VIRULENCE TO TOMATO OF IRANIAN ISOLATES OF *RALSTONIA SOLANACEARUM*

A. Safaie Farahani, S.M. Taghavi and M. Djavaheri

Department of Plant Protection, College of Agriculture, Shiraz University, Shiraz, Iran

SUMMARY

Four strains belonging to biovars 2A and 2T (N2) of *Ralstonia solanacearum* and two mutants impaired in extracellular polysaccharide (EPS) production were studied for their virulence to tomato, colonization of host tissue and survival in the soil. Mutants impaired in EPS biosynthesis were generated using a plasmid containing Tn*5*. Results showed that whereas biovar 2A is more virulent than 2T, they did not differ in the ability to survive in the soil. Susceptibility of various tomato cultivars to *R. solanacearum* strains was evaluated. Results showed that besides the bacterial biovar, the host cultivar is effective in determining disease severity. However, no significant differences were observed in bacterial populations from tomato cultivars with different susceptibility. Mutants defective for EPS production were less virulent than the wilde-type strain, highlighting the role of EPS in pathogenicity. No difference was observed in the colonization of tomato stems by either mutant impaired in EPS production and the wild-type.

Key words: Bacterial wilt, biovars 2A and 2T, tomato, extracellular polysaccharides

INTRODUCTION

Bacterial wilt caused by *Ralstonia solanacearum* is one of the important diseases in tropical and subtropical regions of the world. The bacterium has a wide host range and attacks more than 450 plant species, including economically important crops such as tomato, potato, eggplant, peanut, tobacco and banana (Hayward, 1991). *R. solanacearum* is a soil-borne pathogen that enters the host plant through wound or the area of hairy roots formation. After colonizing the root in the cortex region, the bacterium invades the xylem eventually causing wilt, yellowing, stunting and necrosis in the host plant (Vasse *et al*., 1995).

R. solanacearum is a heterogenous and highly complex species that has been classified into five biovars according to biochemical characteristics. Although the members of biovar 2 have great similarities in their biochemical characteristics, they also are grouped into two biovars, 2T and 2A. Biovar 2T is a new variant (a tropical variant, also called N2) that was discriminated from biovar 2A by some phenotypic traits (Gillings and Fahy, 1993).

R. solanacearum produces several known virulence factors, including extracellular polysaccharides (EPS), and a consortium of plant cell wall-degrading enzymes, such as endoglucanase (EG) and polygalacturonase (PG). Defective mutants in EPS, EG or PG production are less virulent than the wild-type, but the mechanism accounting for this behaviour is not clearly understood (Denny and Baek, 1991; Huang and Allen, 2000; Kao *et al*., 1992; Roberts *et al*., 1988). EPS is a high-molecular weight compound that is formed around the bacterial cell as a slime layer, containing more than 80% galactosamine. (Drigues *et al*., 1985; Whatley, 1988). Denny *et al*. (1998) demonstrated that the severity of wilt in tomato plants inoculated with wild-type isolate is stronger than in those inoculated with a defective mutant in EPS production. Saile *et al*. (1997) studied the role of EG and EPS in virulence of the bacterium on tomato plants, showing that EPS has an important role. In their study, it was shown that EPS-defective mutants colonize tomato stems at a significantly slower rate than the wild-type isolate. In this study, biovars 2A and 2T were compared for their virulence to different tomato cultivars, survival in the soil and in rhizosphere.

MATERIALS AND METHODS

Bacterial strains and preparation of EPS-deficient mutants. Four wild-type strains (Izadiyan and Taghavi, 2011) and two EPS-deficient mutants of *R. solanacearum* were used in this study (Table 1). Strain Rs130 was used to produce EPS-deficient mutants. Competent cells were prepared by the following procedure: a fresh overnight culture diluted *ca.* 1:100 was grown in LB broth at 28°C with shaking at 250 rpm, to an OD_{600} of 0.5. Cells were harvested by centrifuging for 10 min at 4,000 rpm at 4°C. Fifty ml of cold 10 mM NaCl was added and the suspension was centrifuged for 10 min at 4,000 rpm at 4°C. The supernatant was decanted and the pellet resuspended in 25 ml of cold 10 mM NaCl and centrifuged for 10 min at 4,000 rpm at 4°C. After discarding the supernatant, the pellet was resuspended in 2 ml of LB broth containing 10% glycerol. Cells were aliquoted to microfuge tubes,

Corresponding author: S.M. Taghavi
Fax: +98.7112286087
E-mail: mtaghavi@shirazu.ac.ir

Table 1. Wild-type strains and EPS-deficient mutants used in this study.

