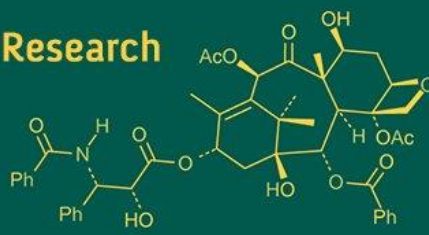
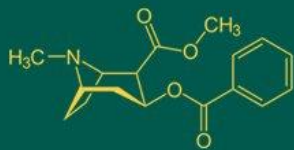


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Molecular identification of pathogen causing leaf rot of onion

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Abstract

A present investigation for identification of pathogen causing leaf rot of onion (*Allium cepa*) was carried out at Department of Plant Pathology, University of Horticultural Sciences Bagalkot. Onion was infested by a new disease called leaf rot, in onion growing areas of Karnataka. The disease is becoming threat to the good yield of onion and causing more damage to the leaves and loss of photosynthesis, which in turn lead to less yield. Hence the identification of the causal agent is very important to manage the disease. The identification of pathogen is done through both biochemical and molecular analysis method.

Keywords: Onion, leaf rot, pathogen identification, biochemical analysis, molecular characterization

Introduction

Initially small water-soaked spots appeared, then became small constricted area and then formed irregular translucent water-soaked oily lesion on leaves. Later became necrotic and hanging down of leaves at constricted area. Lower leaves bent down and necrotic area on the leaves formed and extended. The bent plants at the neck region have been observed. The leaves became sunken and soft at affected area, when the leaves were still green. At severe stage, leaves turned yellow and all the leaves were collapsed to the ground and the plants dies. Freshly cut infected portion of the plant showed milky white bacterial exudate in the ooze test.

Materials and Methods

Identification of bacteria by 16S rRNA methods through PCR technique

Identification of culture by molecular method

PCR based approach was carried out to confirm the identification of the bacterium. The following procedure was followed for identification of unknown bacteria.

Materials required

- Forward and reverse primers (100 ng/μl.)
- 10 mm deoxy nucleotide triphosphates (dNTPs viz., dATP, dGTP, dTTP)
- Taq DNA polymerase
- TAE Buffer 50X
- Nuclease free water
- Taq DNA polymerase buffer (10X)
- Agarose loading dye (6X), ethidium bromide
- DNA size of marker 100 bp etc.

Instruments: Thermal cycler, centrifuge, micropipettes, gloves, pre autoclaved microtips, eppendorf tubes, PCR tubes, electrophoresis unit etc.

Genomic DNA extraction

For extraction of genomic DNA from the test bacterial culture, the single colony from the fresh one day old culture was taken and inoculated in the 50 ml of nutrient broth and kept for the shaking incubation at 28±2 °C for 48h.

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After 48h, 1.5 ml of culture was taken through pipette and transferred into 1.5 ml eppendorf tube. Then centrifuged for 2 min at 14,000 rpm at room temperature and added 550 µl of TE buffer + lysozyme (0.1 mg/ml) and resuspended by pipetting. Incubated the suspension for 30 min at 37 °C and then mixed the contents by flipping tube. Added 80 µl of CTAB/NaCl and mixed by flipping tube. Incubate for 10 min at 65 °C. Added 700 µl of Chloroform/Isoamylalcohol (24: 1) twice. Mixed the contents by flipping tubes for 15 sec. and centrifuged for 5 min at 14,000 rpm at RT. Transferred the aqueous layer (without disturbing or carrying over any of the white middle layer) to new 1.5 ml tube. This step was repeated several times when proteins are taken up with the aqueous layer. Added 2 volume of 2-propanol (Isopropanol) and inverted several times to mix. Centrifuged for 15 min at 4 °C and stored at -20 °C for 3h. Gently drained out the supernatant and carefully added approx. 1 ml ice cold 70 percent ethanol. Collected the DNA by centrifugation for several minutes at 14,000 rpm at RT.

Carefully removed the supernatant and evaporated the remained ethanol in the laminar flow cabin. Dissolved the DNA in 50 µl sterilized distilled water or nuclease free water. Added 6 µl of RNase to DNA solution, to remove the contaminating RNA from precipitate. Dissolved DNA was incubated over night at 4 °C and then utilized the bacterial DNA for molecular identification.

Primers

A representative isolate of the causal agent was subjected to PCR based identification by utilizing the primers of 4 different relevant bacterial pathogens viz., *Acinetobacter haemolyticus*, *Acinetobacter calcoaceticus*, *Enterobacter cloacae* strain ATCC 13047 and *Pectobacterium carotovorum* subsp. *carotovorum*. The primers of these bacteria were designed from nucleotide sequence available in the data base. The primers of relevant bacteria were presented below.

The primers of relevant bacteria were presented below.

