# IDENTIFICATION OF CAUSAL AGENT OF FOOT AND SHEATH ROT OF RICE IN THE FIELDS OF GUILAN PROVINCE OF IRAN

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***Abstract***

*Foot and sheath rot of rice caused by Pectobacterium carotovorum subsp. carotovorum and Pseudomonas syringae pv. syringae were the important bacterial pathogenof rice in Iran. The disease causes damage on rice which leads to lots of yield in the host plants. During the spring and summer of 2005-2006 different paddy fields regions in Guilan province (Roodsar, Langrud, Lahijan, Rasht, Anzali, Fooman, Soomeesara and Roodbar) were surveyed. Samples were collected from seedling blight and sheath and stalk rot rices. Infected tissues were washed with sterile distilled water and crushed in peptone water. Then 50 µl of the extract were cultured on King's B, NA, CPG and LPGA media containing cyclohexamide (50 µg/ml). After 48 to 72 hours, bacterial colonies were selected and purified. On the basis of morphological, physiological, biochemical characteristics, pathogenicity and PCR with specific primers, the isolates were placed in two groups. The first group was 21 strains that caused foot rot, identified as P. c. subsp. carotovorum. The second group was 22 strains that caused sheath rot identified as P. s. pv. syringae. This is the first report of existence of rice bacterial rot on paddy fields in the Guilan province of Iran.*

**Key words:** *Pectobacterium carotovorum* subsp. *carotovorum*, *Pseudomonas syringae* pv. *syringae*. **s**heath and foot rot, rice

## INTRODUCTION

Rice (*Oryza sativa* L.) is the primary food grain consumed by almost half of the world's population, making it the most important food crop currently produced. (Cottyn *et al.*, 2001). More than 70 diseases caused by fungi, bacteria, viruses or nematodes have been recorded on rice. *Pectobacterium carotovorum* is a representative species that incites soft rot on a wide range of plants, although other bacteria such as *P. chrysanthemi* and *P. fluorescens* can also be associated with the disease. However, *P. s.* pv. *syringae*, a pathovar capable of causing disease on more than 200 different plant species seems quite distant from the definition. Host specificity appears to be evident for *P. s.* pv. *syringae* strains infecting grasses and beans. However, the assessment of host specificity and virulence of strains either for identification or characterization purposes requires standardized procedures, as different responses can be obtained by adopting different techniques and some plant species appear more suitable than others for defining the virulence of the strains (Yessad-Carreau *et al*., 1994). PCR-based marker techniques have been employed extensively for confirming genotypes of organisms at the level of species and population. The PCR method requires little biological material and provides a rapid method for screening large sample sizes. PCR markers have been developed using either arbitrary primers or specifically designed primers from known DNA information such as repetitive sequences. The objectives of the present research, isolation of causal agent of foot and sheath rot of rice on rice in the Guilan province and identification of isolates by biochemical, nutritional, pathogenicity and PCR method.

## MATERIALS AND METHODS

**Bacterial isolation from sheath, stem and collar**
During spring and summer of 2005–2006, rice samples were collected from different paddy fields regions in Guilan province were surveyed and samples were collected from stem sheath and collar rot. Isolations were made from infected tissues. From each field, four replicas of 50 rice disease seedling and mature plants were collected at random. Individual infected tissues were cut in 3–5 ml of sterile distilled water with a homogenizer and 100 µl of homogenate were streaked on LPGA, CPG and King's B mediums, with 50 µg /ml of actidion. At least 50 samples were tested from each field. From each infected tissue sample, five single colonies were isolated and one strain / field was selected as a representative strain for this study. For long-term storage, the purified strains were grown in peptone sucrose and frozen at –80°C in 20% glycerol. The strains were revived on Luria Pepton (LP) (Difco) medium for biochemical tests, DNA isolation and pathogenicity tests.

**Biochemical and physiological tests** Strains were characterized based on the following tests: Gram, oxidative/fermentative test, production of fluorescent pigment on King's B medium, hypersensitive reaction (HR) in tobacco and geranium leaves, oxidase test, levan formation, catalase, urease, gelatin liquefaction,

litmus milk, salt tolerance (5% and 7%) and gas formation from glucose. In addition, tests for arginine dehydrolase, hydrogen sulfide production from peptone, reducing substance from sucrose, tyrosinase casein hydrolase, nitrate reduction, indole production, 2-keto gluconate oxidation lecitinase, starch hydrolysis, phenylalanine deaminase, esculin and Tween 80 hydrolysis and optimal growth temperature were conducted (Schaad *et al.,* 2001). The presence of DNase was tested on DNA agar (Diagonistic Pasteur, France). Carbohydrate utilization using Ayer basal medium was carried out and the results were recorded daily upto 2–8 days.

