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Methods for screening ginger (*Zingiber officinale* Rosc.) for bacterial wilt resistance

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ABSTRACT: Currently the bacterial wilt management depends on selection of disease free seed rhizomes, rhizome treatment by hot air or hot water or rhizome solarization, periodical roguing of infected plants and crop rotation with non-host plants to reduce the disease causing potential of soil. Though effective in disinfecting the ginger rhizomes from *Ralstonia solanacearum*, none of these strategies practically arrested the spread of disease in the field during peak monsoon season, which is highly congenial for horizontal disease spread across the region. Exploitation of host resistance for management of bacterial wilt can be one of the important ecofriendly disease control strategies. To locate resistance against bacterial wilt, a reliable screening procedure becomes vital. Three *in vivo* methods viz., pseudostem inoculation, soil inoculation, rhizome inoculation and a novel *in vitro* method i.e., direct incorporation of bacterial cells in the medium when the plantlets are 2-3 leaf stage were evaluated by using different concentration of bacterial cells. Among the different methods evaluated, pseudostem inoculation resulted in wilting of plants in 5-7 days, followed by the soil inoculation method in 7-10 days, rhizome inoculation method in 45-60 days and the *in vitro* method in 10-14 days. Interestingly, the *in vitro* method did not result in typical wilting of plants, where the inoculated plants showed only yellowing. One of the observations during the standardization was the occurrence of 'disease escapes' among the inoculated plants during the first round of screening, which succumb to disease upon repeated inoculation of pathogen. The PCR assay confirmed the absence of the pathogen in the soil around the uninfected plants, which necessitated the need for three rounds of inoculation for reliable screening for bacterial wilt resistance. Since the soil inoculation closely mimics the natural condition where disease occurs, it is recommended from this study for screening ginger for bacterial wilt resistance. Any surviving plants after three rounds of selection can be further validated through the *in vitro* method standardized in this work.

Key words: Bacterial wilt, *Ralstonia solanacearum*, *in vitro* screening, disease escapes, PCR

Bacterial wilt caused by *R. solanacearum* (Smith) Yabuuchi is a disease widely distributed in tropical, sub-tropical and temperate regions worldwide. *R. solanacearum* -incited wilt disease is one of the major production constraints of ginger in small and marginal farming communities (Kumar and Sarma, 2004). Host range of the pathogen is very wide and ginger is one among the several hundred important hosts of the pathogen. Bacterial wilt of ginger is reported from India, China, Japan, Indonesia, Philippines, Hawaii and many other ginger-growing countries. In India, the disease is found in Kerala, Karnataka, Himachal Pradesh, Sikkim, West Bengal, Assam and other North Eastern states. The disease out-break is very severe during peak monsoon, causing total crop loss. The diseased clump of ginger releases bacterial cells in the form of bacterial ooze, which infects the adjacent roots of other clumps in the field. The strain causing bacterial wilt of ginger in India belongs either to biovar 3 or 4; the former being the most virulent in India (Kumar and Sarma, 2004). The pathogen is primarily rhizome-borne and is believed to be transmitted to many ginger-growing areas through latently infected rhizomes. Secondary spread within the field and neighbouring localities is through rain splashes and runoff water (Kumar *et al.,* 2004). Selection of pathogen free planting material, rhizome treatment by heat, antibiotics, strict phytosanitation, rotation with non-host crops

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are among the recommended strategies for field control of bacterial wilt of ginger (Kumar and Hayward, 2005). However, the disease still poses threat to ginger cultivation in many states, particularly in North Eastern states of India. Varieties resistant to bacterial wilt disease have been reported in many solanaceous vegetables like tomato, chilli and eggplant. Locating resistance for bacterial wilt in ginger requires an efficient and reliable disease screening method. The screening methodology should be easy to adopt, applicable to large number of germplasm and planting material in short time and should be unambiguous in nature. The methodology that closely mimics the natural condition would be advantageous for identifying stable and genuine resistance in ginger. The surviving plants in the pathogen-inoculated soil or natural field soil can be further subjected to pathogen detection assays to confirm the survival of the pathogen in the rhizosphere of the surviving plants. Such an assay would further authenticate the true resistance of the plant against wilt pathogen. Microbiological, serological and molecular methods are available for the detection of bacterial wilt pathogen in soil. Among the methods, PCR based molecular method is not only reliable but is also highly precise. In order to standardize a reliable screening procedure for bacterial wilt resistance in ginger, three different *in vivo* methods *viz*., pseudostem inoculation, rhizome inoculation, soil inoculation and an *in vitro* method were compared. The incidence of disease escapes among the infected plants in the pathogen-inoculated soil during screening assay was studied in detail. Several inoculation techniques have been recommended earlier for evaluating resistance of plants to bacterial wilt caused by *R. solanacearum* (Winstead and Kelman, 1952, Kelman 1953, Li and Tan, 1984, Tan *et al.,* 1994). These include stem inoculation, hypodermic injection and root inoculation (Kelman, 1953). Soaking seed in bacterial suspension ($6x10^8$ cfu ml^{-1}) for 30 minutes is also a useful inoculation technique (Li and Tan, 1984). Infected soil placed in pots or other containers can also be used as a source of inoculum. In the present work four different methods were compared for evaluating germplasm of ginger for bacterial wilt resistance.

