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# PCR based detection of bacterial wilt pathogen, *Ralstonia solanacearum* in ginger rhizomes and soil collected from bacterial wilt affected field

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**Abstract**

PCR based detection of bacterial wilt pathogen in rhizomes and soil has been described. Bacterial wilt pathogen, *Ralstonia solanacearum* was PCR detected in apparently healthy rhizomes and soil collected from fields in endemic area. The protocol offers an indexing method for bacterial wilt pathogen in seed rhizomes and soil. The study emphasizes the need for imposition of restriction in the movement of such rhizomes from endemic locations to non traditional areas.

## Introduction

Bacterial wilt of ginger is an important production constraints and it is widely distributed in most tropical and subtropical regions (Kumar *et al* 2004). The causative organism, *Ralstonia solanacearum (Pseudomonas solanacearum* Smith) is a soil and plant inhabiting bacterium which affects many monocotyledonous and dicotyledonous plants (Hayward 1991). The bacterial wilt is characterized by bacterial entry into the host followed by its multiplication and movement through the xylem vessels of the host plant. In the process, they interfere with the translocation of water and nutrients which in turn results in drooping, wilting and death of the above ground parts of the plants. In the case of ginger, the first noticeable symptom of bacterial wilt is downward curling of leaves due to loss of turgidity and within 3-4 days the leaves dry up. The affected rhizome starts rotting and putrefying due to attack of saprophytic soil microorganisms. The rotted rhizomes emit foul smell and the affected plants die within 2-3 weeks. Bacterial wilt is highly favoured by the environmental conditions such as high rainfall and warm weather that are important predisposing conditions for the disease development. Rhizome borne inoculum is primarily responsible for the initiation of the disease in the field which further spread horizontally across the field due to incessant rain and conducive weather. It is speculated that the rhizomes collected from previously diseased field carry the inoculum to new locations as well as to next season. PCR based method has been developed for the detection of the bacterial wilt pathogen in soil and rhizome (Kumar & Anandaraj 2006). Method for detection of bacterial wilt pathogen on symptomless tuber has been reported (Janse, 1988). In the recent years several advancements have been made for the detection of bacterial wilt pathogen in environmental samples (Schaad 2002; Seal 1995, 1997, Kumar & Anandaraj, 2006). Primers specific for PCR based detection of *R. solanacearum* in plant and soil samples have been reported (Ito *et al.* 1998). We describe here

a rapid and sensitive method for indexing bacterial wilt pathogen in ginger rhizomes and soil that involves isolation of total DNA from ginger vascular tissue and soil followed by a PCR based detection using universal *R. solanacearum* specific primers.

## Materials and methods

### *Collection of rhizomes*

The mature ginger rhizomes and soil were aseptically collected in polypropylene bags from various bacterial wilt affected fields across a bacterial wilt endemic (Wyanad district) and non endemic location (Kozhikode) in Kerala.

### *Extraction of bacterial cells from rhizome surface and isolation of total DNA from surface microflora*

Ginger rhizome was shaken with 50 ml of PBS (Phosphate buffer saline, NaCl-8.0; KCl-0.3; $Na_2HPO_4$ 1.44; KH2$PO_4$ 0.24; Distilled $H_2O$-1000 ml pH 7.4) for about 30 min at 300 rpm in a reciprocal shaker. The supernatant was collected and centrifuged at 10000g for about 30 min. and the pellet was resuspended in 5 ml of PBS and preserved at -80°C until further use. One milliliter of the suspension was used for DNA extraction by modifying the protocol reported by Kumar *et al*. (2004).

One mL of soil suspension was centrifuged at 10000 g for 10 minute at 4°C and the soil pellet was collected and washed twice by centrifuging at 10000 g for 10 minute at 4°C. The pellet was added with 675 µl of extraction buffer [Tris-Cl (pH 8) 100mM; EDTA (pH 8) 100 mM; $Na_2HPO_4$ 100 mM; NaCl 1.4M; CTAB 1%] incubated at 37°C for 30 minute with intermittent shaking. The suspension was added with SDS (75 µl of 20 %) and incubated at 65°C for 2 h with end over end inversion. The suspension was centrifuged at 6000 g for 10 minute at 28°C and the clean solution collected in a new tube. The suspension was once extracted with equal volume of Phenol: Chloroform: IAA (25:24:1) and the aqueous phase was removed to a new tube and extracted once with equal volume of Chloroform: IAA (24:1). The aqueous phase was collected in a tube and DNA precipitated in 0.6 volume of ice-cold isopropanol for about 1 h. The DNA was recovered at 10000 g for 10 min at room temperature and washed once with 70% ethanol and DNA was pelleted by centrifugation at 10000 rpm for 5 min. and was finally dissolved 100 µl of TE (10:0.1) and reconstituted at 50 ng $\mu l^{-1}$