Strain	Relevant characteristics	EPS rating	Viscosity (sec)	References
Rs130	Wild-type, biovar 2A	+++	115.7	Izadiyan and Taghavi, 2011
Rs111	Wild-type, biovar 2A	+++	113.4	Izadiyan and Taghavi, 2011
Rs145	Wild-type, biovar 2T	+++	116.6	Izadiyan and Taghavi, 2011
Rs169	Wild-type, biovar 2T	+++	114.1	Izadiyan and Taghavi, 2011
Rs130-1	Rs130::Tn*5*, EPS^i Nx^r Km^r	+	9.6	This study
Rs130-2	Rs130::Tn*5*, EPS^i Nx^r Km^r	+	8.9	This study

Nx^r and Km^r designate resistance to nalidixic acid and kanamycin, respectively. EPS, extracellular polysaccharide; EPS^i, impaired in EPS production. Viscosity (sec) shows time for supernatants of cultures grown in BG broth to pass through a viscometer.

frozen rapidly in a dry ice-ethanol bath and stored at –70°C until use. Plasmid PBS1 (Denny *et al*., 1988) was transferred into the bacterium using electroporation so that Tn*5* transposase present in the plasmid can acts as a mutagenic agent. Electroporation was carried out using a Gene Pulser II (Bio-Rad, USA). Cuvettes were placed on ice to cool. Electrocompetent cells were thawed, and 50 µl of a suspension added to microfuge tubes placed on ice. Two µl of plasmid were added and, after mixing, plasmids and cells were transferred to the cuvette. After electroporation carried out at 200 Ω, 2.5 kV and 25 µF, 0.5 ml of LB were immediately added to the cuvette and the contents transferred to the sterile tube for a 4 h growth with shaking at 28°C. The tubes were spun for 10 min at 10,000 rpm. The supernatant was discarded, the cells were resuspended in 50 µl of LB broth, plated on NA medium (with antibiotics) for incubated at 28°C for 3 days.

Production of EPS in culture. For determining if EPS were present in *R. solanacearum* strains as a capsule, bacterial cells were removed from BGT plates, smeared and examined with a Tyler's modification of Anthony's method (Conn *et al*., 1957). *Klebsiella pneumoniae* was used as a positive control. The viscosity of culture supernatants was an indication of the relative amount of EPS produced in culture. Cells were removed by centrifugation from cultures grown for 3-4 days in BG broth (with antibiotics) and the viscosity of the supernatant was determined with a viscometer. Measurments were made at room temperature and corrected for the viscosity of water.

Plant growth and bacterial inoculation. Tomato seeds (cvs Super majar, Super ch, Queen, Peto early, Super strain B, Early orbano, Lesto, and Sun) were surface-disinfected with 70% ethanol for 1 min, followed by 1.0% sodium hypochlorite (20% household bleach) for four min and two washes in distilled water. To prepare bacterial inoculum, *R. solanacearum* strains were streaked from frozen stocks on TZC plates (Kelman, 1954), then grown in Boucher's minimal medium (Boucher *et al*., 1985), containing 0.1% (w/v) yeast extract and 0.2% (w/v) citric acid (pH adjusted to 7).

Stems of tomato plants (Table 2) at the four-leaf stage were injected with 20 µl of a bacterial suspension (OD_{600}=1). The severity of wilting was determined at different time points and the final assessment was made 21 days post inoculation (dpi). Symptom severity was scored with an arbitrary scale from 0 to 4 : 0 for healthy plants, 1, 2, 3 and 4 for 1-25, 26-50, 51-75 and 76-100% of wilted leaves (Roberts *et al*., 1988).

Survival of *R. solanacearum* in the soil. To determine the survival of *R. solanacearum* strains Rs130, Rs145, RS130-1 and Rs130-2, sterile soils were inoculated with a bacterial suspensions of 10^8 CFU/gr. Soil samples (1 g) collected 2, 4 and 6 weeks post inoculation were suspended in sterile water, serially diluted and plated on semi-selective SMSA medium (Nesmith and Jenkins, 1983). Bacterial colonies were counted after incubation at 28°C for three days.

