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ASSESSMENT OF INOCULATION TECHNIQUES SUITABILITY FOR DETERMINATION OF TOMATO PLANTS RESISTANCE TO BACTERIAL SPECK (*PSEUDOMONAS SYRINGAE* PV. *TOMATO*)

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Abstract

Four procedures applied for screening of tomato seedlings for resistance to bacterial speck showed that plants of 13 tomato cultigens exhibited a large amount of variation in response to *Pseudomonas syringae* pv. *tomato* (Pst) infection. The inoculation with spraying leaves on plants and leaves detached from the plants with water bacterial suspension gave the most homogeneous and consistent results. Two other methods accomplished by rubbing leaf surface and dipping young seedlings in a suspension of Pst were found not useful for the distinction of resistant and susceptible genotypes due to low repeatability of results. Low (2–5%) surviving stand of resistant cv. ‘Ontario 7710’ was also observed after application of both methods. Good correlation between cultigen susceptibility and disease symptoms intensity on the first four true leaves appeared. Out of 12 susceptible cultigens tested, 11 had the third leaf most infected.

Key words: *Lycopersicon esculentum*, cultigens, bacterial speck, resistance evaluation

Introduction

In recent years, bacterial speck of tomato, caused by *Pseudomonas syringae* pv. *tomato*, has become a major disease in most tomato growing areas of Poland. The disease may result in considerable economic losses caused by low fruit quality and plants productivity (Macias 1997). Currently, there are no commercial tomato cultivars resistant to bacterial speck grown in Poland. Management of the disease has been limited to the application of copper compounds and in some countries to

antibiotics. However, these practices have not been sufficiently effective (Jones and Jones 1989, Ramos et al. 1989, Da Silva and Lopes 1995, Pernezny et al. 1995). Development of cultivars with resistance to bacterial speck would be a valuable contribution to growers. Progress, however, depends on the availability of an effective technique to identify resistant germplasm and progeny at the seedling growth stage. Recent studies (Kozik and Sobiczewski 2000, Kozik et al. 2006) have demonstrated the range of differences in bacterial speck symptom intensity is caused by various *P. syringae* pv. *tomato* strains (race 0) isolated in Poland. However, it was also found that out of four available *P. syringae* pv. *tomato* strains tested, only Pst 3 gave homogeneous and consistent results. Additionally, a monogenic dominant (Pilovsky and Zutra 1982, Pitblado and MacNeill 1983) and incompletely dominant resistance (Kozik 2002) identified in cv. 'Ontario 7710' led to the assumption that a general improvement may be possible using breeding strategies. Another important prerequisite for successful breeding are methods which recognize genetic differences in bacterial speck resistance in the early development stages of plant growth. Although molecular markers for resistance genes against bacterial speck have been found (Martin et al. 1991, Carland and Staskawicz 1993), methods based on inoculation of plants are still commonly used in selection of breeding material to bacterial speck. Therefore, the purpose of work reported here was to compare different inoculation procedures based on reactions of resistant and susceptible cultigens at different growth stages to *P. syringae* pv. *tomato* infection at various inoculum levels.

Materials and methods

Bacteria and plant material

The bacterial strain Pst 3 of *P. syringae* pv. *tomato*, previously determined as race 0 (Kozik and Sobiczewski 2000, deposited at the Collection of Plant Pathogens of the Institute of Plant Protection, Poznań, Poland) was used in all tests. For preparation of inoculum, bacteria were incubated at 24°C on nutrient agar medium supplemented with 1% glucose. The inoculum was prepared by washing 24-hour-old cultures of bacteria with sterile distilled water. Concentration of bacteria in the suspension was adjusted to 10^8 and 10^7 cfu per 1 ml determined spectrophotometrically and by serial dilution plating method. Before inoculation of tomato plants, the strain was checked with hypersensitivity test on tobacco cv. 'Samsun' according to Klement (1963).

The following 11 cultigens (cultivars, breeding lines) were used in all experiments: 'Atma', 'Beta 40', 'Kirys', 'Lima', 'Mory 33', 'Mospomor', 'Movione', 'New Yorker', 'Riposta', 'Warszawski Płaski', 'Venture'. For comparison two cultigens: 'Ontario 7710' and A 100 were used as resistant and susceptible checks, respectively.

Experimental conditions and disease evaluation

10 days after sowing, 15 tomato seedlings of each cultigen were transplanted into 10 cm diameter plastic pots containing sphagnum peat substrate (Kronen Mix) and maintained on benches in a greenhouse. The study consisted of three experiments with three replications each.

