

## DETECTION OF LATENT INFECTIONS OF *RALSTONIA SOLANACEARUM* BIOVAR 2, RACE 3 IN TOMATO CROPS

E. Stefani<sup>1</sup>, S. Giosuè<sup>2</sup> and U. Mazzucchi<sup>3</sup>

<sup>1</sup> Dipartimento di Scienze Agrarie, Università di Modena e Reggio Emilia, Viale J.F. Kennedy 17, 42100 Reggio Emilia, Italy

<sup>2</sup> Istituto di Entomologia e Patologia Vegetale, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29100 Piacenza, Italy

<sup>3</sup> Dipartimento di Scienze e Tecnologie Agroambientali, Università di Bologna, Viale G. Fanin 42, 40127 Bologna, Italy

### SUMMARY

A protocol was set up to detect latent infections of *Ralstonia solanacearum* biovar 2, race 3 in field tomatoes. For two growing seasons, healthy tomato plants were inoculated with a virulent strain, and monitored for between 7 and 55 days. Final extracts used for direct isolation on Kelman's medium and for indirect immunofluorescence staining were prepared from 1 cm segments collected from the base of the lowest side shoots. Reisolations were identified by colony morphology, PCR, IFAS, and pathogenicity tests on tomato plantlets. Reisolation was successful from 18 days onwards, with a frequency that constantly increased in the following weeks. Samples prepared by mixing one latently infected segment with increasing numbers of healthy segments revealed a sensitivity threshold of 1:999. This non-destructive protocol was shown to be appropriate for monitoring in the open field.

*Key words:* Brown rot, field monitoring, *Pseudomonas solanacearum*, detection, mandatory control.

### INTRODUCTION

*Ralstonia solanacearum* is a complex bacterial species divided into races, biovars and, recently, genomovars, phylotypes and sequevars (Fegan and Prior, 2005). Its host range is quite wide and distribution is almost worldwide, mainly in warmer and more humid regions. Race 3 is highly virulent on potatoes, tomatoes and other solanaceous crops and, recently, outbreaks of wilt caused by this race have been described on *Pelargonium* spp. (Hudelson *et al.*, 2002; Janse *et al.*, 2004). Its temperature optimum is lower than that of other races and this might partly explain the establishment, over the last decade, of potato brown rot foci in Belgium, France, Germany, Hungary, Italy, the Netherlands, Portugal, Spain, Slovakia and the UK. The occurrence of brown

rot was transient, since single cases were observed and put under eradication or eradicated (International Society for Infectious Diseases, 2004). To prevent the introduction of *R. solanacearum* to new territories and to limit its spread, the European Union Council issued Directive 98/57 for mandatory control (European Community, 1998). The measures prescribed include systematic monitoring of tomato and potato crops, including tubers (Art. 2), which, in the event of detection, must be followed by additional monitoring of secondary host plants. This monitoring must be adequate to detect both visible and latent infections. As regards latent infections, the Directive provides a detailed description of the protocol for potato tubers but not for tomato or other plant species.

This paper describes a non-destructive analysis protocol to detect latent infections in adult tomato plants, thus allowing multiple monitoring of tomato fields over the whole growing season without affecting crop productivity.

### MATERIALS AND METHODS

**Bacterial culture and direct isolation (DI).** *R. solanacearum* strain PD2762, biovar 2, race 3, isolated from potato was used. It was grown for 48 h at 27°C on Kelman's selective nutrient agar (TTC) (Kelman, 1954), and transferring only the virulent-phenotype colonies. The colonies were selected for colour and morphology and then purified on the same medium.

**Identification.** This was based on generating an amplicon of 288 pb with the primers OLI-1 and Y-2 (PCR) (Seal *et al.*, 1993), on pathogenicity on 5-leaf Money-maker tomato plants and on cell fluorescence with indirect immunofluorescence staining (IFAS) (European Community, 1998). The anti-*R. solanacearum* PD511 antiserum from tomato, race 1, with a titre of 1:9000, was used, assessed on 30 ml of suspension of the homologous strain at a concentration of 10<sup>6</sup> cells ml<sup>-1</sup>. The antiserum has been previously tested on several races 3, biovar 2 strains (Anonymous, 2000; Elphinstone *et al.*, 2000).

