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Biocontrol potential of rhizobacterial isolates against *Xanthomonas axonopodis* pv. *citri*, an incitant of Citrus bacterial canker

ADAM KAMEI[1], S. DUTTA[2], R. DANGMEI[3] AND D. C. KHATUA[4]
[1,2,3&4]*Department of Plant Pathology, Faculty of Agriculture*
[2]*AICRP on Vegetable Crops, Directorate of Research, Bidhan Chandra Krishi Viswavidyalaya, Kalyani, West Bengal 741235*

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Twenty six rhizobacteria were isolated from rhizospheres of different citrus and cabbage plants collected from Gangetic Alluvial regions of West Bengal, to develop effective biocontrol approaches for management of Citrus bacterial canker disease. The bacterial isolates were evaluated for their antagonistic activity against *Xanthomonas axonopodis* pv. *citri*(*Xac*). The antagonists were selected based on their inhibition capability against *Xac*. The antagonist bacterial isolate CT-1 was identified as potent antagonist of *Xac* by *in-vitro* antagonism technique. Inoculation of CT-1 in citrus leaf, followed by challenge inoculation of *Xac* after 24 h at a ratio of 8:1 (CT-1: *Xac*) caused maximum reduction (72 %) of *Xac* induced susceptible reaction (SR). In mono-inoculation of *Xac* a population of 1.9 X 10^9 cfu/cm^2 of citrus leaf was reached within 7 days. At this stage the susceptible reaction was fully developed on citrus leaf. Among the three different concentrations evaluated, 8:1 (CT-1: *Xac*) was most effective in protection of susceptible reaction when challenged at 24 h by *Xac*. Therefore, the antagonistic properties of these rhizobacteria can be exploited for potential biological control against Citrus bacterial canker pathogen.

Key words: Biological control, citrus canker, rhizobacteria

INTRODUCTION

Citrus bacterial canker is one of the most dreaded disease of citrus, in tropics and sub-tropics affecting all types of important citrus crops but more severe infection occur on Acid lime (*Citrus aurantifolia*) which is incited by *Xanthomonas axonopodis* pv. *citri* (*Xac*). Using copper-based bactericides is a standard method to control Citrus bacterial canker disease worldwide (Leite and Mohan, 1990). In countries where canker is present, integrated systems of compatible cultural practices and phytosanitary measures consisting of resistant hosts, removal of inoculums sources, properly designed windbreak systems, timely application of protective copper containing and antibiotic sprays are generally the most effective means of disease management (Das, 2003). Because of prolonged and continuous use of chemicals in disease management the pathogen has developed resistance. Therefore, the search for alternatives to chemical control of plant disease, especially biological control, has gained momentum in recent years (Compant *et al.*, 2005). The control of pathogens by biological agents has not been practiced on a large scale, but many experiments have shown the potential for further development as a promising approach in responding to disease control and consumers' health concerns. In this research work, we have sought to isolate from the rhizosphere so that some new antagonistic isolates that exhibit a highly inhibitory effect against plant pathogens can be obtained.

[1]Email : kameiadam.03@gmail.com

MATERIALS AND METHODS

Isolation of rhizobacteria

The soil samples were collected from rhizosphere of different citrus and cabbage plants. Ten gram of soil from each sample was crushed in a sterilized mortar with the help of a pestle and shaken with 100 ml of sterilized distilled water for 10-20 minutes to obtain a suspension. The suspension thus obtained was used for the isolation of rhizobacteria by dilution plating method using King's B medium (KB medium). The colony forming units (cfu) were counted on the plates after 48 h of growth at 28^{0}C (±2^{0}C) and the morphologically different colonies from each medium were picked up and purified.

In-vitro antagonism

One millilitre of pathogenic bacterial suspension (0.1 OD) was poured on plate with 25 ml of nutrient agar (40-45ºC) and spot inoculated with 48 h grown fluorescent pseudomonad isolates. After 3 days incubation at 26±1ºC the diameter of the inhibition zone was recorded.

Studies of population dynamics of Xac and rhizobacteria in plants

CT-1 was inoculated on citrus plants by syringe infiltration method (Klement, 1963) in pots. The challenge inoculation was done with *Xac* at 24 h after inoculation with CT-1 at the same inoculation area. Co-inoculation with CT-1 and *Xac* served as 0 hour challenge inoculation. The mono-inoculated, co-inoculated and challenge inoculated leaves were crushed in sterilized motor and pestle and serial dilution were made aseptically and population of both *Xac* and CT-1 were enumerated through dilution plating method.

