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Phenotypic and Biochemical Characterization of *Pectobacterium carotovorum* sub sp *carotovorum*, the Incitant of Onion Soft Rot

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A B S T R A C T

Onion (*Allium cepa* L.) is one of the most important commercial vegetable crops which have significant nutritional value to the human diet besides possessing medicinal properties. It is affected by various diseases among which soft rot incited by *Pectobacterium carotovorum* subsp *carotovorum* (Pcc) is an economically important disease in the field and storage. In the present study, survey carried out in Madurai, Perambalur and Salem districts of Tamil Nadu revealed that the maximum incidence of onion soft rot (63.25 %) was noticed in Muthampatti of Salem district of Tamil Nadu whereas lowest incidence (16.39 %) was noticed in Arasakulam of Madurai district. Ten bacterial isolates were isolated from symptomatic onion which produced grey and convex slimy colonies on nutrient agar medium. Biochemical characterization revealed that all the isolates were tested positive for potato soft rot test, catalase production, OF test, gelatin liquefaction, KOH solubility, methyl red test, and growth in 5% NaCl and negative for gram reaction, oxidase and gas formation. *In vitro* pathogenicity tests showed that, the isolate PCC 6 was found to be high virulent which developed typical water-soaked symptoms in onion bulbs after 2 days and soft rot symptoms were expressed on 4 days after inoculation. The presence of deep pits and cavities zone in agar plates confirmed that all the isolates were *Pectobacterium* spp. Based on the phenotypic, and biochemical characterization, the isolates were identified as *Pectobacterium carotovorum* subsp. *carotovorum*.

Introduction

Onion (*Allium cepa*) is one of the important food ingredients widely used in our day-to-day life for culinary purpose (Virginia, 2006). Among the various constraints in onion cultivation, bacterial soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum* is an important disease which causes economical loss to the farmers (Agrios, 2006). The disease is most common in storage or transit; (Sherf and Macnab, 1986). However, this disease can develop on onions in the field before harvest, after heavy rains and also when the leaves dry (kim *et al.*, 2002). *Pectobacterium carotovorum* is a species of Gram negative, facultatively anaerobic, rod-shaped bacteria (Whitehead *et al.*, 2002). *P. carotovorum* subspecies *carotovorum* is a phytopathogenic

enterobacterium which is responsible for soft rot, a disease which cause tissue maceration by secreting large amounts of plant cell wall degrading enzymes (PCWDE) which are linked to the type II secretion system (T2SS) (Toth *et al.,* 2003). The disease show characteristic water soaked, slimy rotten appearance of infected plant tissue. A foul smelling viscous fluid oozes from the neck when the infected bulbs are squeezed. In the field, the youngest leaves or the entire leaves of affected plants are bleached and wilted. Characterization and identification of pectolytic erwinia are based on biochemical and phenotypic characteristics (De Boer and Kelman, 2000) and recently molecular techniques have also been applied. *P. carotovorum* subsp. *carotovorum* has been previously isolated from potato producing areas in Iran (Soltani-Nejad *et al.,* 2005). *P. atrosepticum* also has been reported in south-west Iran, but the identification was based only on biochemical and physiological characteristics (Kreig *et al.,* 1984). The bacterial isolates originated from single colonies were tested for their ability to cause soft rot on potato tubers following standard procedure (Lelliot *et al.,* 1984).The biochemical or genetic characteristics of *P. carotovorum* strains show diversity among the same subspecies (Nabhan *et al.,* 2006). The aim of the study is to make identification and characterization of the causal agent of bacterial soft rot of onion by using morphological and biochemical methods.

Materials and Methods

Survey and collection of pathogen

A survey was conducted in onion growing areas to assess the soft rot disease incidence in Tamil Nadu. Infected onion bulb with soft rot symptoms were collected from the field and market of Madurai, Salem and Perambalur districts of Tamil Nadu. The soft rot disease incidence was assessed by counting the number of affected plants / total number of plants in each plot (25 m^2). In each area, three fields were assessed and the mean disease incidence was calculated. The collected samples were used for further studies.