MATERIALS AND METHODS

Strain used: Phenotypic characters of *R. solanacearum* indicating its virulence such as irregularly shaped fluidal colonies with spiral pink centre identified on CPG medium (gL^{-1} Cassamino acid 1; Peptone 10; Glucose 5) amended with 2, 3, 5, Triphenyl tetrazolium chloride (50mg/ml) were considered as important traits before inoculating the plants. Ginger strain of *R. solanacearum*-GRS Tms, a biovar 3 was used in the study.

Inoculum Production and Cultural Conditions: Virulent colonies of *R. solanacearum* as identified on CPG medium was inoculated in 100ml of Sucrose Peptone Broth (gL^{-1} Sucrose 20; Peptone 5; Mg SO_4 $7H_2O$ 0.25; K_2H PO_4 0.5 pH 7.2) and incubated at 28°C with constant agitation of 150 rpm in an orbital shaker incubator. Bacterial cells after sufficient growth (OD_{600} 1.00) were pelleted (8000 rpm/ 20min/4°C) and resuspended in 100 ml sterile distilled water.

Cultivar used: Ginger cultivar '*Himachal*' was used in the study along with 690 other ginger germplasm accessions obtained from germplasm conservatory of IISR, Calicut and Nagaland University, Medziphema.

Inoculation Procedure

Pseudostem inoculation: Bacterial inoculum (100μl @10^8 cfu ml^{-1}) prepared in sterile distilled water was placed with the help of micropipette inside the bottom most leaf sheath of 45 day-old ginger plants after making pin pricks with sterile needle. The inoculated plants were kept in green house at 28°C. After the third day onwards, the plants were monitored for symptoms of yellowing and wilting. Data on wilt incidence and number of days needed to express wilt were recorded.

Soil inoculation method: Bacterial inoculum (100μl @ 10^8 cfu ml^{-1}) prepared in sterile distilled water was poured in potting mixture containing soil, sand and farmyard manure @ 2:1:1 proportion (10 kg) planted with ginger. Plants of age 45 days were inoculated, watered regularly and kept in green house. The data on disease incidence and number of days to express wilt was recorded.

Rhizome inoculation: Seed rhizomes (25g) with at least two emerging sprouts were immersed and soaked in bacterial suspension (10^8 cfu ml^{-1}). After 2 hours, the rhizome was removed on to a sheet of absorbent paper to drain the excess moisture. Rhizomes thus inoculated were planted in soil. The data on disease incidence and number of days to express wilt was recorded as mentioned above.

***In vitro* method:** Plantlets of 2-3-leaf stage developed through tissue culture were directly inoculated with different concentrations of bacterial inoculum. Briefly, decimal dilutions of *R. solanacearum* prepared in sterile distilled water were poured directly into the base of the tissue-cultured plantlets maintained in 250 ml glass bottles on MS medium. After inoculation the bottles were maintained in at 28±2°C. Disease symptom observed on the plantlets was recorded and the data on number of days needed for manifesting the symptoms of yellowing of leaves was recorded.

Determination of minimum pathogenic concentration required of *R. solanacearum* for causing wilt in ginger: Decimal concentration of bacterium viz., 0, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9 cfu ml^{-1} were prepared as water suspension and inoculated in ginger plants by adopting pseudostem inoculation, soil inoculation and *in vitro* inoculation methods. Only three concentrations such as 10^6, 10^7, 10^8 cfu ml^{-1} were tested in rhizome inoculation method as others yielded erratic disease incidence in a preliminary trial (Information not given). The data on disease incidence and number of days to express wilt was recorded.