### *PEG purification*

DNA was further purified by PEG purification. Briefly 40 µl of PEG solution [PEG 8000 Sigma P4463 30 % w/v; NaCl 1.6 M] was added to DNA and incubated for 1-15 hr h at 0°C. DNA was recovered by centrifugation at 12,000 g at 4°C for about 20 minutes and pellet washed once with 70% alcohol and the final pellet was resuspended in 50 µl TE buffer after drying in vacuum to remove the traces of alcohol and reconstituted at 50 ng µl-$_1$

### *Isolation of DNA from rhizomes*

Rhizome (500 mg) was pre-chilled at -80°C for 24 h. The rhizome was ground in pre-heated (60°C) DNA extraction buffer [Tris-Cl (pH 8) 100 mM; EDTA (pH 8) 20 mM; $NaCl_2$ M; CTAB 2%; ß-Mercaptoethanol 0.3%; PVP 1%] in a pre-chilled pestle & mortar for 10 min. The whole macerate was transferred to clean and sterile microfuge tubes and incubated at 60°C in a water bath for 1 hr. with occasional mixing. The tissue was extracted with equal volume of Phenol: Chloroform: IAA (25:24:1) and centrifuged at 10000 g for 10 min. at 4°C. The aqueous phase was transferred to a new tube and extracted once with equal volume of Chloroform: IAA (24:1). The aqueous phase was collected in a new tube and DNA precipitated for about one hour in 0.7 volume of ice-cold isopropanol. The DNA was collected by centrifugation at 10000 g for 10 min at room temperature. Finally the DNA pellet was dissolved in 200 µl of TE buffer. The DNA was further purified by salt and alcohol precipitation. Briefly, DNA was added with 1/10th volume of Sodium acetate (3 M, pH 5.2) and 2.5 volume of ice cold isopropanol and allowed for precipitation at 30 minutes. The precipitated

DNA was collected by centrifugation (12000 g/10 min/4°C) and the pellet was washed once with 70% ethanol and dried until the traces of alcohol removed. The pellet was finally dissolved 60 µl of TE (10:0.1) and reconstituted at 50 ng $\mu l^{-1}$

### *Detection of Ralstonia solanacearum using species-specific primers*

Ralstonia solanacearum was detected by PCR as reported by Kumar and Anandaraj (2006). PCR amplification was performed in 10 µl reaction volumes in a thermal cycler (Eppendorf, Mastercycler gradient) using soil or rhizome DNA as template. The test samples were compared with positive (pure DNA of *R. solanacearum*) and negative control. Amplified products were loaded in a 2 % agarose gel containing 1.0 $\mu g/mL^{-1}$ of EtBr and visualized in gel documentation system (Alpha Imager 2000, USA).

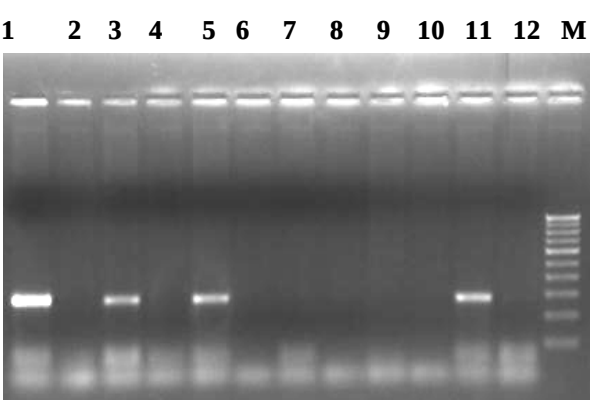
## Results and discussion

Indirect evidences have been reported for the rhizome transmission of the bacterium in ginger rhizomes and potato tuber. Occurrence of genetically identical virulent lineage of biovar 3 across the well separated geographical location in India is attributed to rhizome transmission of the bacterium (Kumar et al 2004). Such evidences have been reported in Indonesia in ginger rhizomes (Supriadi 2000). Bacterial wilt outbreak in ginger is mainly due to planting of latently infected or externally contaminated rhizomes collected from the bacterial affected field which poses risk of transmitting the bacterium to newer areas. However, direct evidence on the role of rhizome borne inoculum in bacterial wilt incidence is very scanty. Therefore, one of the objectives of the present investigation was to know the health status of the rhizomes collected endemic area for bacterial wilt. The crop loss in these localities goes up to 50-100% owing to multitude of reasons such as the heavy rainfall, undulating topography to ease the movement of water from affected field to other area, proximity of the fields to each other, lack of internal quarantine regulations and use of apparently healthy rhizomes collected from bacterial wilt affected fields