Interaction between rhizobacteria (CT-1) and Xanthomonas axonopodis pv.citri in citrus plants

Potted citrus plants were used in the experiment. Fluorescent *Pseudomonas* spp. (CT-1; 0.5 X 10^{-7} cfu/ml) were used for pre-inoculation and challenge inoculation was done with *Xac* in the ratio of 1:1, 2:1 and 8:1 (CT-1: *Xac*) at challenge time intervals of 0 and 24 h. The inoculations were done by the syringe infiltration method (Klement, 1963) during 8 am to 11 am when most of the stomata remained open. Water soaked areas (about 2 cm) were developed on the adaxial site of the leaf by injecting desired amount of bacterial suspension with the help of a dispo van 2 ml syringe fitted with 22 gauge hypodermic needles. The challenge inoculations were done at the same spot on the pre-inoculated leaves. The leaves inoculated with *Xac* alone served as control. The study was carried out during June-July when the temperature range between 28-37^{0}C and humidity was around 70-80%. Disease severity was recorded 2,4, 5 and 7 days after challenge inoculation following the scale described by Hoa (1991). Percentage reduction in susceptible reaction was calculated with the following formula of Verma *et al.* (1994).

$$\text{Reduction (\%)} = \frac{\text{Grade obtained}}{\text{Maximum grade}} \times 100 - 100$$

Statistical analysis

The data collected were subjected to appropriate statistical analysis using software package SPSS Statistical Tool 16.00

RESULTS AND DISCUSSION

Isolation of rhizospheric bacteria of citrus were made on King's B medium. Twenty six rhizobacterial isolates were obtained. All the bacteria were screened for their *in vitro* antagonistic activity against *Xanthomonas axonopodis* pv. *citri* pathogens. Among these, CT-1 isolates showed potential antagonistic activity against the tested bacterial pathogen.

In-vitro antagonism of rhizospheric bacteria against bacterial plant pathogen and biochemical identification

Perusal of the data in Table 1 indicated that all the ten rhizobacterial isolates showed antagonistic activity under *in vitro* condition. Among the isolates, CT-1 was found to be the most effective isolate giving an inhibition zone of 14 mm. The antagonistic nature of some strains of bacteria *viz.*, *Pseudomonas syringae*, *Erwinia herbicola*, *Bacillus subtilis* and *Pseudomonas fluorescence* isolated from citrus phylloplane were reported to be antagonistic *in vitro* to the canker pathogen (Ota, 1983; Goto *et al.,* 1979; Kalita *et al.,* 1996). Based on the biochemical characterization following the identification scheme of Bossis *et al.*, (2000) re-

Table 1 : *In-vitro* antagonistic activity of the rhizobacterial isolates against bacterial plant pathogen

Name of the isolates	Isolated from the Rhizosphere of	*X.a.* pv. *citri*
CT-1	Citrus	14
CT-2	Citrus	13
CT-3	Citrus	9
CB-2	Cabbage	7
PCT-2	Pummelo	11
PCT-5	Pummelo	7
CB-2	Cabbage	6
PCT-7	Pummelo	8
CT-7	Citrus	7

vealed that all the antagonistic isolates were *fluorescent Pseudomonads*.

Population dynamics of Xac and rhizobacteria in plants

Perusal of the data presented in Table. 2 showed that in mono-inoculation of *Xac* a population of 1.9 X 10^9 cfu/cm^2 of citrus leaf was reached within 7 days. At this stage the susceptible reaction was fully developed on citrus leaf. Profuse bacterial oozing was found to be observed from 4th day onward after inoculation of *Xac.* The population of rhizobacteria under mono-inoculation condition remained almost the same level up to 5 days and afterwards declining trend was observed in citrus leaf. In challenge inoculation condition (CT-1 challenged by *Xac*), steady increase in population of CT-1 was observed. Under challenged inoculation condition, the rate of multiplication of *Xac* was found to be comparatively slower as compared to *Xac* mono-inoculation condition. This was also evident from the interaction studies (between CT-1 and *Xac* at 1:1, 4:1 and 8:1 level), where (24-72 %), reduction of susceptible reaction was observed when CT-1 was challenged by *Xac* in citrus plants at 0 and 24 h after inoculation. These observations, thus clearly indicated that reduced rate of multiplication of *Xac* in citrus plant system might be due to pre-colonization, nutrient acquisition and secretion of antimicrobial secondary metabolites in plant pathogen interactions under CT-1 rhizobacteria pre-inoculation condition. The steady increase in CT-1 population under *Xac* challenged inoculation (8.9 X 10^5 cfu at 7 days) as compared to population dynamics of CT-1 under mono-inoculation condition might be due to degradation of host tissues and release of nutrients from host by the patho-

Table 2 : Population dynamics of *Xanthomonas axonopodis* pv. *citri* and CT-1 (Fluorescent Pseudomonads) in mono and challenge inoculation condition (24 h).