Population of *R. solanacearum* in the rhizosphere. The soil of pots containing 4-week-old tomato seedling was inoculated with a bacterial suspension at a concentration of 10^8 CFU/g. Sampling was carried out at 7, 14 and 21 dpi. Seedlings were pulled out from the pots, shaken to remove the soil around the roots, which were then immersed in sterile distilled water and gently shaken for 10 min. Serial dilution were plated on SMSA medium and incubated at 28°C for three days. The average number of bacterial colonies was determined and converted to logarithmic values (Granada and Sequeira, 1983).

Population of *R. solanacearum* in plant stems. The bacterial population in the stems of inoculated plants was determined on the same seedlings used for the assessment of the bacterial population in the rhizosphere. Stems were thoroughly washed, 1 cm piece above the cotyledons was excised, immersed into 1% sodium hypochloride solution for 1 min, then washed twice with sterile distilled water. Stems pieces were immersed into sterile distilled water for 24 h, serial dilution were plated on semi-selective media and developed colonies were counted after 72 h incubation at 28°C (Huang and Allen, 2000).

Statistical analysis. All experimental data were analysed by student's T-test using SAS software version 6.10,

Table 2. Disease index in tomato cultivars inoculated with different strains of *Ralstonia solanacearum* at 21 days post inoculation.

Strain	Disease index in cvs Super majar, Super ch, Queen, Peto early, Super strain B, and Early orbano	Disease index in cvs Lesto and Sun
Rs130	4	3
Rs111	4	3
Rs145	2	1
Rs169	2	1

Symptom severity in plants inoculated with strains belonging to biovar 2A was stronger than in those inoculated with strains belonging to biovar 2T. Cvs Lesto and Sun showed milder symptoms than the other cultivars.

assuming as significant at the level of 5% ($P<0.05$) the difference between treatments

RESULTS

Production of EPS in culture. The viscosity of culture supernatants are likely to be directly related to the amount of EPS produced by each strain. Viscosity measurements separated the wild-type strain (Rs130) from the EPS-deficient mutants (Rs130-1 and Rs130-2) and disclosed the intermediate production of EPS by all of the strains (Table 1).

Pathogenicity tests. Clear-cut differences were observed in the virulence of biovars 2A and 2T. In fact, the symptom severity in plants inoculated with strains Rs130 and Rs111 was stronger than in those inoculated with strains Rs169 and Rs145. By the end of the experiment, disease severity in plants inoculated with strains Rs111 and Rs130 was scored as 3 or 4, whereas a severity level of 1 or 2 was determined for plants inoculated with strains Rs169 and Rs145 (Table 2). Slight differences in symptom severity of different tomato cultivars were observed. The final disease index was 3 in cvs Sun and Lesto inoculated with biovar 2A, whereas it was scored as 4 in other cultivars. The final disease index in cvs Sun and Lesto plants inoculated with biovar 2T was 1, whereas in other cvs, it was scored 2. Finally, the EPS-deficient mutant was less virulent than the wild-type strain (Fig. 1A).

Survival of *R. solanacearum* in the soil. The population of strain Rs130 reached 2.5×10^6 CFU/g of soil after two weeks, showing a 40-fold decline compared to the original population size, and it further decreased to 1.1×10^6 CFU/g of soil by the fourth week and to 5×10^5 CFU/g of soil by the sixth week. In terms of time of introduction in the soil, Rs130 declined 90- and 200-fold by week 4, and 6, respectively. There was no significant difference in the population size between Rs130 and other strains (Rs130-1, Rs130-2 and Rs145) (Fig. 1B).

Population of *R. solanacearum* in the rhizosphere. At 7 and 14 dpi there was no significant difference between the population of Rs130 and that the other strains (Rs130-1, Rs130-2 and Rs145) but it became significant at 21 dpi. The difference between strain Rs145 and mutants impaired in EPS biosynthesis, strains Rs130-1 and Rs130-2, was no significant in any time point (Fig. 1C).