**Pathogenicity test** Seeds of rice cultivar Khazar were sown in plastic boxes, and 3 weeks later, seedlings were transplanted to 30 cm diameter plastic pots. For *P. s.* pv. *syringae*, bacterial suspensions for inoculations were prepared in 10 ml of sterile distilled water at $1 \times 10^9$ CFU/ml. To test the virulence of the strains, plants with fully expanded leaves were inoculated by the leaf-clipping method. The instrument used to inoculation the rice plant with the bacterial is scissors. Before using the scissors they sterilized by using 70% ethanol. The scissors dipped in the bacterial suspensions and are used to cut inoculate the rice plant. Lesion on leaves were observed at 14 days after inoculation (Backer, 2002). For *P. c.* subsp. *carotovorum*, the sheath, stalk and collar from seedling and mature plants were cut with a sterile scalpel and inoculated with 100 ȝl of bacterial suspension ($1.0 \times 10^9$ CFU/ml). The inoculation site was covered with parafilm (to prevent bacterial inoculum from evaporating) and the plants were kept at 27°C for 4 weeks (Smith and Bartez, 2000). Individual leaves, sheath, stalk and collar were ground in 3 ml of sterile distilled water. The suspensions were then appropriately diluted and 50 µl aliquots spotted on duplicate LPGA and King's B plates. After incubation for 48 h at 28°C, the number of colonies formed on each plate was counted.

**DNA extraction**

For bacterial DNA extraction, the isolates were grown overnight, in nutrient broth (Merck, Darmstadt, Germany), at 26°C and the DNA was extracted as described by Martins *et al.,* 2005. One tube of 1.5 ml was used to centrifuge the cells at 13,000 × g for 5 min and the pellet was suspended in 200 µl Tris 0.1 mol/L and added with 200 µl of lysis solution (NaOH 0.2 N and 1% SDS), mixed and added 700 µl of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v), homogenized and centrifuged 10 min at 13,000 × g. To precipitate DNA, 700 µl of cold isopropanol was added and spinned, washed in 70% ethanol and centrifuged. Precipitated DNA is dried at room temperature and suspended in 100 µl of water. The method described by Ausubel *et al.* (1996) was performed comparing 30 strains. The samples from the both methods were electrophoresed on 1.5% agarose gels, stained with ethidium bromide and photographed under UV.

**Primers for P. s. pv. syringae and P. c. subsp. carotovorum**

Primers PSF, 5'-AGCCGTAGGGGAACCTGCGG-'3 and PSR 5'-TGACTGCCAAGGCATCCACC-'3 were designed for *P. s.* pv. *syringae* (Manceau and Horvais, 1997) and primers EXPCCR 5'-GCCGTAATTGCC TACCTGCTTAAG-3' and EXPCCF 5' GAACTT-CGCACCGCCGACCTTCTA-3' were designed for *P. c.* subsp. *carotovorum* (Kang *et al.,* 2003).

**PCR conditions for amplification and electrophoresis** Amplification was carried out in a 25 µl volume in 0.5 ml microtube using a Hybaid programmable thermal controller. Each 25 µl PCR reaction mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl2, 200 µM of each nucleotide (dATP, dCTP, dGTP and dTTP), 0.25 µM of each primer, 100 ng DNA and 1 U of Taq DNA polymerase (Promega Corp., Madison, WI). A 25 µl sterile, mineral-oil overlay was added to reduce evaporation. DNA amplification was carried out in a PTC-100 programmable DNA thermal cycler (MJ Research, Watertown MA). The amplification was performed as follows: for P. s. pv. syringae, initial 5 min 94°C denaturation; 45 cycles of 1 min 94°C, 1 min 52°C, 1 min 72°C; and 5 min 72°C extension, for P. c. subsp. carotovorum, initial 1 min 94°C denaturation; 45 cycles of 1 min 94°C, 1 min 36°C, 2 min 72°C; and 5 min 72°C extension. Amplified fragments were separated in 1.5% agarose gel using TBE buffer and were visualized and photographed using a Gel Documentation System, GDS 8000 (BioRad., California, USA), after staining with ethidium bromide.