Detection of pathogens in inoculated soil: In order to confirm the presence of the pathogen, *R. solanacearum*, in surviving plants, PCR based method was adopted. The soil from the surviving plants was subjected to DNA isolation by soil DNA isolation kit (Mo Bio Inc., USA). The isolated DNA was quantified and quality checked before using them as template DNA in PCR. The total bacterial community genomic DNA from soil was primed with Rs specific primers in the PCR. Reaction volume (25μl) contained PCR buffer (Mo Bio, USA), $MgCl_2$: 1.5mM (Promega Corporation, USA), dNTP mix:0. 05mM (Mo Bio, USA), Polymerase enzyme: 0.5u, Template DNA: 100ng, BSA: 10mg, Primers (Opina *et al.*, 1997): 20pmoles each (Rs Primer1: 5'-gTC gCC gTC AAC TCA CTT TCC-3'; Rs Primer2: 5'-gTC gCC gTC AgC AAT gCg gAA TCg-3'). PCR was performed in Eppendorf master cycler gradient thermal cycler with initial denaturation at 94°C for 3 min, annealed at 53°C for 1 min and extended at 72°C for 1.5 min, followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72°C for 15 s and the final extension 72°C for 5 min. The final PCR products were resolved in 2.0 % agarose in 1x Tris Acetate EDTA buffer at 4°C for 6 hours at 4V/cm. The gel was stained with ethidium bromide and photographed on an UV transilluminator and the results were documented in Alpha imager for analysis. The presence of 281 bp amplicon in the sample confirmed the presence of *R. solanacearum.*

RESULTS AND DISCUSSION

Exploitation of host resistance for management of bacterial wilt can be one of the effective disease management strategies. Need for improved screening techniques to evaluate promising and resistant germplasm of crop plants against bacterial wilt have been emphasized (Hayward, 1991). Such a screening procedure should be rapid, reliable and easy to carryout even in resource-poor laboratories (Mehan *et al.,* 1995). A reliable screening methodology for identifying resistant ginger germplasm against bacterial wilt was developed and validated using large collection of germplasm as well as using *in vitro* plantlets. Critical population of *R. solanacearum* to cause wilt in ginger was worked out. The minimum pathogenic concentration varied depending on the method of inoculation. For pseudostem inoculation method, the minimum pathogenic concentration of *R. solanacearum* for wilting of all the inoculated plants was found to be $3x10^4$ per plant as against soil inoculation and rhizome inoculation methods which required at least 10^8cfu g^{-1} of soil or per milliliter of suspension to cause complete wilt (Table 1). The rhizome inoculation method was found to yield very erratic results as the inoculated plants failed to give rise to diseased plants on many occasions (Data not presented). Besides, the method required at least 45-60 days depending on the inoculum concentration for the first symptoms of the wilt to manifest. The *in vitro* method was found to be very sensitive as the yellowing of the ginger plants could be seen even with a concentration of 1 cfu ml^{-1}. The bacterium selectively colonized on the roots of *in vitro* plants as a slimy bacterial mass.

Three *in vivo* methods viz., pseudostem inoculation- where the pathogen was delivered into

Table 1. Relationship between concentration of bacterial cells, wilt incidence and incidence of disease escapes

Inoculum concentration (cfu/ml)	Pseudostem inoculation		Soil Inoculation		*In vitro* method	
	Wilt (%)	Escapes (%)	Wilt (%)	Escapes (%)	Yellowing (%)	Escapes (%)
$3X10^9$	100 (5)	0	100 (7)	0	100 (7)	0
$3X10^8$	100 (6)	0	100 (12)	0	100 (7)	0
$3X10^7$	100 (6)	0	30 (12)	70	100 (8)	0
$3X10^6$	100 (7)	0	30 (13)	70	100 (10)	0
$3X10^5$	100 (7)	0	10(14)	90	100 (12)	0
$3X10^4$	80 (8)	20	0	100	100 (12)	0
$3X10^3$	80 (8)	20	0	100	100 (15)	0
$3X10^2$	80 (9)	20	0	100	100 (15)	0
0	0	-	0	-	0	-

Data in the parenthesis denotes number of days to express first symptoms of wilt

All experiments were repeated at least once

Note: Only three concentrations viz., 10^8, 10^6, and 10^4cfu ml^{-1} were tested in rhizome inoculation method. All inoculated plants wilted in 10^8cfu ml^{-1}. The other two concentration was found to yield erratic wilt incidence ranging from 0-100% within the replication.

the vascular elements directly after making a prick, soil inoculation-where the pathogen was delivered into the soil around the rhizome and roots, rhizome inoculation- where the pathogen was delivered onto the rhizomes and a novel *in vitro* method where the bacterial cells were directly incorporated in the medium when the *in vitro* grown plantlets are 2-3 leaf stage were compared for screening ginger germplasm for bacterial wilt resistance.