In the first experiment three methods of inoculation on 'Kirys', A 100 and 'Ontario 7710' were performed:

method I – spraying leaves on plants with bacterial suspension using hand sprayer,

method II – rubbing upper leaf area with cheese cloth dipped in bacterial suspension,

method III – spraying detached leaves with bacterial suspension using hand sprayer; leaf petiole of detached leaves were placed in Erlenmayer flasks with 200 ml of water.

In the second experiment two methods of inoculation on 'Kirys' and A 100 were used: method I (see above) and another method of dipping seedlings in bacterial suspension for 1–2 s (method IV).

In the third experiment inoculation with method I was used on the first four true leaves of all 13 cultigens. The lesions of bacterial speck were counted separately on each leaf.

Methods I, II and III were applied to plants at the fourth true leaf stage, and method IV was applied to seedlings when the first true leaf was initiated. After inoculation the plants were kept under a plastic cover to maintain a relative humidity of 100%. After four–five days the cover was removed and therefore humidity fluctuated between 50 and 70%. The temperature set points were 27°C day/21°C night. Disease intensity was assessed two weeks after inoculation. The lesions of bacterial speck on inoculated leaves per plant were counted, then an overall rating for the plot was presented.

The selected colonies were used for studies according to Koch postulates.

All data were subjected to analysis of variance and separation of means was made using Student's test at 5% of probability.

Results

Checking strain Pst 3 pathogenicity according to Klement (1963) before all inoculation tests of tomato plants/leaves gave positive hypersensitive reaction on tobacco leaves cv. 'Samsun'.

In the first experiment, when leaves on plants and detached leaves were inoculated with bacterial suspension (methods I and III, respectively), a high density of very small specks distributed over leaf surfaces was noticed on the susceptible cultigens ('Kirys', A 100) at the first observation four days after inoculation. The

spots were surrounded by a chlorotic halo. After next four days, small specks started to form smaller or larger necroses.

When the leaves were rubbed with a cheese cloth dipped in bacteria suspension (method II), the first larger water soaked spots, followed by spreading of necrotic areas over entire leaf surface, were observed at the same time. Simultaneously, control plants treated with water showed similar tissue injury, but no initial symptoms in the form of dark-brown spots with chlorotic halo. Therefore inoculation sites in the first test were compared with the number of spots taken from the first date of scoring.

A significant interaction between inoculation procedure and cultigen was obtained (Table 1). All three cultigens used in the first experiment expressed different disease incidence depending on inoculation method, however similar tendency was observed in general. The highest disease severity appeared on all tested cultigens after the rub inoculation procedure (method II). There were no significant differences in quantity of spots on 'Ontario 7710' regardless of inoculation method. Two other cultigens tested 'Kirys' and A 100 showed significantly higher number of spots when the rub method was applied in comparison to both spray inoculation methods.

Table 1

Effect of inoculation method on incidence of bacterial speck (*Pseudomonas syringae* pv. *tomato*) in three cultigens of tomato plants at four–five true leaf stage (inoculated with bacterial suspension 10^8 cfu per 1 ml) (experiment 1)

Inoculation method	Cultigens		
	'Kirys'	A 100	'Ontario 7710'
I	45.2 Ba	55.6 Ba	2.3 Aa
II	167.4 Bb	156.1 Bb	31.2 Aa
III	52.5 Ba	66.3 Ba	4.8 Aa

I – spraying with bacterial suspension leaves on plants, II – rubbing upper leaf area with cheese cloth dipped in bacterial suspension, III – spraying with bacterial suspension leaves detached of plant.

Numbers in each column followed by the same small letter and numbers in each line followed by the same capital letter are not significantly different ($\alpha = 0.05$).

In the second experiment, reaction of cultigens to spray inoculation of plants at the four–five leaf stage (method I) and to dip inoculation of seedlings (method IV) was also much different (Table 2). At the cotyledonary stage (method IV) all seedlings of susceptible cultigens 'Kirys' and A 100 were killed at both concentrations (10^7 and 10^8 cfu per 1 ml) while the plants of resistant 'Ontario 7710' survived only in 2 and 5%, respectively. When the plants were inoculated with higher bacteria concentration (10^8 cfu per 1 ml) at the four–five leaf stage (method I) 'Ontario 7710' was only slightly infected, but 'Kirys' and A 100 showed very severe disease symptoms. In case of lower bacteria concentration (10^7 cfu per 1 ml) no symptoms were noticed on 'Ontario 7710'. Although plants of A 100 and 'Kirys' manifested higher infestation than 'Ontario 7710,' several plants showing no symptoms ("escapes") occurred at the concentration of 10^7 cfu per 1 ml.