Successful management of bacterial wilt of ginger largely depends on use of pathogen free rhizomes as planting material. Several methods have been proposed for detection of bacterial wilt pathogen in soil, planting material and irrigation water (Caruso et al 1998; Kumar et al 2002; Priou, 2001; Priou et al 1999; Yung and Wang 2000). Apparently good looking rhizomes collected from the fields of Wyanad district were indexed for bacterium using PCR mediated assay. Utility of PCR based assay for detection and diagnosis of bacterial pathogens is reported (Hadidi et al 1995; Henson and French 1993; Opina et al 1997). Twelve of the 30 rhizome samples collected from endemic location showed positive amplicon of size 280 bp specific for *R. solanacearum* and three of ten samples collected from non endemic location in Calicut produced positive amplicon for *R. solanacearum* (Fig.1, 2).

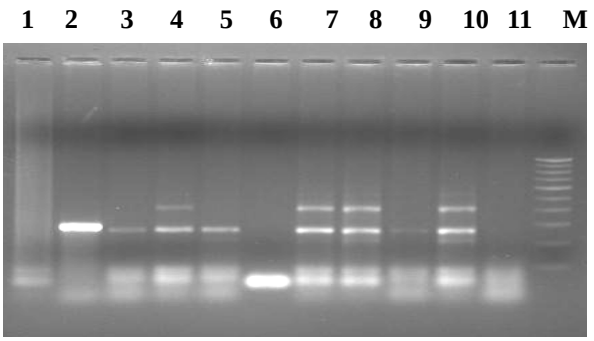
The soil collected from the vicinity of the healthy rhizomes collected from the bacterial wilt affected bed also tested positive for the bacterium. Three sample soils were found to

**Fig. 1.** PCR based detection of *Ralstonia solanacearum* in soil collected from bacterial wilt endemic area in Kerala




Lane 1. Positive control, Lane 2. Madal-1, Lane 3. Alathur-3, Lane 4. Surabhikavala, Lane 5. Padichira, Lane 6. Edamala, Lane 7. Pulpally, Lane 8. Madal-2, Lane 9. Mullankolly, Lane 10. Pulpally 1, Lane 11.Allathur-2, Lane 12. Alathur 1, Lane M. 100bp ladder DNA size marker

**Fig.2.** PCR based detection of *Ralstonia solanacearum* in rhizomes collected from bacterial wilt endemic area in Kerala




Lane 1. Negative control, Lane 2. Positive control, Lane 3. NT-4a, Lane 4. NT-4b, Lane 5. NT-4c, Lane 6. NT-4d, Lane 7. NS-3, Lane 8.NS-2, Lane 9. NS-1, Lane 10. NU-1, Lane 11.NU-2. Lane 12. 100bp ladder DNA size marker

be contaminated with *R. solanacearum*. These results indicated the risk associated with the use of apparently healthy rhizomes collected from the diseased area in spreading the disease to newer locations. Survival of the bacterial wilt pathogen in soil has been reported (Granada and Sequeira 1983; Graham and Lloyd 1979a; Graham *et al* 1979b). Incidence of crop loss in Kodagu district has been reported which was attributed to planting of rhizomes from unknown and unauthorized sources (Kumar unpublished) from endemic locations in Wyanad district of Kerala (Personal communication from farmer). The recent outbreak of bacterial wilt in the Northeastern state of Nagaland was also attributed to the distribution of contaminated rhizomes from unauthorized source (Personal communication from Dr. D.L. Daiho, Pathologist, Nagaland University). The study revealed the utility of PCR mediated assay for rapid indexing of the ginger rhizomes and field soil. The study underscores the importance of avoiding the rhizome material from diseased field for planting purpose.