**Experimental field.** This was planted for two consecutive years with tomato cv Moneymaker, in the Veneto Agricoltura Experimental Centre (Rosolina, Rovigo, Northern Italy), in soil below sea level, close to the Venice lagoon. Drainage water was collected in a "salt water" canal and pumped into the Canal Bianco close to the mouth of the river. A ditch and a boundary fence together with the surrounding water ensured isolation of the field, thus reducing the risk of environmental contamination. The experiment was authorized and supervised by the Regional Phytosanitary Service.

The plant were transplanted (130 × 25 cm) on April 27th in 3 rows of 40 plants each and inoculated on 2 and 29 July in two successive years. Inoculation was done on 2 July by placing a 50 ml drop of bacterial suspension ( $10^8$  cells ml<sup>-1</sup>), from colonies grown 48 h on TTC medium, in a transverse cut into the xylem made with a scalpel on the collar (2-3 cm above the ground). Control plants were similarly inoculated with distilled water.

**Side shoots and petioles.** Side shoots and petioles were removed from symptomless plants with a sterile scalpel in correspondence with their insertion point on the main stem at different heights from the ground. They were collected in the months of July, August and September at different times after inoculation (Table 1). In the laboratory, each shoot (or petiole) was washed in running tap water, surface-sterilized with cotton wool dipped in 70% ethanol and then air-dried. Sterile scalpels were used to cut 1 cm segments after removing a portion of a few millimeters from the base.

**Final concentrate.** Each segment was placed in a Stomacher bag (16 × 10 cm) and crushed with a hammer before adding 3-5 ml of sterile distilled water (SDW). After resting 15 min at room temperature (27-30°C) the suspension was filtered through a double layer of sterile gauze and the filtrate was used for DI. Each plate was streaked with 10 µl of suspension.

**Acropetal colonization of the stems.** Two symptomless 130 cm-high tomato plants were lifted on 2 September. After cutting the petioles and the shoots at the base, the main stem was washed and surface-sterilized as above. Starting from 15 cm above the inoculation point, 1 cm segments were cut at 10 cm intervals along the stem up to a height of 115 cm. The individual segments were used to prepare final concentrates (see above) to be used for DI.

**Latently infected stem segments.** Tomato plants with 5-6 leaves were grown in the greenhouse at 18-28°C and inoculated with *R. solanacearum* through wounds at the base. Twenty days after inoculation, 10 cm symptomless segments were cut from the main stem and 0.5 cm slices were cut from the ends of each segment. A 10 cm segment was considered to be latently infected when IFAS revealed the presence of fluorescent cell colonies at both ends. The latently infected 10 cm segments were cut with a scalpel into 1 cm segments. Then, 1 cm segments were prepared from the control plants.

**Sensitivity threshold.** This was assessed by preparing multiple samples consisting of one latently infected segment mixed with 49, 99, 199, 299, 499 and 999 healthy segments, taken from not inoculated plants. The different samples were tested for *R. solanacearum*.

**Isolation from multiple samples.** Multiple samples were placed in flask together with a sufficient volume of SDW to cover the segments on the bottom. The flask was placed on a rotary shaker (120 rpm) at room temperature for two hours. The washing liquid was filtered through double sterile gauze and the filtrate was centrifuged for 15 min at 10,000 g at 8-10°C. The pellet was resuspended in 1 ml of SDW (final concentrate) and used to prepare 10<sup>-1</sup> and 10<sup>-2</sup> dilutions with SDW. The final concentrate and the ten-fold dilutions were used for DI, IFAS and PCR.

**Table 1.** Results of testing lateral shoots of symptomless field tomato plants, cv Moneymaker, inoculated 7-55 days earlier with a virulent strain of *R. solanacearum* biovar 2, race 3. Data show a positive correlation between time (d.p.i.), successful re-isolation and shoot height (cm).

Days after inoculation	Frequency of successful reisolation	Heights of shoots with <i>R. solanacearum</i>
0-7	0.00	-
8-14	0.00	-
15-21	0.14	15, 18
22-28	0.12	12, 23
29-35	0.25	10, 15, 23, 32, 38
36-42	0.47	6, 8, 8, 12, 12, 12, 21, 26
43-49	0.30	8, 12, 18, 35, 37, 37,
50-55	0.79	0, 11, 12, 12, 13, 14, 15, 18, 19, 23, 24

**Data analysis.** Data on successful re-isolation frequencies, calculated as the number of reisolations divided by the number of sampled shoots in each day after inoculation, were grouped for the number of weeks after inoculation and fitted by a power model in the form:  $y = ax^b$ , where  $y$  = frequency of reisolations,  $x$  = number of weeks after inoculation and  $a, b$  = equation parameters (Clewer and Scarisbrick, 2001).

