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Biochemical and Molecular Characterization of *Erwinia* Species Causing Tip-over Disease of Banana

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ABSTRACT

Tip-over disease caused by pectolytic Erwinias is becoming a serious threat to banana plantations. Several workers in the past have reported it to be caused by *Erwinia carotovora* subsp *carotovora, Erwinia carotovora* subsp *atroseptica,* and *Erwinia chrysanthemi.* Morphological, biochemical and molecular studies were carried out to identify the exact cause of the disease. Of the nine isolates from different agro climatic regions of Karnataka and Andhra Pradesh, two isolates were similar to *Erwinia chrysanthemi* and the remaining isolates were similar to *Erwinia carotovora subsp carotovora*. Further, restriction fragment length polymorphism also showed the presence of two groups. Polymorphic banding pattern was obtained using *Alu I* and *Rsa I* enzymes indicating variation among isolates. It was found that *Erwinia carotovora* subsp *carotovora* is distributed in moderate climatic conditions where as *Erwinia chrysanthemi* is distributed in warmer regions. Thus the distribution and spread of *Erwinia* species is found to be influenced by environmental conditions.

Key words : Banana, Biochemical characterization, Erwinia, RFLP, Tip-over disease.

. The genus *Erwinia* is proposed by Winslow for Gram negative, non-spore forming, peritrichous, fermentative, rod shaped bacteria and it belongs to the family Enterobacteriaceae. Among the pectolytic Erwinias, *Erwinia carotovora* subsp *carotovora* seems to be more ubiquitous and is widely distributed in the world with broad host range, while the strains may exhibit variation in pathogenicity to plants. *Erwinia chrysanthemi,* another pectolytic Erwinia causing soft rot and wilt diseases, is also widely distributed in many temperate and tropical areas.

Tip-over or Bacterial heart rot of Banana is one among the diseases caused by pectolytic Erwinias and is becoming serious threat to banana plantations. There is a controversy over the exact identity of the causal bacterium. Several workers in the past have reported it to be *Erwinia carotovora* subsp *carotovora, Erwinia carotovora* subsp *atroseptica,* and *Erwinia chrysanthemi* from across the world (Dickey and Victoria, 1980; Choi *et al.,*1988). From India Edward *et al.* (1973) and Lakshmanan and Mohan (1986) have reported the causal organism as *Erwinia carotovora.* But chattopadhyay and Mukherjee (1986) from west Bengal attributed *Erwinia chrysanthemi* as the causal agent.

Classical techniques like morphological, cultural, biochemical and physiological studies help to characterize the pathogen to some extent. Development of selective media like CVP (Crystal Violet Pectate) minimizes the problem of sensitivity. Immunological techniques have been developed and their efficiency is increased by culture on selective medium. But the serological tests are complicated because of high number of serogroups with in *Erwinia carotovora.* New molecular tools like hybridization probes have been demonstrated to be very efficient in plant disease diagnosis and some probes have been developed for pectolytic Erwinias. However, the use of such probes for routine tests is limited by the relatively low signal of the non radioactive labeling. PCR and RFLP not only enhance the sensitivity of detection but also to find out strainal variation if any and their distribution in various geographical areas.

Here, we studied the biochemical and physiological characterization of *Erwinia* species as well as molecular characterization of the same. We collected the *Erwinia* isolates causing tip-over disease in banana from different agro climatic regions of Karnataka and Andhra Pradesh. In addition to conventional techniques for identification of the bacterium, we used six restriction enzymes in restriction enzyme analysis of whole genomic DNA of different isolates.

MATERIAL AND METHODS

Bacterial isolates: All the nine isolates of *Erwinia* spp. from banana collected form different parts of Karnataka and Andhra Pradesh designated as I_1 (Vemgal, Kolar district), I_2 (Doddabelavangala, Bangalore district), I_3 (Kanakapura, Bangalore district), I_4 (Chikballapur, Kolar district), I_5 (Nelamangala, Bangalore district), I_6 (Kyathsandra, Tumkur district), I_7 (Bijapur, Bijapur district), I_8 (Gowribidanur, Kolar district) and I_9 (Kovvur, W.G. Dt. A.P.) were used.

Morphological and staining characters:

The morphological characteristics such as cell shape, size, gram reaction, flagellar, capsule and spore staining were studied as described by Society of American Bacteriologists (Schaad and Stall, 1988).