Multiplication of *Ralstonia solanacearum* in tomato stems. No direct correlation was observed between the estimated number of bacteria within plant stems and their virulence. In fact, no significant difference was observed between the population of strains Rs130 and Rs145 (data not shown). No significant difference was also registered in the bacterial population in tomato stem between the wild-type and the EPS mutant strains at any sampling time (Fig. 1D). Since seedlings of cvs Sun and Lesto showed less wilting symptoms than the other tomato cultivars (Table 1), the concentration of strain Rs130 in the stems of cvs Lesto, Sun, Queen and Super CH was assessed, showing that there was no significant difference in the bacterial population in any of the tomato cultivars (data not shown).

DISCUSSION

The survival of EPS-deficient mutants in the soil was not different from the wild-type strain Rs130, demonstrating that EPS did not have any role in the survival of the bacterium in the soil. Bacterial populations increased over the time in the rhizosphere of tomato. The absence of significant difference between the colonization of wild-type and mutant strains at 7 and 14 dpi indicates that a change in EPS production did not affect bacterial chemotaxis. Saile *et al.* (1997) reported that invasion of tomato roots seems to be non-specific. They also showed that 4 h post soil inoculation with *R. solanacearum*, bacteria such as *E. coli* were also detected in the root cortex. Other studies showed that various bacteria are present in root, stem, seed and fruit of healthy-appearing plants as previously reported (Di Fiore and Del Gallo, 1995; Kloepper *et al*., 1992).

At 21 dpi, a significant difference was observed in the rhizosphere population of strain Rs130 compared to that of the other strains (Rs130-1, Rs130-2 and Rs145). At 21 dpi, inoculated plants with Rs130 showed complete wilting. So that, it can be speculated that the difference in the rhizosphere population between Rs130 and other strains is due to bacterial release from plant roots and it is not relative to colonization of root.

Although there was a difference in the virulence of biovars 2A and 2T, this was not associated with differences in bacterial population within plant stems. Mutants Rs130-1 and Rs130-2 were less virulent to tomato compared to the wild-type strain, which demonstrates a role for EPS in virulence.

Monitoring of bacterial population in tomato stems showed that wild-type and mutant strains impaired in EPS production could similarly colonize the xylem, which can lead to the conclusion that EPS has no role in bacterial multiplication and that defective mutants in EPS biosynthesis can multiply as well as wild-type strain. Saile *et al.* (1997) studied the localization of *R. solanacearum* in the plant using immune fluorescence microscopy concluding that severe wilting symptoms appear only when the bacterium colonizes the whole stem. They also found that, unlike the wild-type strain, the mutants unable to produce EPS did not spread well in the plant stem. Other studies

