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Detection of *Ralstonia solanacearum* in ginger rhizomes using post-enrichment NCM-ELISA

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Abstract

Bacterial wilt, caused by *Ralstonia solanacearum*, is an important production constraint in ginger. The pathogen is seed rhizome borne and the low level of inoculum is very difficult to be detected and monitored by conventional methods. Pathogen detection and selection of healthy planting material is an important prerequisite for the production of a healthy crop of ginger in the field. In the present work we have evaluated the suitability of NCM-ELISA kit, developed at International Potato Center (CIP), Lima, Peru, for detecting bacterial wilt pathogen in ginger. The result indicated that the antibodies developed at CIP for potato strain of *R. solanacearum* was sensitive enough to detect *R. solanacearum* from ginger, chilli, *Chromolaena* and tomato. The sensitivity of the kit was determined to be 42 cells per ml of ginger extract when ELISA was performed after enrichment in selective medium. We have also tested the specificity of antibodies and found that the antibodies were specific for *R. solanacearum*. The extraction protocol (citrate buffer at pH 5.6) developed for potato was found to be suitable for ginger also.

Key words: bacterial wilt, ginger, NCM-ELISA, *Ralstonia solanacearum*, rhizome borne disease.

Introduction

Bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* 1996 is one of the important diseases that limits the production of ginger in India and many other tropical countries. Bacterial wilt is particularly severe in countries like India, Indonesia, Malaysia and China because of unregulated movement of latently infected seed rhizomes which is mainly responsible for the spread of the disease (Hayward 1991). Ginger is cultivated in virgin soil or in soil after a reasonable period of fallow. Incidence of wilt noticed in such fields, clearly reveals the rhizome-borne nature of *R. solanacearum* in ginger. Since there are no chemicals or biocontrol agents presently available for the management of bacterial wilt, the main practical approach to ensure a healthy ginger crop is through the planting of bacterial wilt - free seed rhizomes.

Many sensitive detection techniques have been developed for monitoring pathogens in seed tubers of potato (Seal & Elphinstone 1994; Priou *et al.* 1999b). However, no such technique is available for testing ginger seed rhizomes for the presence of *R. solanacearum* in India. The technique should be sensitive enough to detect a very low level of inoculum in the seed rhizome and also the technique should be easy to be performed even in poorly equipped

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laboratories. In the present work we have tested the suitability of the post-enrichment NCM-ELISA kit developed at the International Potato Centre (CIP), Lima, Peru for the detection of *R. solanacearum* in latently infected potato tubers, to detect the pathogen in ginger seed rhizome. The objectives of the work were to test (i) the ability of antibodies produced at CIP against potato isolates of *R. solanacearum* to detect isolates of *R. solanacearum* from ginger and other hosts and (ii) the suitability of the pathogen extraction protocol developed for potato tubers to ginger rhizome.

Materials and methods

R. solanacearum *strains*

Twenty strains of *Ralstonia solanacearum* were isolated from ginger, tomato and *Chromolaena* (Table 1). These isolates belonged to biovar III with the exception of some isolates obtained from Sikkim that were aberrant biovar III because they did not utilize dulcitol as a carbon source. The ginger strains were isolated from the major ginger growing locations in Kerala, Assam, Sikkim, West Bengal and Andhra Pradesh. Typical colonies of *R. solanacearum* (white fluidal colony with pink centre) from pure cultures on Kelman's medium (Kelman 1954) were maintained at 4° C in sterile distilled water and also in 20% glycerol at - 80° C.

NCM-ELISA

In order to know the specificity of the antibodies produced at CIP to recognize ginger isolates, pure cultures of 20 ginger isolates of *R. solanacearum* (Table 1) were tested using NCM-ELISA described by Priou & Gutarra (1998).

An 8 x 12 cm of 0.45 mm pore size nitro cellulose membrabe (NCM) (Biorad) was immersed for 5 minutes in 30 ml Tris Buffered Saline buffer (TBS) (0.02 mol l^{-1} Tris-HCl [pH 7.5], 0.05 mol l^{-1} NaCl, 0.01% NaN_3) for NCM-ELISA. Twenty µl of the samples were put on the membrane manually, transferred to a dry filter paper and air-dried for at least 60 minutes. The NCM dotted with the samples was incubated for 1 h in 30 ml of the blocking solution (2% non-fat powdered milk in TBS buffer) in a petridish (15 cm dia.), with gentle rotary agitation (50 rpm) and the membrane was incubated for 2 h with gentle agitation in 30 ml of the antibody solution (100 ml of *R. solanacearum*-specific antiserum diluted 1 : 1000 was added to another 30 ml of the same blocking solution). The membrane was washed to remove the unbound *R. solanacearum* antibodies with 30 ml T-TBS (TBS with 0.05% Tween-20) three times for 3 minutes each with constant agitation at 100 rpm. Then, the membrane was incubated for 1 h with gentle agitation with 30 ml of the conjugated solution, containing goat-antirabbit antibodies conjugated to alkaline phosphatase (Biorad), diluted 1 : 4000 in 30 ml of the blocking solution. The membrane was rinsed three times for 3 minutes each with T-TBS, with constant agitation (100 rpm) to remove the unbound conjugate. During the last washing, the colour development (substrate) solution was prepared by adding drop by drop while agitating, first 100 ml of NBT (p-nitro blue tetrazolium) solution and then 100 ml of BCIP (p-toluidine salt of 5-bromo, 4-chloro, 3-indolyl phosphate) solution in a dark flask containing 25 ml of substrate buffer (0.1 mol l^{-1} Tris base, 0.1 mol l^{-1} NaCl, 0.005 mol l^{-1} $MgCl_2$, $6H_2O$, pH 9.6). The membrane was then incubated with 25 ml of the substrate solution with gentle agitation for 5 to 30 minutes. The reaction was stopped by discarding the substrate solution and by rinsing the membrane thoroughly with tap water. The membrane was then placed on filter paper sheets to dry.