Physiological and biochemical characteristics:

The physiological and biochemical characteristics of all the strains were studied for pectate degradation, potato soft rot, gelatin liquefaction, acetoin production, sensitivity to erythromycin, gas from glucose, indole production, reducing substances from sucrose, growth at 36^{0}C and 39^{0}C and acid production from various substances like d-lactose, trehalose, maltose, cellobiose, etc. The tests were conducted as per the methods described by Dickey and Victoria, 1980 and Schaad and Stall, 1988.

Chromosomal DNA isolation:

Bacteria were grown overnight at 28^{o}C in Luria Broth (Sambrook *et al.,*1989). 50 ml of overnight culture was micro centrifuged and the DNA was extracted by using a modification of the method of Klotz and Zimm (1972). The bacteria were resuspended and washed in 15ml of TE buffer (pH 8.0) with 1% SDS, lysed with lysozyme. (10mM Tris, 1mM EDTA (pH 8.0)). The mixture was deproteinized by sequential phenol and chloroform-iso amyl alcohol (24:1 vol/vol) extraction. The DNA was precipitated in ethanol, resuspended in TE buffer and quantified by spectrophotometry at 260nm.

Restriction enzyme analysis of genomic DNA:

Six restriction enzymes *viz.,* BamH I, Hind III, Alu I, Rsa I, Hae III, Hpa II were used. 10 μg of DNA was digested with 1μl of all the six restriction enzymes in separate experiments and incubated at 37^{o}C for 1hr, 2hr and overnight duration in a 25μl volume. The digested samples were analyzed by agarose gel electrophoresis.

RESULTS AND DISCUSSION

Comparison of cultural characteristics of the bacteria isolated from different places:

Even though most of the isolates *viz.,* I_1 to I_6 and I_8 showed similar cultural characteristics, two isolates (I_7 and I_9) showed slight differences. Most of the isolates yielded bacterial colonies that were creamish yellow, mucoid, glistening, convex, round to irregular in shape. The isolate I_7, the bacterium isolated from diseased plant material obtained from Bijapur showed dark yellow colonies which are round to irregular in shape, moderately mucoid and slightly convex in nature. The isolate I_9 also showed slight difference in colony characteristics.

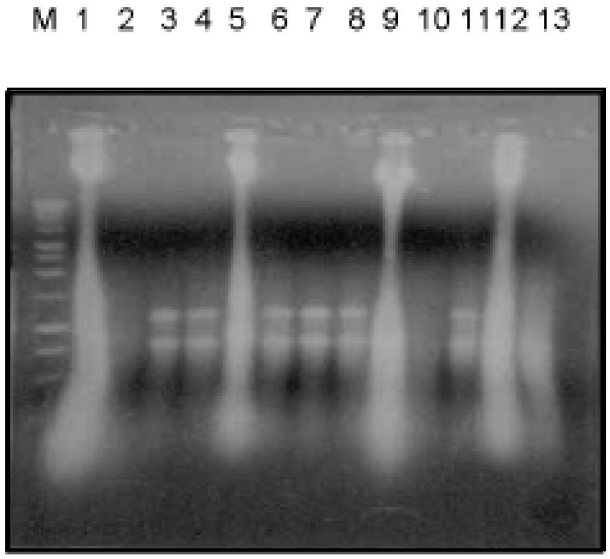
Morphological and biochemical characteristics:

The results of various morphological and biochemical tests are presented in Table-I. All the isolates were rod shaped, gram negative, capsulated, peritrichously flagellated, non spore forming and non acid fast. All the isolates were similar for the following biochemical characters *viz.,* positive for pectate degradation on CVP medium, growth at 36^{0}C and 39^{0}C, catalase production and negative for indole production. Further they gave positive result for the production of acid from various sugars like lactose, trehalose, maltose and cellobiose. All the isolates caused soft rot of inoculated potato slices.

The isolates showed significant differences for the other biochemical characteristics mentioned in Table-I. All the isolates except I_7 and I_9 showed similar result for the biochemical characteristics studied. However isolate I_9 showed slight difference with regard to sensitivity to erythromycin and reducing sugars. The isolates (I_1 to I_6 and I_8) except I_7 and I_9 produced acetoin, gas from glucose and acid from sugars like lactose, trehalose, maltose and cellobiose. They were negative for gelatin liquefaction and insensitive to erythromycin.