Isolation of soft rot bacteria

Soft rot bacterial isolates were isolated from different onion samples by "Streak plate" technique as described by Mortensen (1997) and Kim *et al.,* (2002). Nutrient agar (NA) medium was used for the isolation of soft rot bacteria. A small part from the margin of rotted tissues of the infected onion bulbs were removed with a scalpel and were surface disinfected with 70% ethanol for 2-3 min. Sterilized samples were washed several times in sterilized water to remove the residues. The samples were placed in petridishes containing sterilized water and were crushed with a sterile scalpel. After crushing, the petridishes were kept undisturbed for 10-15 min to release the bacteria associated with rotted tissues. One loop full of resulting suspension (water containing bacteria) was streaked on the solidified NA medium in each plate. The plates were incubated at 30˚C for 48 hr (Rahman *et al.,* 2017). Characteristic individual bacterial colonies that appeared on NA medium were picked up using a bacterial loop and transferred to another plate. Purification of bacterial colony was done by re-streaking of single colony on another fresh plate.

Pathogenecity test

The pathogenecity test was conducted under *in vitro* conditions in onion bulb. Wounds were made with 0.25 G syringe needle in the onion bulb to inoculate the pathogen. The bacterial inoculum was obtained from 2 days old culture on NA broth incubated at 30˚C

and adjusted to 10^7 Cfu/ml. The bacterial culture @ 100 µL was inoculated longitudinally from the neck part and transversely from the outer to inner part of the onion bulb. Inoculated onion bulbs were maintained at 30˚C for 7 day and examined daily for development of symptoms (Abd-Alla *et al.,* 2010). Sterile water was used as a negative control. The bacteria from the infected bulbs were re-isolated and the characters were compared with the original isolate and the Koch's postulates were proved.

Biochemical characterization

A series of biochemical tests were performed for characterization of the isolated pathogen using the protocol already described. The biochemical tests were a. Potato soft rot test b. fermentation of glucose test (OF test) (Hugh and Leifson, 1953), c. Gram reaction (Suslow *et al.,* 1982), d. oxidase reaction (Kavocs, 1956), e. Catalase production (Hayward, 1992), f. Gelatin liquefaction test (Schaad, 1988), g. Urease production (Schaad, 1988), h. Methyl red test, i. Gas formation (Hugh and Leifson, 1953). In crystal violate pectate test, the detection of soft rot erwini as depends on the characteristic deep pits or cavities formed by colonies of the bacteria in Crystal violate pectate medium.

Statistical analysis

Experimental datas were statistically analyzed using analysis of variance (ANOVA) and the SPSS version 17.0. The treatment means were separated at 5% significance level using Duncan's Multiple Range Test (DMRT).

Results and Discussion

Survey and isolation of pathogen

A survey carried out in Madurai, Perambalur and Salem districts of Tamil Nadu revealed that the maximum incidence of onion soft rot (63.25 %) was noticed in Muthampatti of Salem district of Tamil Nadu whereas lowest incidence (16.39 %) was noticed in Arasakulam of Madurai district.

Ten isolates of *Pectobacterium carotovorum* subsp *carotovorum* was isolated from infected onion from various field and market regions. The isolates produced creamy white slimy colonies in nutrient agar which was maintained in pure culture for further studies (Table 1 & 2).

Pathogenecity test

All the isolates recovered from diseased onion were individually inoculated into healthy onion *in vitro* by wound inoculation method. Among these ten isolates, PCC 6 was found to be high virulent and PCC 1 was found to be least virulent.

The isolate produced water soaked lesion in onion 2 days after inoculation and typical soft rot symptoms appeared after 4 days (Fig 1a and 1b).

Biochemical characterization of the pathogen

The results of the biochemical characterization revealed that all the isolates were tested positive for potato soft rot test, catalase production, OF test, gelatin liquefaction, KOH solubility, methyl red test, and Growth in 5% NaCl and negative for gram reaction, oxidase and gas formation (Table 3).