NCM-ELISA of ooze from infected plants

Twenty ml of bacterial oozes obtained from different infected hosts were dot-blotted on nitrocellulose membrane and the ELISA was performed as described above. The test was also performed with extracts from healthy ginger samples from different localities as well as from healthy plants adjacent to severely wilted plants.

Sensitivity of the test

In order to test the presence of low numbers of the bacterium, enrichment was performed by

Table 1. Isolates of *Ralstonia solanacearum* tested using CIP NCM-ELISA kit

Isolate	Crop	Location	Biovar	Serological Reaction
GRS Tms	Ginger	Thamarassery, Kerala	III	+
GRS Ktm1	Ginger	Kothamangalam, Kerala	III	+
GRS Pul	Ginger	Pulpally, Kerala	III	+
GRS Vyt	Ginger	Vythiri, Kerala	III	+
GRS Ktm2	Ginger	Kothamangalam, Kerala	III	+
GRS EW	Ginger	Sikkim	III dul$^-$	+
GRS SW 18	Ginger	Sikkim	III dul$^-$	+
GRS NWR	Ginger	Sikkim	III dul$^-$	+
GRS ISPS	Ginger	Sikkim	III dul$^-$	+
GRS White	Ginger	Sikkim	III dul$^-$	+
GRS Zah	Ginger	Zahirabad, Andhra Pradesh	III	-
GRS Mdm	Ginger	Madakimalai, Kerala	III	-
GRS Jor	Ginger	Jorhat, Assam	NT	-
GRS Kki	Ginger	Kahikuchi, Assam	III	+
GRS Soma	Ginger	Vythiri, Kerala	III	+
GRS Tms M1	Ginger, Spontaneous EPS$^-$ mutant	Thamarassery, Kerala	III	+
GRS Vyt Rif^R	Ginger, Spontaneous rifamycin resistant mutant	Vythiri, Kerala	III	+
GRS Tms M2	Ginger, Spontaneous EPS$^-$ mutant	Thamarassery, Kerala	III	+
TRS Cal	Tomato	Peruvannamuzhi, Kerala	III	+
ERS Cal	*Chromolaena*	Peruvannamuzhi, Kerala	III	+

+ Purple colour comparable to that of positive control (loaded with 20 ml of a water suspension of *R. solanacearum* potato strain CIP204, biovar 2A, at 10^8 cells ml^{-1}), - No colour development, NT: Not tested

incubating the extract in selective medium (Elphinstone *et al.* 1996) at 30° C with constant agitation (Priou *et al.* 1999a & b). A water suspension of *R. solanacearum* strain GRS Km1 (Biovar III) at 4.15 x 10^8 cells per ml was serially diluted until a final concentration of 0.0415 cells per ml of ginger extract was obtained. Five hundred ml of diluted *R. solanacearum* suspension was added to 500 ml of SMSA. The broth was incubated at 30° C with constant agitation for three days. One set of tubes was frozen away immediately after addition of bacterial cells and also after every 24 h upto 72 h. Twenty ml of the culture was dot-blotted on to NCM and the assay was performed as described earlier. The extract of ginger rhizome in citrate buffer without any enrichment was included as a check.

Specificity of NCM-ELISA

Specificity of Rs. antibodies raised against potato strain of *R. solanacearum* was tested using 121 bacterial isolates obtained from Bacterial Repository, Indian Institute of Spices Research, Calicut. These isolates were isolated from rhizosphere soils or infected plants of ginger, black pepper and cardamom from states of Kerala, Karnataka, Tamil Nadu, Sikkim, West Bengal, Himachal Pradesh and Assam. A loopful of bacterium was inoculated in 5 ml of nutrient broth for 12-15 h at 28° C with constant agitation and incubated. Well grown culture (20 ml) was dot blotted on to nitrocellulose membrane and ELISA was performed as mentioned above.