## RESULTS

**Biochemical and physiological test** For *P. s.* pv. *syringae*, all strains (21 strains) were Gram, oxidase, catalase, pectinase, arginine dihydrolase negative, the strains were able to produce syringomycin and showed ice nucleation activity. All strains produced HR on tobacco and geranium and could hydrolyze gelatin. Presence of DNase was tested on DNA agar. For *P. c.* subsp. *carotovorum*, all strains (22 strains) were Gram, oxidase, urease, phosphatase, arginine dihydrolase, sensitive to erythromycin and copper nitrate negative. Strains of *P. c.* subsp. *carotovorum* was able to utilize glucose under anaerobic conditions. All strains could hydrolyze casein. Presence of DNase was tested on DNA agar.

**Pathogenicity test** All strains of *P. c.* subsp. *Carotovorum* caused foot rot, on the rice plant after four weeks. The primary symptoms are yellow leaves and dark – brown tiller decay. The stalk becomes soft and rots and has an unpleasant odor. At advanced stage, so many tillers decay. Foot rot was observed from maximum tillering to reproduction. All strains of *P. s.* pv. *syringae* caused bud rot and leaf sheath rots on

seedling and mature plants, respectively. These symptoms did not occur in the control.

**Detection of *P. s.* pv. *syringae* and *P. c.* subsp. *carotovorum* by direct PCR** All isolates of *P. s.* pv. *syringae* were identified by specific primers PSF and PSR. On agarose gel electrophoresis 1.5%, isolates produced a band 558 bp and all isolates *P. c.* subsp. *carotovorum* produced a band 550 bp (expected size). The bands of isolates were similar with isolates standards of CFBP 3077 for *P. s.* pv. *syringae* (Figure 1) and CFBP 1356 for *P. carotovora* subsp. *carotovora* (Figure 2). Based on the phenotypic pathogenicity and PCR tests, the causal agent of bacterial sheath rot was identified *P. s.* pv. *syringae* and causal agent of bacterial collar rot was classified as *P. c.* subsp. *carotovorum*.

## DISCUSSION

Based on morphological, phenotypical, nutritional characteristic, pathogenicity tests and PCR using specific primers, we identified causal agent of foot and sheath rot of rice as *P. c.* subsp. *carotovorum* and *P. s.* pv. *syringae*, respectively. All the strains of *P. c.* subsp. *carotovorum* and *P. s.* pv. *syringae* produced foot and sheath rot on rice. No significant differences were observed in the degree of disease symptoms. These results suggest that strains isolated from different fields do not differ in their degree of virulence. This is the first report of bacterial foot and sheath rot of rice in the north region of Iran. The *Rir1b* gene of rice is one of a set of putative defense genes whose transcripts accumulate upon inoculation of rice with the non-host pathogen *P. s.* pv. *syringae* (Schaffrath *et al.*, 2000). The specific detection of *P. c.* subsp. *carotovorum* using molecular techniques has been hampered by the complexity among strains associated with other subspecies. To address this problem, a URP-PCR (Kang *et al*., 2003) fingerprinting approach was used to find DNA sequences specific to *P. c.* subsp. *carotovorum*. A PCR polymorphic band shared only in *P. c.* subsp. *carotovorum* strains was cloned and used as a probe (pECC2F), which detected only *P. c.* subsp. *carotovorum* and *P. c.* subsp. *wasabiae* strains. *P. s.* pv. *syringae* is recognized as an important rice pathogen causing both sheath rot and seedling rot (Uematsu *et al.*, 1976). Further research that elucidates the mechanisms eliciting this genetic diversity is needed. An understanding of the ecology of natural microbial communities should lead to a more efficient deployment of bacterial populations for disease management. Study on the biological control of bacterial blight on rice by antagonistic strains in different parts of Iran and use of resistant cultivars could be a case study for future research.

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**Figure 1:** Agarose gel electrophoresis of products from polymerase chain reaction (PCR) performed on DNA 16S of Pseudomonas syringae pv. syringae isolates, M, 100 bp DNA marker; lane 1 is positive control (P. s. pv. syringae CFBP 3077) showing the amplification the approximately 558 bp; lane 2, control negative (distilled water); lanes 3 to 12, strains of P. s. pv. syringae isolated from sheath rot of rice.