Crystal violate pectate test

All the isolates produced deep pits and cavities zone in crystal violet pectate (CVP) medium which confirmed the isolates as *Pectobacterium* sp. (Fig. 2).

Table.1 Origin and collection of isolates used in this study

S.No	Place of collection	Districts	Isolate name	Geo co-ordinates		Percent disease incidence (%)*
				Latitude	Longitude	
1	Arasakulam	Madurai	PCC1	9.79N	78.79E	16.39
2	Aaviyur	Madurai	PCC2	9.98N	78.11E	23.96
3	Anukkur	Perambalur	PCC3	11.66N	78.48E	21.68
4	Keelaperambalur	Perambalur	PCC4	11.08N	79.57E	20.29
5	Valappady	Salem	PCC5	11.03N	78.03E	21.55
6	Muthampatti	Salem	PCC6	11.25N	78.11E	63.25
7	Thukkiyampalayam	Salem	PCC7	11.22N	78.04E	25.41
8	Othakadai (Market)	Madurai	PCC8	9.35N	78.16E	26.26
9	Simakkal (Market)	Madurai	PCC9	9.36N	78.19E	30.56
10	Maatuthavani (Market)	Madurai	PCC10	9.48N	78.84E	17.97
CD (p=0.05)						0.84

*Mean of three replications

Table.2 Cultural characters of different isolates of *Pectobacterium carotovorum* subsp *carotovorum*

Isolate	Colony colour	Appearance
PCC1	Creamy white	Slimy
PCC2	Grayish white	Slimy
PCC3	Creamy white	Slimy
PCC4	white	Slimy
PCC5	Creamy white	Slimy
PCC6	Creamy white	Slimy
PCC7	Creamy white	Slimy
PCC8	Creamy white	Slimy
PCC9	Grayish creamy white	Slimy
PCC10	Grayish creamy white	Slimy

Table.3 Biochemical characteristics of soft rot bacteria isolated from Onion

Isolates	Biochemical test									
	Gram reaction	Potato soft rot	Catal ase	Oxidase	OF test	Gelatin liquefaction	KOH solubility	Methyl red test	Gas formation	Growth in 5%Nacl
PCC1	-	+	+	-	+	+	+	+	-	+
PCC2	-	+	+	-	+	+	+	+	-	+
PCC3	-	+	+	-	+	+	+	+	-	+
PCC4	-	+	+	-	+	+	+	+	-	+
PCC5	-	+	+	-	+	+	+	+	-	+
PCC6	-	+	+	-	+	+	+	+	-	+
PCC7	-	+	+	-	+	+	+	+	-	+
PCC8	-	+	+	-	+	+	+	+	-	+
PCC9	-	+	+	-	+	+	+	+	-	+
PCC10	-	+	+	-	+	+	+	+	-	+