Results and discussion

The results obtained clearly indicated the ability of the antibodies included in the kit to detect

Table 2. Specificity of NCM-ELISA kit developed for detection of *Ralstonia solanacearum*

Source	No. of isolates tested	No. of typical *R. solanacearum*	No. of isolates closely related to *R. solanacearum**	No. of isolates showed positive reaction
Ginger (*Zingiber officinale*)	60	20	18	38
Black pepper (*Piper nigrum*)	51	0	0	9
Cardamom (*Elettaria cardamomum*)	1	0	0	0
Ageratum (*Ageratum conyzoides*)	2	0	2	0
Tomato (*Lycopersicon esculautum*)	1	1	0	1
Potato (*Solanum tuberosum*)	1	1	0	1
Chromolaena (*Chromolaena odorata*)	1	1	0	1
Capsicum (*Capsicum annuum*)	1	1	0	1
Teak (*Tectona grandis*)	1	0	1	1
Neem compost	2	0	0	0
Total	121	24	21	52

* Based on colonies developed on Kelman's medium

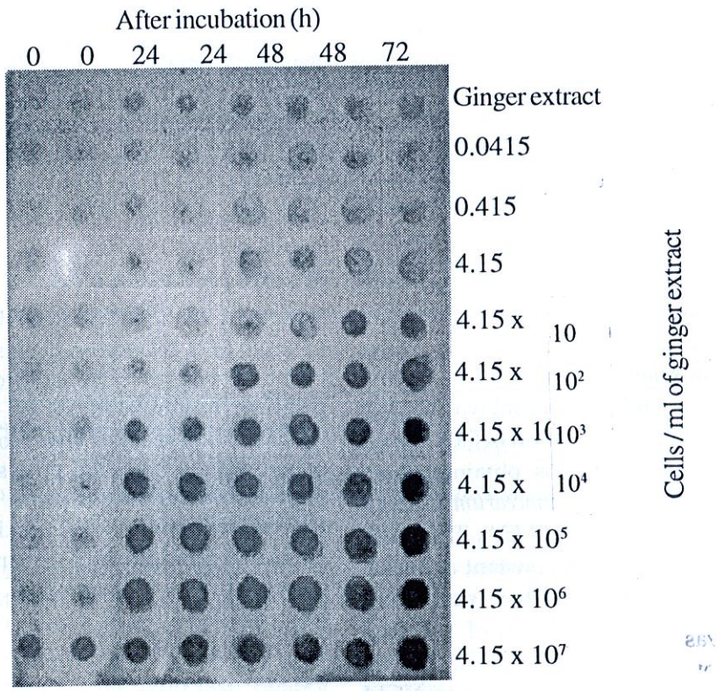

Fig. 1. NCM-ELISA after enrichment in selective medium

strains of *R. solanacearum* isolated from ginger, tomato and *Chromolaena* (Table 1). The colour development was comparable to that of positive control strips provided with the kit, loaded with water suspensions of *R. solanacearum* at 10^8 to 10^5 cells ml^{-1}. It is interesting to note that the antibodies could also detect aberrant isolates with dulcitol negative phenotype. Bacterial ooze from all the seven locations had a positive reaction, which testify for the suitability of the antibodies to detect *R. solanacearum* in plant material. The kit could detect *R. solanacearum* not only from ginger but also from tomato, *Chromolaena* and *Ageratum*.

The results of the specificity of NCM-ELISA kit are presented in Table 2. It is clear from the data that the ginger strains tested positively with the antibodies. Of the 50 ginger isolates assayed, 38 of them gave positive reaction. Among the 38 isolates 20 are typical *R. solanacearum* as identified on Kelman's medium, the remaining 18 isolates were very closely related to *R. solanacearum* in colony characters. Nine of the black pepper isolates had given positive reaction with *R. solanacearum* specific antibodies. These bacteria could be closely related to *R. solanacearum*. Other isolates, which gave positive reaction, were obtained from bacterial wilt affected teak, tomato, *Capsicum*, *Chromolaena*, potato and *Ageratum*. Except the isolates obtained from *Ageratum*, all others tested positive with NCM-ELISA.

The sensitivity of the assay is presented in Fig 1. The non-enriched extract when dot-blotted, could detect the bacteria at a concentration of 7.7×10^5 cells per ml of extract. However, the kit could detect as low as 41.5 cells of bacteria in the ginger extract when the extract was incubated in selective medium for 72 h at 30° C, while for potato extracts a sensitivity of 2-10 cells ml^{-1} w obtained after only 48 h of enrichment (Priou *et al.* 1999a & b). When the kit was evaluated using field samples, positive result was noticed with typically wilted samples and also with extracts dot blotted from healthy rhizomes collected from plants adjacent to the wilted plants. Several detection techniques have been suggested for detection of *R. solanacearum* from plant materials. Some of the techniques do not require isolation, culturing and purification of bacterium (Black & Elphinstone 1997). Such methods are advantageous as very often it is difficult to isolate or purify *R. solanacearum* from ginger, soil and other agricultural samples. Enzyme linked immunosorbent assay (ELISA) is one of the direct detection methods that can be performed in resource poor laboratories with limited facilities (Seal 1997). In conclusion, our results demonstrate that the kit developed at CIP for potato tubers could be used for monitoring *R. solanacearum* in latently infected seed rhizomes of ginger with a fair degree of accuracy.

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