Organism	Primer name	Sequence	No. of base pairs	Product length
1) <i>Acinetobacter haemolyticus</i>	AHF	F 5'CAGGGGATCTTCGGACCTTG-3'	20	256 bp
	AHR	R 3'-GCCTCCTCCTCGCTTAAAGT-5'	20	
2) <i>Acinetobacter calcoaceticus</i>	ACF	F 5'-TGCTACTGATCTTAGCGGCG-3'	20	372 bp
	ACR	R 3'-AAGTAGCCTCCTCCTCGCTT-5'	20	
3) <i>Enterobacter cloacae</i> strain ATCC 13047	ECF	F 5'-AAGAAGCACCGGCTAACTCC-3'	20	351 bp
	ECR	R 3'-AAGGGCACAACCTCCCAAAA-5'	20	
4) <i>Pectobacterium carotovorum</i> subsp. <i>Carotovorum</i>	PCF	F 5'-AAGCACTTTCAGCGAGGAGG-3'	20	416 bp
	PCR	R 3'-CTCAAGGGCACAACCTCCAA-5'	20	

PCR reaction

The PCR amplification was done with 4 primers from related bacterial pathogens, after confirmation the single primer was used for the isolates of different surveyed districts.

PCR amplification of 16S rRNA primers were performed using 100 ng DNA template 1X PCR buffer 200 µM dNTPs mix 0.4 µM primers and 1 U Taq DNA polymerase (Merck, Bangalore, India) and sterilized sterile distilled water to make final volume of 20 µL. PCR amplification was conducted with a thermocycler (Eppendorf, Vapo project, Germany). The amplification was carried out with an initial 94 °C for 4 min, followed by 30 cycles of 30 seconds, denaturation at 94 °C 45 seconds of primer annealing at 55 °C, 1 min extension at 72 °C. The final extension step was for 10 min at 72 °C.

Gel electrophoresis

One percent gel was prepared by adding 1gm of agarose to 100ml of 1x TAE buffer and kept in the micro wave oven for about 1-2 min for melting, then allowed to cool. After mild cooling of agarose, added 0.5 µl of ethidium bromide, then poured to the gel tray for solidification. A comb was placed on the gel to make the wells, after solidification the comb were taken out. Gel was taken out side from the gel casting unit and tank buffer (1x TAE) was poured into electrophoresis unit, then placed the gel in the electrophoresis unit. The DNA along with the dye and primers were added into the wells through micropipette and

the temperature was set at 100 °C for 35 minutes. After 35 minutes the gel was taken and kept for the gel documentation and the gel image was taken in the Gensys software in Department of Biotechnology, COH-Bagalkote.

Results

16S rRNA identification of bacteria causing leaf rot disease of onion through PCR technique

The molecular characterization was carried out by standardized procedure of 16S rRNA technique. Overnight grown pure culture of test bacteria was inoculated to nutrient broth and kept for the incubation in a shaker cum incubator at 28±2 °C for 48 hours and then subjected for molecular identification. A representative isolate of the causal agent was subjected to PCR based identification by utilizing the primers of 4 different relevant bacterial pathogens viz., *Acinetobacter haemolyticus*, *Acinetobacter calcoaceticus*, *Enterobacter cloacae* strain ATCC 13047 and *Pectobacterium carotovorum* subsp. *carotovorum*. The primers of these bacteria were designed from nucleotide sequence available in the data base. Successful amplification of PCR product was observed at 493bp as evident by gel electrophoresis, which shown similarity with *Enterobacter cloacae*. Then used the specific primer of *Enterobacter cloacae* with one representative isolate from each surveyed districts (BGE-1, VE-6, GDE-7, DHE-5 and CHE-8). All isolates were amplified at same base pairs, similar to earlier one. The gel image representing bands of *E. cloacae* is depicted in Plate 1 and 2.

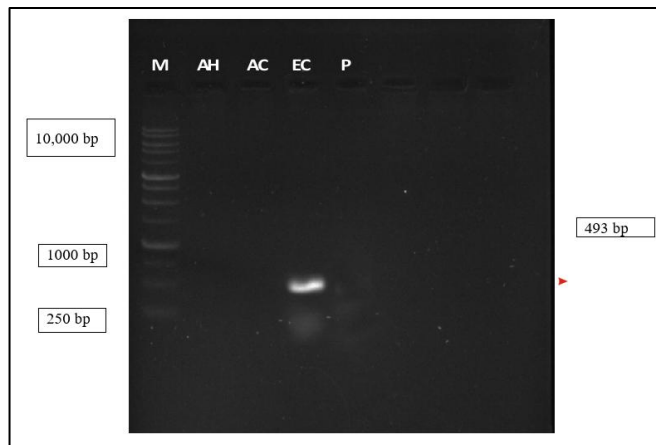


Plate 1: 16srRNA identification of leaf rot causing bacteria of onion through PCR technique

a) 16srRNA identification of leaf rot causing bacteria of onion through PCR technique