The number of re-isolations in each day after inoculation in shoots at different heights above the soil (0 to 76 cm) were grouped in classes 10 cm wide, and the frequency of re-isolations was calculated for each class dividing the number of positive re-isolations by the number of shoots at the same height. The relationship between the frequency of successful re-isolations and the height above the soil of sampled shoots was described by a third order polynomial model. Regression analyses were performed using the procedure of SPSS ver. 11.0 (SPSS Inc., Chicago, Illinois, USA). Data reliability was evaluated through regression analysis of variance, the standard error of parameters, the coefficient of determination ( $R^2$ ) and the distribution of residuals against the independent variable.

## RESULTS

Several tomato plants inoculated with *R. solanacearum* did not show any wilting symptom during the experiments. This may be due to the inoculation technique and to plant age. Root inoculation under controlled conditions usually led to wilting and death of almost 100% of plants (data not shown).

The side shoots and leaf petioles of symptomless plants were monitored for the endophytic presence of *R. solanacearum* in July, August and September on different numbers of days after inoculation. The results of the reisolations from the base of the side shoots at different heights above the soils are shown in Table 1. The first latent infections were detected after 20 and 21 days in shoots 18 and 15 cm above the ground respectively. The highest frequency of infection was always in shoots

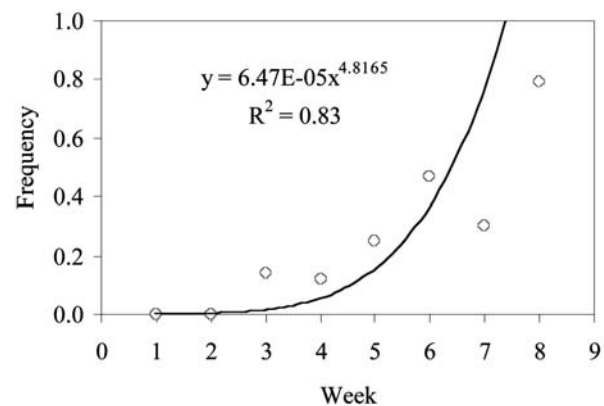
**Table 2.** Frequency of re-isolation of *R. solanacearum* biovar 2, race 3 for the lowest shoots at different intervals of height above the soil.

Height (cm)	Successful re-isolation (n.)	Shoots (n.)	Frequency
0-10	6	36	0.17
10-20	17	36	0.47
20-30	6	16	0.38
30-40	5	19	0.26
40-50	0	5	0.00
>50	0	5	0.00

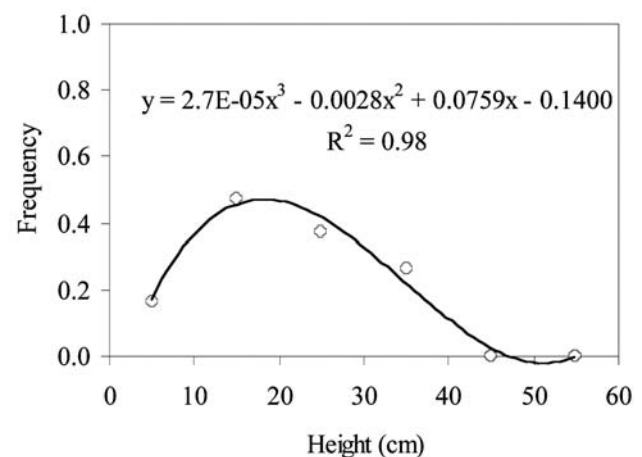
8-20 cm high. Detection was also successful for shoots between 21-30 cm and 31-40 cm high, but not in shoots more than 38 cm high.

The frequency of successful re-isolation increased as the number of weeks after inoculation increased, reaching the maximum (79%) after 8 weeks (Table 2). The highest frequency of re-isolation was between 10 and 20 cm above the soil (17 out of 36 shoots sampled at this height, independently from the day after inoculation) (Table 2), then the frequency decreased as the height increased.