Comparison of different methods is furnished in table 2. Among the different methods evaluated, pseudostem inoculation resulted in wilting of plants in 5-7 days, followed by the soil inoculation method with in 7-10 days; rhizome inoculation method in 60-120 days and the *in vitro* method in 10-15 days. Interestingly, the *in vitro* method did not result in typical wilting of plants where the inoculated plants showed only yellowing (Fig.1). Traditionally bacterial wilt screening for resistance is done in naturally infected fields. Though easy to do, this method is known to produce 'disease escapes' among the surviving plants. Leaf bioassay and a severity scale index were developed as a rapid and reliable technique for the varietal screening of anthurium germplasm against bacterial wilt caused by *R. solanacearum* (Kiran, 1997). Under field conditions, each infected plant, irrespective of the plant type, is known to release hundreds of thousands of cells in to soil, which in turn infect roots of other healthy plants in the proximity of the infected plants. This infection cycle results in huge population of *R. solanacearum* in the soil. It is likely that the healthy plant roots are exposed to such a large population of the bacterial cells in the field during peak monsoon season. This necessitates the need for high population of bacterial cells in the screening programme.

One of the observations during the standardization was the occurrence of 'disease escapes' among the inoculated plants during the first round of screening which later succumbed to wilt upon repeated inoculation of pathogen (Table 3). Among the methods evaluated, stem inoculation where the bacterial cells were directly delivered in to the plant was found to express disease, depends upon the concentration of bacterial cells inoculated. At lower inoculum level (below 10^4cfu $plant^{-1}$) 20% of the inoculated plants could not express wilt. Similarly, plants were found to escape infection below a 3 $x10^4$ cfu ml^{-1} of inoculum with other methods as well. Absence of wilt expression among the susceptible cultivars could be due to the inherent microbiological differences in rhizosphere and other environmental variables prevailing in the inoculated niche. The pathogen could not be detected in soils around the surviving plants (Fig 2), which confirmed that the non-symptomatic plants were not really resistant to the pathogen. PCR

Table 2. Comparison of different methods of screening ginger for bacterial wilt disease

Method	Minimum Pathogenic Concentration of *R. solanacearum* for wilting of all the inoculated plants	Number of days for symptoms expression	Nature of symptoms	Remarks
Stem inoculation after pinprick	3 x 10^5 cfu ml^{-1}	5-7	Typical wilt	Rapid assay but depend on injury, which can influence the outcome of the screening
Soil inoculation	3 x 10^8 cfu g^{-1}	7-10	Typical wilt	Repeated inoculation with pathogen is needed, suitable for large scale screening in short time, Highly dependable
Rhizome inoculation	3 x 10^8 cfu ml^{-1}	45-60	Typical wilt	Long incubation period. Highly erratic in reproducing the disease, not dependable for screening programme.
Direct inoculation in tissue cultured medium	*1 cfu ml^{-1}	10-14	Only yellowing, No wilting	Screening of large collection is possible

*10^2 cfu/bottle of 100 ml medium with 20 plantlets

Table 3. Response of ginger plants to soil inoculation with bacterial wilt pathogen, *Ralstonia solanacearum*

*Screening rounds	Accessions screened	Accessions wilted	Percentage escapes in relation to the number of plants inoculated
First round (10^7 cfu g^{-1} of soil)	40	33	17.5
Second round (10^7 cfu g^{-1} of soil)	7	4	7.5
Third round (10^7 cfu g^{-1} of soil)	3	3	0.0

*The interval between two rounds of inoculation was 30 days

Fig. 1. Response of ginger plantlets to *R. solanacearum in vitro*
1. Uninoculated plantlets, 2. *R. solanacearum* 10^9 cfu ml^{-1} 3. *R. solanacearum* 1 cfu ml^{-1}

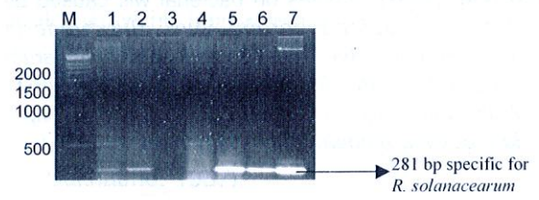

Fig. 2. Detection of *Ralstonia solanacearum* in surviving ginger rhizosphere
M. DNA size marker (500bp ladder)
Lane 1, 2 & 4: Soil DNA from pots where all the plants succumbed to wilt
Lane 3: Soil DNA from pots where disease escapes were found,
Lane 5 & 6: Soil DNA from wilt affected ginger from fields as positive control;
Lane 7: DNA from pure culture of *R. solanacearum* as positive control

s confirmed the absence of the pathogen in oil around the uninfected plants, which sitated three rounds of inoculation for reliable ning for bacterial wilt resistance. While dering the ease, large-scale adoption in short and simplicity over other methods, the soil ation is recommended from this study, for ning germplasm for bacterial wilt resistance in .

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