Days after inoculation	cfu / cm^2 of citrus leaf area						
	Xac alone		CT-1 alone		CT-1 challenged with *Xac* (24 h)		
	Population	Ooze test	Population	Ooze test	*Xac*	CT-1	Ooze test
0	$^{f}2.23 \times 10^5$	-	4.5×10^4	-		4.3×10^4	-
1	$^{e}1.73 \times 10^6$	-	5.8×10^4	-	3.17×10^5	8.3×10^4	-
2	$^{d}9.17 \times 10^6$	+	6.2×10^4	-	9.30×10^5	2.1×10^5	-
3	$^{c}5.47 \times 10^7$	++	5.7×10^4	-	2.77×10^6	5.8×10^5	-
4	$^{b}9.47 \times 10^8$	++++	5.1×10^4	-	6.90×10^6	1.1×10^5	+
5	$^{a}2.10 \times 10^9$	++++	4.2×10^4	-	4.70×10^7	4.2×10^5	++
7	$^{a}1.90 \times 10^9$	++++	3.4×10^4	-	8.9×10^7	8.9×10^5	+++

gens and their utilization by CT-1 in host-pathogen and antagonist interaction. Sinclair *et al.* (1970) found 10 fold increased in carbohydrates, amino acids and proteins in the intercellular fluid of susceptible pepper plant after inoculation with *Xanthomonas vesicatoria.* Similarly, Borah *et al.* (2000) observed that the population of Plb (*Bacillus* sp.) increased in the presence of *Xanthomonas axonopodis* pv. *vignae-radiatae* (*Xav*) in susceptible cultivar and simultaneously reduced *Xav* population. Rhizobacteria and saprophytes present on the host surface could not multiply due to non-availability of certain essential nutrient, however, due to diseased condition, substantial increase in nutrient release takes place and help in fast multiplication of bacteria in host surface (Chowdhury and Verma, 1980).

Table 3 : Interaction between rhizobacteria and *Xac* in pre and challenged inoculation in citrus plant.

Treatments		Time of challenge (h)	Ratio	Disease Grade (Days after challenge inoculation)				Per cent reduction in Disease severity
Pre-inoculation	Challenge inoculation			2	4	5	7	
CT-1	*Xac*	0	8:1	0	1.4	2.0	2.4	52
CT-1	*Xac*	0	4:1	0	2.2	3.2	3.6	28
CT-1	*Xac*	0	1:1	0	2.8	3.8	4.4	12
CT-1	*Xac*	24	8:1	0	0.8	1.2	1.4	72
CT-1	*Xac*	24	4:1	0	1.2	2.2	2.6	48
CT-1	*Xac*	24	1:1	0	1.8	2.6	3.8	24
Control	*Xac*	-	-	0	3.4	4.2	5.0	-

Interaction between rhizobacteria and Xac in pre- and challenged inoculation in citrus plant

Perusal of the data presented (Table. 3), indicated that pre-inoculation of CT-1 (fluorescent pseudomonad) followed by challenge inoculation of *Xac* after 24 h at a ratio of 8:1 (CT-1 : *Xac*) caused maximum reduction (72%) of *X. a.* pv. *citri* induced susceptible reaction (SR). The reduction in SR was also recorded in challenge inoculation of *Xac* at 0 and 24 h at all ratios tested. Colonization of sites, production of antimicrobial secondary metabolites , induction of resistance and or competition for nutrients may be the likely explanation for the reduction of pathogen population and manifestation of susceptible reaction following pre-inoculation of antagonists on citrus plant system. The 24 h time gap further helped the antagonists for colonization, triggering the host defence system and production of sufficient antimicrobial secondary metabolites for combating against the pathogen. Similar observations were made by Sinha and Verma (1984), Bora *et al.* (1993) and Borah *et al.* (2000). Thus from the present observation, it may be concluded that, among the three concentrations evaluated, 8:1 (CT-1: *Xac*) was the most effective in protection of susceptible reaction when challenged at 24 h by *Xac.* Pre-inoculation of Plb followed by challenge inoculation of *Xav* after 24 h at a ratio of 1:2(Plb:*Xav*) could effectively control the bacterial leaf spot of mungbean caused by *Xav* (Borah *et al.,* 2000). Sinha (1981) also reported almost 100% protection against bacterial blight of cotton at 2:1 ratio (Plb: *Xam*).

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