M-Marker

AH-Acenitobacter haemolyticus

AC-Acenitobacter calcoaceticus

EC-Enterobacter cloacae strain 13047

P -Pectobacterium carotovorum subsp. Carotovorum

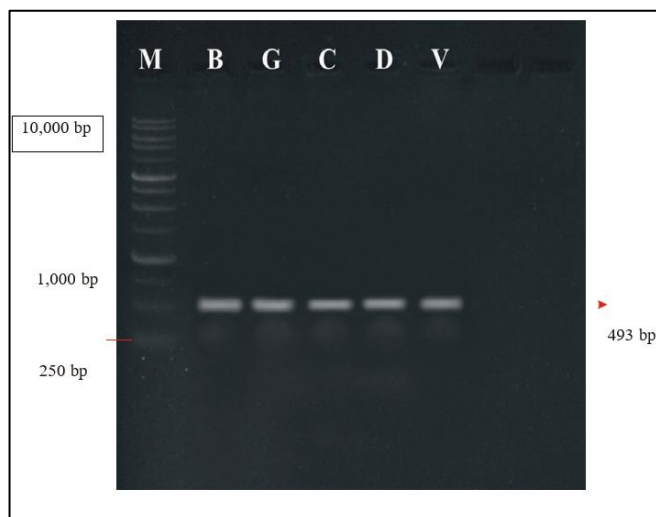


Plate 2: 16srRNA identification of leaf rot causing bacteria of onion through PCR technique

b) 16srRNA identification of different isolates of leaf rot causing bacteria

M-Marker

B-Bagalkot

G-Gadag

C-Chitradurga

D-Dharwad

V-Vijayapura

Discussion

Molecular characterization and PCR based diagnosis of leaf rot disease

The molecular characterization was carried out by standardised procedure of 16s rRNA technique. A representative isolate of the causal agent was subjected to PCR based identification by utilizing the primers of 4 different relevant bacterial pathogens. The primers of these bacteria were designed from nucleotide sequence available

in the data base. Successful amplification of PCR product was observed at 493 bp as evident by gel electrophoresis, which showed similarity with *Enterobacter cloacae*. Through this technique, the test bacterium was confirmed as *Enterobacter cloacae*. Then used the specific primer of *Enterobacter cloacae* with one representative isolate from each surveyed districts. All isolate took for molecular identification were confirmed as *Enterobacter cloacae*. To the best of our knowledge, this is the first report of *Enterobacter cloacae* causing leaf rot of onion in India.

Kiran Kumar *et al.*, (2017) ^[3] were conducted an experiment on isolation and identification of *Enterobacter cloacae* from onion (*Allium cepa* L.) leaf spot disease. They isolated the bacterial colonies on nutrient agar (NA) from infected leaves. They took pure single colonies which on gram staining, expressed as gram negative. Koch's postulate was proved on onion seedlings. They reconfirmed the culture as *E. cloacae* by molecular based identification through specific primers (Forward-16S RF-AGA GTT TGA TCG TGG, CTC AG, Reverse 16S RP-CGG TTA CCT TGT TAC GAC TT). To their knowledge, this is the first report of bacteria causing leaf spot of onion.

Hauben *et al.* (1998) ^[2] identified the phylogenetic position of phytopathogens within the *Enterobacteriaceae*. The almost complete 16S rDNA sequences of twenty-nine plant-associated strains, representing species of the genera *Erwinia*, *Pantoea* and *Enterobacter* were determined and compared with those of other members of the *Enterobacteriaceae*. The species of the genus *Pantoea*, included in this study, form a monophyletic unit (cluster IV), closely related with *Erwinia*, whereas the three phytopathogenic species of the genus *Enterobacter* are scattered among the genera *Citrobacter* and *Klebsiella*.

Hafiz *et al.* (2013) ^[1] were conducted research on molecular and pathogenic diversity of *Erwinia carotovora* Spp. *atroseptica* associated with potato blackleg and soft rot. The isolates were identified as *Eca* by biochemical tests and their identity was further confirmed by polymerase chain reaction (PCR) using subspecies-specific primers *Eca* 1F and *Eca* 2R which amplified the *Eca*-specific band of 690 bp.

Identification and characterization of *Enterobacter* complex of mulberry wilt disease in China, was done based on biologic metabolic profile, fatty acid methyl esterase analysis (FAME) and sequence analysis of partial 16S rDNA and *rpoB* genes. The 4 strains were identified as members of genus *Enterobacter*. The causal organism for mulberry wilt disease was *Enterobacter cloacae*. However, this study has found that other *Enterobacter* spp. (*Enterobacter asburie*, *Enterobacter morri* and *Enterobacter* spp.) also cause mulberry wilt disease (Wang *et al.* 2010) ^[4].

Conclusion

The pathogenicity test was proved that, the bacterium is pathogenic on onion seedlings, as isolated bacteria produced the peculiar disease symptoms of leaf rot disease.

Through 16S rRNA molecular analysis, the test bacterium was identified as *Enterobacter cloacae*, which was amplified at 493bp.

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