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## Reference

Caruso, P, Llop, P., Palomo, J.L., Garcia, P., Morente C. and M.M.Lopez 1998 Evaluation of methods for detection of potato seed contamination by *Ralstonia solanacearum*. In: Bacterial wilt disease-Molecular and Ecological Aspects, P.Prior, C.Allen and J.Elphinstone (eds) Springer Verlag, Berlin, Germany, p 128-132.

Graham, J. and Lloyd A.B 1979a Survival of potato strain (race 3) of *Pseudomonas solanacearum* in the deeper soil layers. Australian Journal of Agricultural Research, 30: 489-496.

Graham, J., Jones, D.A. and Lloyd, A.B 1979b Survival of *Pseudomonas solanacearum* Race 3 in plant debris and latently infected potato tubers. Phytopathology, 69:1100-1102.

Granada, G.A. and Sequeira L. 1983 Survival of *Pseudomonas solanacearum* in soil, rhizosphere and plant roots. Canadian Journal of Microbiology, 29: 433-440.

Hadidi, A., Lenyl. and E.V.Podlecksis 1995 Polymerase chain reaction technology in Plant Pathology. Molecular Methods in Plant Pathology, p 10-15.

Hayward, A.C 1991 Biology and Epidemeology of bacterial wilt caused by *Pseudomonas solanacearum*. Annual Review of Phytopathology, 29: 65-87.

Henson, J.M. and R.French 1993 The Polymerase Chain Reaction and plant disease diagnosis. Annual Review of Phytopathology, 31:81-109.

Ito, S., Ushijima, Fuji, T., Tanaka, S., Kameya-Iwaki, M., Yoshiwara. and F.Kishi 1998 Detection of viable cells of *Ralstonia solanacearum* on soil using semi selective medium and a PCR technique. Journal of Phytopathology, 146: 379-384.

Janse, J.D 1988 A detection method for *Pseudomo-*

*nas solanacearum* in symptomless potato tubers and some data on its sensitivity and specificity. Bulletin OEPP, 18: 343-351.

Kumar, A., Sarma, Y.R. and S.Priou 2002 Detection of bacterial wilt pathogen, *Ralstonia solanacearum* in ginger rhizomes using post-enrichment NCM ELISA, Journal Of Spices and Aromatic crops, 11: 35-40.

Kumar, A, Sarma, Y.R. and Anandaraj M 2004 Evaluation genetic diversity of *Ralstonia solanacearum* causing bacterial wilt of ginger using Rep-PCR and RFLP-PCR Current Science, 87(11):1555-1561

Kumar, A and Anandaraj M 2006 Method for isolation of soil DNA and PCR based detection of ginger wilt pathogen, *Ralstonia solanacearum*, Indian Phytopathology 59(2): 154-160

Opina, N.F., Tanver, G., Holloway, J.F., Wang, T.H., Li, R., Lee. and Wang 1997 Detection of *R. solanacearum* by PCR. A novel method for development of species and strain specific DNA probes and PCR primers for identifying *Burkholderia solanacearum*. Journal of Molecular Biology and Biotechnology, 5:19-33.

Priou, S.L 2001 Instruction Manual for DAS-ELISA Kit for the detection of *Ralstonia solanacearum* in soil, International Potato Centre (CIP), Lima, Peru.

Priou, S.L., Gutarra, H, Fernandez. and Alley P 1999 Sensitive detection of *Ralstonia solanacearum* in latently infected potato tubers and soil by post enrichment ELISA-CIP Programme Report 1997-98 International Potato Centre, Lima, Peru, p 111-121.

Schaad, N.W 2002 Use of probable real-time PCR for same-day on-site field diagnosis of bacterial disease. Phytopathology, 92:110.

Seal, S.E 1995 PCR- based detection and characterization of *Pseudomonas solanacearum* for use in less developed countries, EPPO Buletin, 25:227-231.

Seal, S.E 1997 Molecular methods for detection and discrimination of *Ralstonia solanacearum*. In: Bacterial Wilt Disease: Molecular and Ecological Aspects, P.Prior; C.Allen and J.Elphinston (Eds) p: 103-108.

Supriadi, K. Mulya, and D. Sitepu 2000 Strategy for controlling wilt disease of ginger caused by *Pseudomonas solanacearum*. Jurnal Penelitian dan Pengembangan Pertanian 19(3): 106-111

Yung-An L. and Chi-Chung Wang 2000 The design of specific primers for the detection of *Ralstonia solanacearum* in soil samples by Polymerase chain reaction. Botanica Bulletin Academica Sincia, 41:121-128.