acetate, 20mM Sodium acetate, 1mM EDTA, 1% SDS, 0.002% Beta Mercaptoethanol, 1M L-Ascorbic Acid) by vigorous pipetting and incubated at 37 °C for 40 min. Afterwards 100 µl of 5M NaCl was added and mixed thoroughly. Subsequently, 80 µl of CTAB was added, mixed thoroughly and incubated for 20 min at 65°C. Chloroform and isoamyl alcohol in the ratio of 24:1 was added in to the tube, mixed thoroughly and centrifuged for 10 min at 13,000 rpm at 4 °C. 400 µl of clear supernatant was transferred into new vial and equal volume of chloroform was added. The tube is mixed vigorously (so that it becomes milky solution) and centrifuged at 13000 rpm for 10 min. The aqueous, viscous supernatant was transferred to a fresh tube (about 300 µl), 0.6 volume isopropanol added and centrifuged for 10 min at 13,000 rpm to precipitate the nucleic acids. The supernatant discarded and resulting pellet washed with 70 per cent ethanol. The pellet was dried and dissolved in 40 µl of HPLC water.

Phylotype analysis

Phylotype identification of strain was done as described (Fegan and Prior, 2005; Prior and Fegan, 2005). Phylotype specific multiplex PCR (Pmx-PCR) was carried out in 20 µl final volume of reaction mixture, containing 1µl of templete DNA, 2 µl of 2mM dNTP, 0.5 µM of primers (Nmult: 21: 1F, Nmult:21:2F, Nmult:22:InF, Nmult:23: AF and 0.5 µM of the primers 759 and 760 (Opina et al., 1997), 2 µl of 10x PCR buffer, 1 unit of *Taq* polymerase and autoclaved double distilled water to make up the volume 20 µl with following programme: 96°C for 5 min. followed by 35 cycles of 94°C for 5 sec. 59°C for 30 sec. and synthesis at 72°C for 30 sec. The final extension allowed for 10 min. at 72°C in thermo cycler (EPGradientS, Eppendorf) . The PCR product was resolved in 1% agarose gel to analyze the amplification. This Pmx-PCR amplifies the 280-bp "universal" *R. solanacearum* specific reference band plus following phylotype-specific PCR products: a 144-bp amplicon from phylotype I strains; a 372-bp amplicon from phylotype II strains; a 91-bp amplicon from phylotype III strains; and a 213-bp amplicon from phylotype IV strains.

Results and discussion

Bacterial strain collection and their identification

In this studies, bacterial wilt infected brinjal stems were collected in *Kharif* 2016 season from wilt affected areas of Bhubaneswar of Orissa state in India. A total of five bacterial culture were recovered from wilt affected brijal stems. On Kelman's (1954) TZC agar medium, these strains yielded typical virulent type colonies, which were cream coloured, irregularly shaped, highly fluidal with pink pigmentation in the centre (Figure 1d). These characters were consistent with *R. solanacearum* as described by Kelman (1954) on TZC agar medium. Total genomic DNA of all the culture was extracted and subjected to PCR amplification using the *R. solanacearum* specific universal primer pair 759/760. An expected single 280-bp fragment (Opina et al., 1997) amplified in all the strains (Fig. 1C), which further confirmed the identity of these strains as *R. solanacearum*.

Sequence analysis

One PCR positive samples Out of five samples amplified with primer pair 759/760 was PCR clean up followed by sequencing ABI 3130 Genetic Analyzer (Applied Biosystems, USA). The sequence was blast in NCBI data base and found 100% similarity with *Ralostonia solacearum* strain RS-lpxC-DOB-1 (AB910593) infecting *Solanum lycopersicum* (Tomato) in Karnataka and the sequence was submitted in NCBI data base under Acc. No. KY393266.

Phylotype identification

Phylotype specific multiplex PCR revealed that all the five strains from Bhubaneswar belonged to phylotype I as a 144-bp amplicon was observed in all the strains when Pmx-PCR products of these strains were subjected to electrophoresis on 1% agarose gel (Fig. 1D). In multiplex PCR all five samples were amplified with 280 bp and 144 bp amplification (Fig. 1E) with all five specific primers. The result confirmed that all the five infected samples were Ralostania solanacearum Phylotype I only.

As per report *Ralostonia solanacerous* infecting brinjal in Bihar, Goa, Maharastra, Kerala, Karnataka, Andman is Phylotype I but the disease was also reported from Orissa, Himachal Pradesh and Jharkhand (Chandrashekara et al., 2012; Dinesh et al., 2010) but phylotype of the disease is unknown in this area. In this study, the disease infected samples collected from Bhubaneswar district of Orissa in India and confirmed the samples were infected with bacterium by TZC media reaction and the bacterium was *Ralostonia solanacerous* by 280 bp amplification with universal primers 759/760. The bacterial sequence is highly matching (100%) with reported strain of bacteria infecting tomato in Karnataka. It was also confirmed that the unknown bacterium in Orissa belong to Phylotype I by amplification of 144 bp using specific universal primer and not belong to Phylotype II, III and IV because it's not amplified with specific universal primers in multiples PCR . On the basis of above finding we can concluded that the wilted eggplant samples collected from Bhubaneswar districts of Orissa is *Ralostonia solanecerous* Phylotype I.

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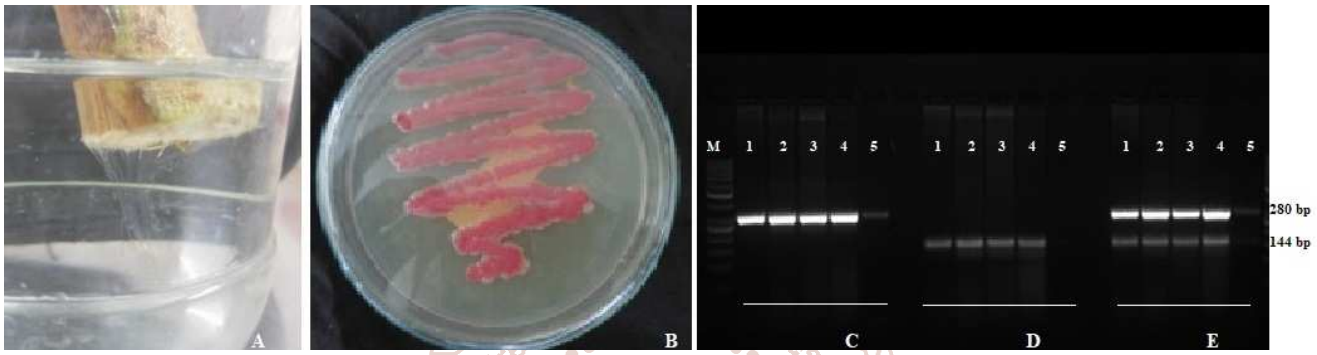

Figure1. Bacterial wilt of eggplant caused by *R. solanacearum* showing (A) bacterial streaming in clear water from stem of infected plant (B) typical *R. solanacearum* colonies on TZC agar medium and PCR amplification of five infected samples from Orissa using (C) *R. solanacearum* specific universal primer pair 759/760 (amplicon: 280bp) (D) Phylotype I specific primers (E) Phylotype specific multiplex PCR (Lane M = 1 kb ladder, lane 1-5 = strains of *R. solanacearum*).