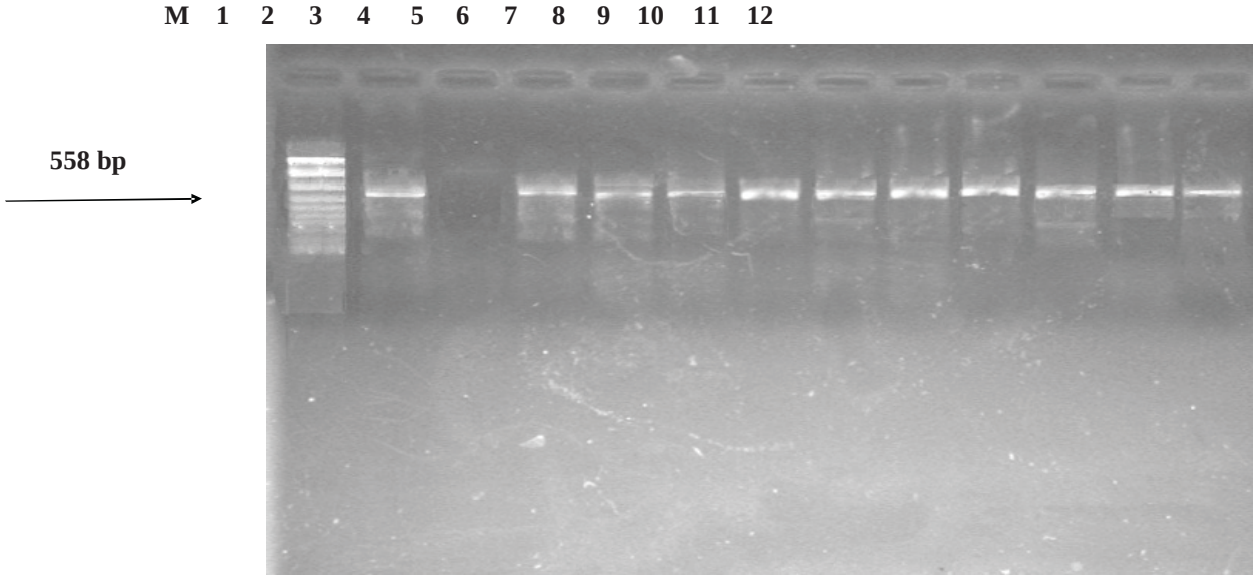
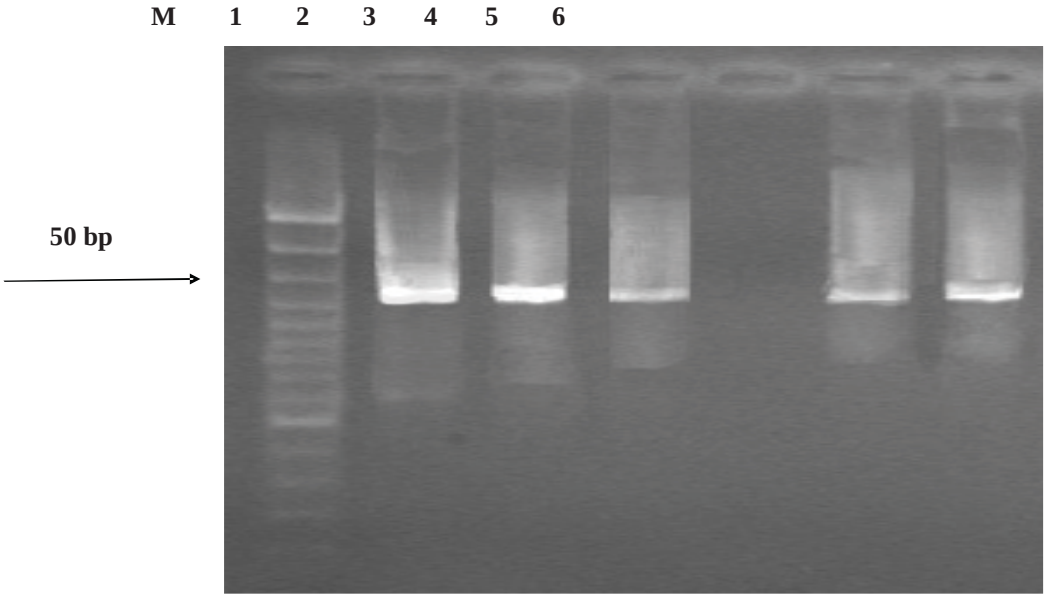



**Figure 2:** Agarose gel electrophoresis of products from polymerase chain reaction (PCR) performed on DNA 16S of *Pectobacterium carotovorum* subsp. *carotovorum* isolates, M, 100 bp DNA marker; lane 1 is positive control (*P. c.* subsp. *carotovorum* CFBP 1356), showing the amplification the approximately 550 bp; lane 2, 3, 5 and 6 were strains of *P. c.* subsp. *carotovorum* isolated from stalk rot of rice.

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