Isolate I_9 showed slight difference from all other isolates of Karnataka. The isolate was positive for reducing substances from sucrose and showed weak reaction to acid production from lactose and sensitive to erythromycin. It produced acid from lactose after 4 days where as all other isolates produced acid with in 48hrs. Isolate I_7 from Bijapur showed marked difference in all the biochemical characteristics. It was positive to gelatin liquefaction, sensitive to erythromycin, reducing substances from sucrose and was negative to acetoin production, gas production from glucose and a weak positive reaction to production of acid from various sugars like lactose, trehalose, maltose and cellobiose.

Fig I: Restriction fragment length analysis profiles of all the isolates with *Alu I* enzyme



Lanes :
M: Marker
1 and 2: undigested and digested DNA from I_1 respectively
3 and 4: digested DNA from I_2 and I_3 respectively
5 and 6: undigested and digested DNA from I_4 respectively
7 and 8: digested DNA from I_5 and I_6 respectively
9 and 10: undigested and digested DNA from I_7 respectively
11: digested DNA from I_8
12 and 13: undigested and digested DNA from I_9 respectively

Similar results have been reported in the past. Choi *et al* (1988) studied seven bacterial isolates causing soft rot of banana and reported three isolates as *Erwinia carotovora subsp carotovora* and four as *Pseudomonas cichorii*. Hassanzadeh (1990) isolated 10 strains of *Erwinia carotovora* from diseased banana trees in different locations in chabahar and compared with an isolate of rotted cucumber fruit. Biochemical and pathogenicity tests indicated that the isolates are intermediate between *Erwinia carotovora* and *Erwinia chrysanthemi.*

Chromosomal DNA isolation:

Good amount of genomic DNA was obtained from all the isolates and the concentration was measured by ultraviolet spectrophotometry.

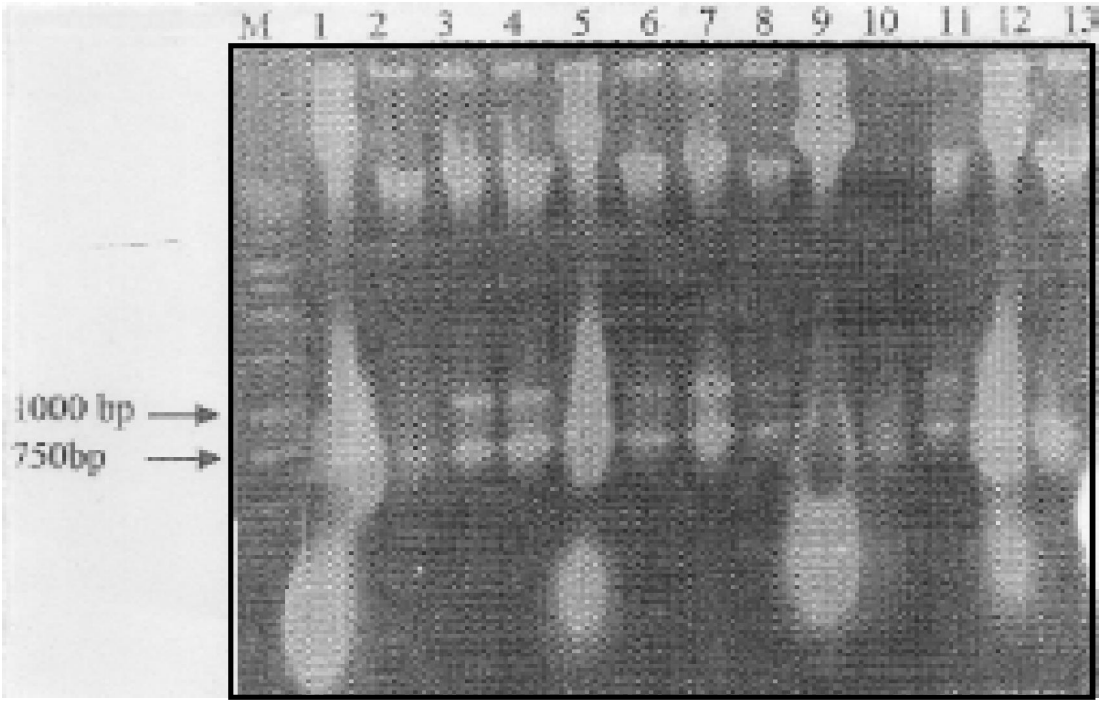
RFLP analysis:

An RFLP analysis was undertaken on the Erwinia isolates from Tip-over disease affected banana plants by digesting the genomic DNA with several enzymes. Of all these restriction enzymes BamH I and Hind III did not give any polymorphism. Hae III and Hpa II also did not show any bands but there was a streak formation in all the isolates. Conversely two enzymes Alu I and Rsa I gave polymorphic banding pattern among the isolates. Combined result of these two restriction enzymes gave RFLP groups.

