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 pathogens of 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. sheath 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
dehydrogenase, hydrogen sulfide production from peptone,
reducing substance from sucrose, tyrosinase casein
hydrolase, nitrate reduction, indole production, 2-keto
gluconate oxidation lecininase, 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 (Diagnostic Pasteur, France).
Carbohydrate utilization using Ayer basal medium was
carried out and the results were recorded daily up to 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.
syringae*, bacterial suspensions for inoculations were
prepared in 10 ml of sterile distilled water at
1 x 10^5 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 µl of bacterial suspension
(1.0 x 10^3 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 centrifuged the cells at 13,000 x 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 x g. To
precipitate DNA, 700 µl of cold isopropanol was added
and spun, 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’-AGCCGTAGGGGAACCTGCG-3’ and
PSR 5’-TGACTGCAAAGCCTCACC-3’ were designed
for *P. s. pv. syringae* (Manceau and Horvais,
1997) and primers EXPCCR 5’-GCCGTAATTGCC
TACCTGTTAAG-3’ and EXPCCF 5’-GAACCT-
GCACCGCCGACCTCTCA-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 Hybird
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 sepa-
rated in 1.5% agarose gel using TBE buffer and were
visualized and photographed using a Gel Documenta-
tion 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 dihydrolease 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 dihydrolease,
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. Caro-
tovorum* 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 rot 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
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. carotovorum* produced a band 558 bp and all isolates *P. c. subsp. syringae* PSR. On agarose gel electrophoresis 1.5%, isolates sheath rot was identified by direct PCR (Figure 1) and CFBP 1356 for *P. carotovora* subsp. *carotovorum* (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.

**REFERENCES**


Accepted for publication on April 28, 2008
**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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