The relationship between frequency of re-isolations and weeks after inoculation was satisfactorily fitted ( $R^2=0.83$ ) by the power model shown in Fig. 1, and the relationship between frequency of re-isolation and height of shoots above the soil by the 3<sup>rd</sup> order polynomial regression ( $R^2=0.98$ ) (Fig. 2). The regression analysis of variance was significant ( $P=0.002$  and  $P=0.03$ ) in both cases, showing that the highest part of total variance was



**Fig. 1.** Relationship between frequency of re-isolation of *R. solanacearum* on tomato plants, cv Moneymaker, and number of weeks after inoculation.



**Fig. 2.** Relationship between frequency of re-isolation of *R. solanacearum* on tomato plants, cv Moneymaker, and height of shoots above the soil.

accounted by the regression model; the standard errors of equation parameters were lower than parameter values and the residuals were randomly distributed (not shown).

*R. solanacearum* was successfully reisolated from leaf petioles after 41 days, in only 2 out of 37 cases, one at soil level and the other at a height of 6 cm. After 48 days, reisolation was not successful even for petioles 18-22 cm from the ground.

*R. solanacearum* was detected with IFAS and DI and confirmed by PCR on the pure cultures, and in all multiple, symptomless stem samples including the 1:999 mixture, i.e. down to a dilution of  $10^{-3}$ .

Approximately 2 months after inoculation of the stem base, the two whole plants monitored showed signs of wilting of the basal leaves and severe loss of turgor in the apical shoot. Brown discoloration of xylem tissues in the base of the main stem spread upwards in the form of streaks. *R. solanacearum* was successfully reisolated only up to a height of 55 cm from the inoculation point, from main stem segments with no discoloration. *R. solanacearum*-like colonies were isolated on Kelman's agar plates from stem segments collected at greater heights and up to 115 cm, but these were identified neither by PCR nor pathogenicity tests.

## DISCUSSION

Approximately 3 weeks after inoculation, the first latent infections were detected at the base of the lowest side shoots at a height of 15-18 cm from the ground. Over the following weeks, reisolation from symptomless plants was successful from shoots up to a maximum of 38 cm from the ground. The highest frequency of detection was constantly observed for shoots 18-21 cm from the ground. The frequency of reisolation was much lower and later for petioles on the main stem. This is evidence of the sensitivity and reliability of the results. Natural *R. solanacearum* inoculation generally occurs in the roots (Kelman, 1953). In young tomato plants, after colonizing the cortex and crossing the endodermic barrier, the bacteria invade the xylem and within a few hours spread upwards to the collar and the base of the stem (Vasse *et al.*, 1995). Little is known about the pathogenesis and movement of *R. solanacearum* in old tomato plants and in side shoots. Endophytic colonization and incubation period largely depend on cultivar, age of host plant and environmental conditions (Kelman, 1953; Hayward, 1991). Reisolation of *R. solanacearum* from the main stem of two plants with severe symptoms, two months after inoculation, was not successful above a height of 55 cm. This finding confirms the results obtained in attempts to reisolate *R. solanacearum* from the base of the lowest side shoots of symptomless plants and underlines the difficulties of DI to detect the pathogen

endophytically. Probably the upward colonization of the stem encounters host-plant barriers, which limit spread from the inoculation point (Nakaho *et al.*, 2000); consequently, absence of the pathogen above a certain height was expected. Moreover, possible presence of *R. solanacearum* in a vital but not culturable form (VBNC), as recently studied by Grey and Steck (2001), may explain the unsuccessful reisolation above a certain height on the main stem of wilting tomato plants.

The frequency of successful reisolation from side shoots increased over time in a more than linear manner up to 55 days after inoculation. As expected, detection of latent infections using the basal section of the lowest side shoots increased with time. Assuming that natural infections of field tomato plants occur during the first irrigation, e.g. at the beginning of June, monitoring should not be carried out before the end of July.

The epidemiology of *R. solanacearum*, as regards host colonization and disease progression, is related to the host cultivar, pathogenic features of the bacterial strain and agro-climatic conditions (Hayward, 1994). All our experiments were done on one cultivar, and using other cultivars or other environments might give different results. The protocol described here has been recommended by the Working Group responsible for setting up diagnostic methods for *R. solanacearum* in the European Union (Anonymous, 2004).

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