Fig.1a Pathogenicity of *Pectobacterium* spp. in Onion

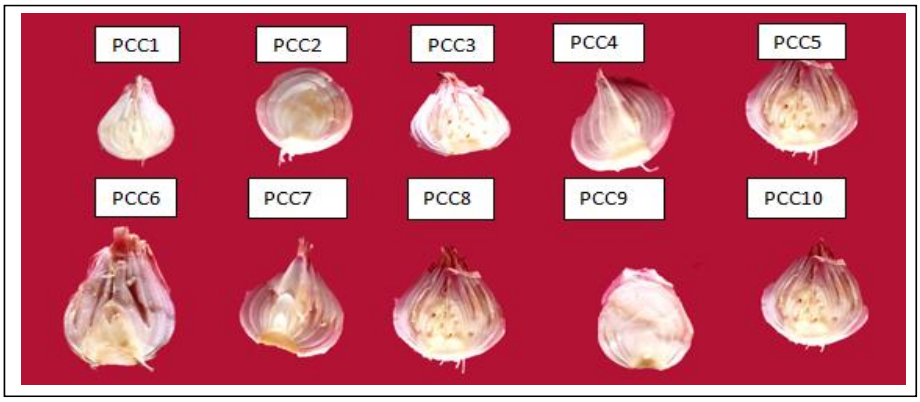


Fig.1b Pathogenicity test of different isolates of onion soft rot

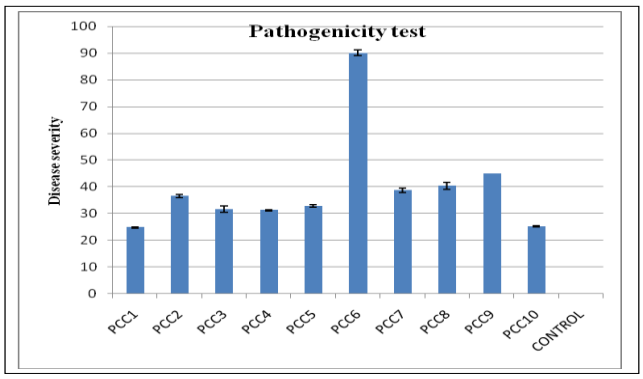
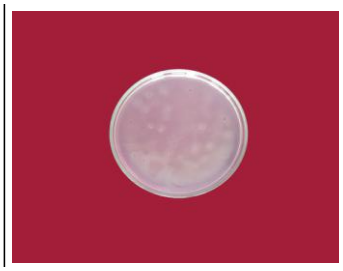


Fig.2 Crystal violet pectate test



In this study, pectolytic bacterial isolates from onion was identified using a combination of phenotypic and biochemical tests. The results are in accordance with Toth *et al.*, 1999; Ma *et al.*, 2007; Baghaee-Ravari *et al.*, 2011. Characterization and identification of pectolytic erwinias are traditionally based on biochemical and phenotypic characteristics and more recently molecular techniques have also been applied by De Boer and Kelman (2000). The most commonly used methods are biochemical tests (Dickey and Kelman, 1988) and pathogenicity tests (Smith and Bartz, 1990). *Pectobacterium* form deep cavities in CVP medium which is a very useful to confirm the pathogen as *Pectobacterium* spp. CVP medium and bioassays on Onion used for pectinolytic

strains selection, connected with use of the genus specific primers Y1/ Y2 (Darrasse *et al.,* 1994), proved to be very useful, cheap and efficient method for identification of *Pectobacterium* spp., (Nazerian *et al.,* 2013). *Erwinia carotovora* subspecies *atroseptica* has been recorded as soft rot pathogen for few vegetables which is restricted to the cool and temperate regions and has a host range limited almost exclusively to potato (Fahy and Persley, 1983, Toth *et al.,* 2003). so this is from the few reports of bacterial rot of onion caused by *Erwinia carotovora* subspecies *atroseptica*. Hence, in this study, survey, collection, isolation and characterization of onion bacterial soft rot was done. The pathogen was isolated from onion bulb showing soft rot symptom and incubated. The identification of the pathogen in our present study based on phenotypic and biochemical characters. All of the bacterial isolates originated from single colonies were tested for their ability to cause soft rot on onion bulbs following standard procedure (Gore *et al.,* 2020). According to the research conducted by Sendhilvel *et al*., (2005), the colonies were greyish white, smooth, round, occur singly and occasionally in short chain. Better growth of the bacterium was observed at 37°C. The isolate of *Pectobacterium carotovora* subsp. *carotovora* showed negative reaction towards gram reaction and oxidase test and gas formation which is in accordance with (Gore *et al.,* 2020) where as it shows positive reaction towards KOH test, catalase test, potato soft rot test, gelatin liquification test, growth in 5% Nacl test, H2S production test, indole production test, OF test and methyl red test. We conclude that the isolated bacterium in the ooze was identified as *Pectobacterium carotovorum* subsp. *carotovorum* and its pathogenicity was established. Thus, from the confirmation of the pathogen, it can be utilized for evaluating the best biocontrol agents for management of this disease.

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