Restriction digestion of the isolates with Alu I enzyme produced different banding pattern among the isolates. Isolates I_1 and I_7 produced no bands, where as other isolates produced distinct bands at 1000bp and 750bp, but the band at 1000bp in case of I_9 was not clear (Fig I). Restriction digestion with Rsa I enzyme also produced distinct banding pattern. Isolates I_2, I_3, I_4, I_5, I_6 and I_8 produced two distinct bands at 1000bp and 750bp. Isolate I_7 has produced only one band at 750bp where as the band at 1000bp was not there. Isolates I_1 and I_9 produced two bands at 1000bp and 750 bp where as the band at 750 bp was not clear (Fig II).

The RFLP analysis indicated that both Alu I and Rsa I enzyme restriction patterns clearly distinguishes I_7 and I_9 from rest of the strains, I_1 falls as the intermediary between first group and second

Fig II: Restriction fragment length analysis profiles of all the isolates with *Rsa I* enzyme



Lanes :
M: Marker
1 and 2: undigested and digested DNA from I_1 respectively
3 and 4: digested DNA from I_2 and I_3 respectively
5 and 6: undigested and digested DNA from I_4 respectively
7 and 8: digested DNA from I_5 and I_6 respectively
9 and 10: undigested and digested DNA from I_7 respectively
11: digested DNA from I_8
12 and 13: undigested and digested DNA from I_9 respectively

group. The results of genomic finger prints proved that the distribution of restriction endonuclease cleavage sites were stable in the bacterial genome. The isolates used in the present study were collected at different places and also showed difference in the biochemical tests. Based on RFLP pattern these isolates may fall under *Erwinia carotovora* and I_7 and I_9 may fall under *Erwinia chrysanthemi.* Similar RFLP grouping was done in *Erwinia carotovora* (Darasse *et al.,* 1994; Fiori *et al.,* 2005; 2002), *Erwinia chrysanthemi, Erwinia amylovora* and *Xanthomonas campestris* and *Xanthomonas graminis* (Tsygankova *et al.,* 2004;).

And it could be inferred that *Erwinia carotovora sub sp. carotova* (isolates I_1 to I_6 and I_8) is distributed in moderate climatic conditions existing in southern parts of Karnataka, while *Erwinia chrysanthemi* (isolats I_7 and I_9) is prevailing in Bijapur and West Godavari districts of Andhra Pradesh, which were comparatively warm regions where the annual rainfall is low with high temperature and low relative humidity. Thus the distribution and spread of *Erwinia* species is influenced by the environmental conditions.

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Table 1. Comparative morphological, physiological and biochemical characteristics of the nine isolates of Erwinia causing tip-over disease of banana.

S.No	Charecteristics	Isolates		
		I_1 to I_6 and I_8	I_7	I_9
I	Morphology			
	Shape	Small rods	Small rods	Small rods
	Occurrence	In singles	In singles	In singles
II	Staining			
	Gram staining	Negative	Negative	Negative
	Spore staining	Non spore forming	Non spore forming	Non spore forming
	Capsule	Noncapsulated	Noncapsulated	Noncapsulated
	Flagella staining	Peritrichously flagellated	Peritrichously flagellated	Peritrichously flagellated
III	Biochemical characteristics			
1	Pectate degradation	+	+	+
2	Potato soft rot	+	V (variable)	+
3	Gelatin liquefaction	-	+	-
4	Acetoin production	+	-	+
5	Sensitivity to erythromycin	-	+	+
6	Gas from glucose	+	-	+
7	Indole production	-	-	-
8	Reducing substances from sucrose	-	+	+
9	Growth at 36^0C and 39^0C	+	+	+
10	Acid from lactose	++ (2 days)	++ (2 days)	+ (weak) (4 days)
11	Trehalose	+=	+	++
12	Maltose	++	+	++
13	Cellobiose	++	+	++
14	Catalase reaction	+	+	+

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