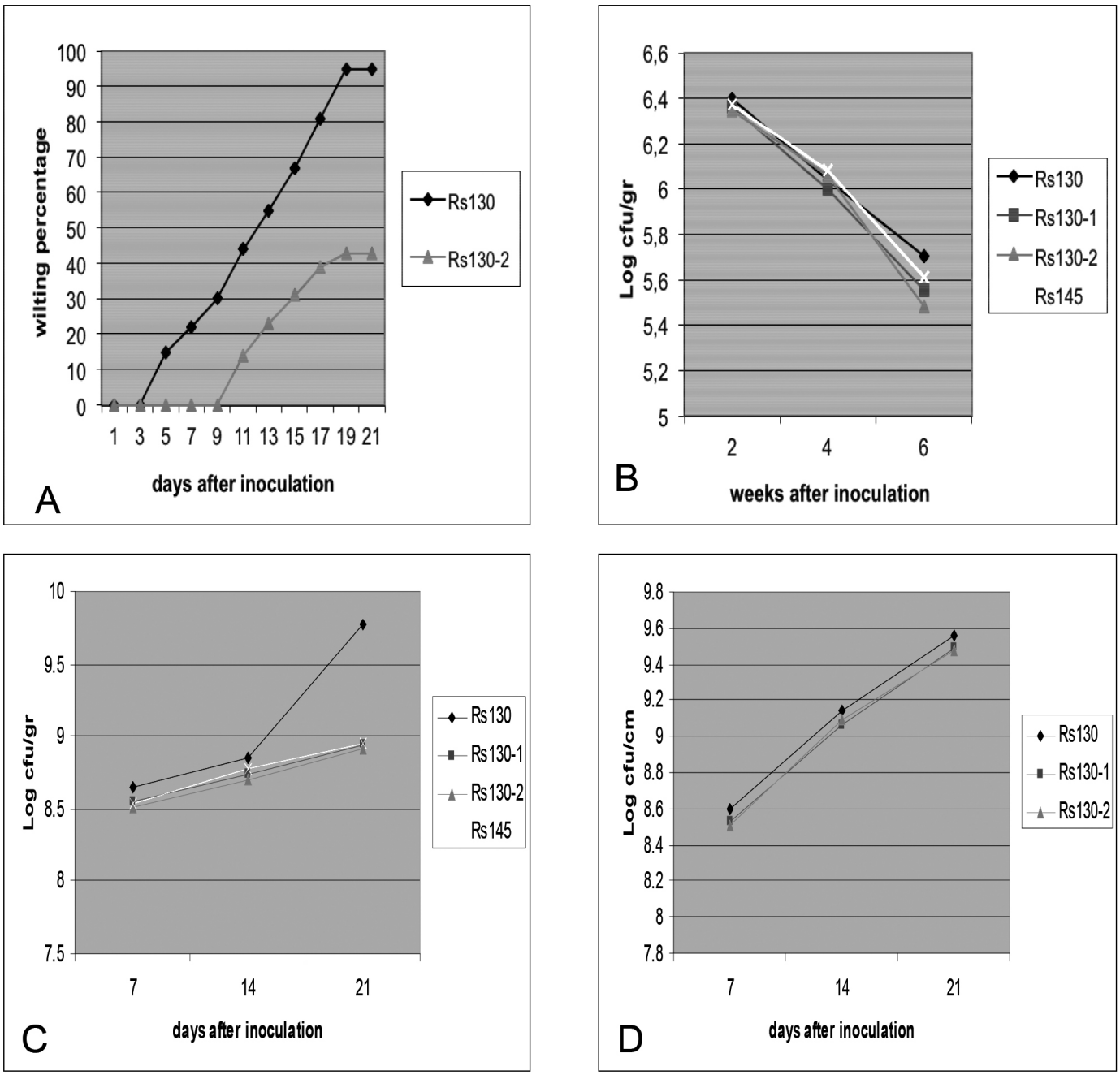

Fig. 1. A. Virulence of strains Rs130 and Rs130-2 on tomato cv. Queen. Three-week-old tomato plants were inoculated by injecting a 2×10^6 CFU/ml bacterial suspension in the stems. Strains Rs130 (wild-type) and Rs130-2 (EPS-deficient mutant) were used as challenging pathogens. B. Population of *R. solanacearum* in the soil. Sterile soils were inoculated with bacterial suspensions of 10^8 CFU/g. No significant difference was observed in the population size of wild-type strains and EPS-deficient mutants in any time points. C. Population of *R. solanacearum* in the rhizosphere of tomato cv. Queen. Initial inoculum concentration was 10^8 CFU/g of soil. In a T-test, only at 21 dpi a significant difference was observed between Rs130 and the other strains (Rs130-1, Rs130-2, and Rs145). D. Average number of *R. solanacearum* in tomato stems of cv. Queen in different points after inoculation with wild-type and mutant strains. This examination was carried out on the same seedlings used for the assessment of the bacterial population in the rhizosphere. No significant difference in bacterial population within the plant stem between wild-type (Rs130) and EPS mutant strains (Rs130-1 and Rs130-2) was observed at any sampling date.

demonstrated that the low severity of wilting induced in tomato by *hrp* mutants in a susceptible cultivar and by wild-type strain in a resistant cultivar is associated with the lack of colonization in the upper half of tomato stem (Prior *et al*., 1996; Trigalet and Demery, 1986). It seems that EPS has a role in bacterial movement and that mutants impaired in EPS production are less virulent than wild-type strain due to hampered movement. The fact that defective mutants in EPS production are less virulent than wild-type strain suggests that EPS has a major role in the virulence of *R. solanacearum*. It seems that induction of wilting symptoms is associated with high production of EPS. In the present study, cvs Sun and Lesto were less susceptible than other cultivars. So, in addition to the bacterial biovar, the cultivar is also effective in determining the severity of wilting symptoms, in accordance to Grimault and Prior (1994) who showed that the susceptibility of different tomato cultivars to bacterial wilt differs.

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