Table 2

Effect of inoculum concentration and inoculation method on incidence of bacterial speck (*Pseudomonas syringae* pv. *tomato*) in three cultigens of tomato plants at four–five true leaf and cotyledon stage (methods I and IV, respectively) (experiment 2)

Cultigen	10 ⁸ cfu per 1 ml		10 ⁷ cfu per 1 ml	
	method I (transplants*)	method IV (cotyledons**)	method I (transplants*)	method IV (cotyledons**)
'Kirys'	58.1 b	100	18.2 b***	100
A 100	62.2 b	100	32.3 c***	100
'Ontario 7710'	5.5 a	98.2	0.0 a	95.4

*Spraying inoculation method (average number of spots per plant).

**Cotyledons dipping technique (percentage of dead plants).

***Occurrence of escapes on susceptible cultigens.

Numbers in each column (method I) followed by the same letter are not significantly different ($\alpha = 0.05$).

In the third experiment, rating of the first four leaves of cultigens sprayed with bacteria suspension at concentration of 10⁸ cfu per 1 ml showed significant interaction between leaf position and cultigen (Table 3). Therefore, it was possible to

Table 3

Severity of bacterial speck (*Pseudomonas syringae* pv. *tomato*) on the first four true leaves of 13 cultigens of tomato plants at four–five true leaf stage (inoculated with bacterial suspension 10⁸ cfu per 1 ml) (experiment 3)

Cultigen	Average number of spots on following leaf				Mean for cultigen
	1	2	3	4	
'Ontario 7710'	0.1 Aa	1.2 Aa	2.4 Aa	0.3 Aa	1.0 a
'Movione'	0.3 Aa	1.1 Aa	7.1 Bbc	1.0 Aa	2.4 ab
'Warszawski Płaski'	0.7 Aab	2.7 Aba	5.0 Bab	1.3 Aa	2.4 ab
'New Yorker'	0.8 Aab	3.1 Aa	6.9 Bbc	1.2 Aa	3.0 ab
'Venture'	0.8 Aab	2.2 Aa	7.7 Bbc	1.7 Aab	3.1 ab
'Mory 33'	1.0 Aab	5.0 Bab	8.2 Cbc	2.7 ABab	4.2 abc
'Riposta'	1.0 Aab	5.6 Bab	10.9 Cc	2.1 Aab	4.9 bc
'Beta 40'	1.5 Aab	8.5 Bbc	7.6 Bbc	3.0 Aab	5.2 bc
'Lima'	1.5 Aab	9.1 Bbc	9.5 Bbc	4.2 Aab	6.1 bc
'Atma'	1.7 Aab	11.5 Bcd	10.0 Bbc	3.7 Aab	6.7 cd
'Kirys'	2.3 Aab	13.6 Bd	15.8 Bd	4.5 Aab	9.1 de
'Mospor'	5.6 Aab	10.6 Bcd	22.4 Ce	6.9 Ab	11.4 ef
A 100	6.2 Ab	19.0 Be	23.9 Ce	5.7 Aab	13.7 f

Numbers in each column followed by the same small letter and numbers in each line followed by the same capital letter are not significantly different ($\alpha = 0.05$).

determine the cultigen resistance related to disease intensity on individual leaves. From among 13 cultigens tested, eight ('Movione', 'Warszawski Płaski', 'New Yorker', 'Venture', 'Mory 33', 'Riposta', 'Mospomor', A 100) showed symptoms of bacterial speck that were significantly higher on the third leaf than on the other leaves. In four other cultigens: 'Beta 40', 'Lima', 'Atma' and 'Kirys', there was no significant difference between the second and the third leaf. 'Ontario 7710' ranked as the most resistant cultigen for all foliar position and cultigens such as 'Mospomor' and A 100 consistently ranked as the most susceptible cultigens for all foliar positions.

Generally, bacterial speck was most severe on the third leaf, than on the second leaf for all cultigens. The lowest degree of disease infestation was observed on the first and the fourth leaf.

Discussion

Studies revealed significant differences in symptom expression depending on inoculation methods, plant age and inoculum concentrations. From among four inoculation methods, spraying leaves with inoculum concentration of 10^8 cfu per 1 ml gave the most homogeneous and consistent results. When the leaves were rubbed with a cheese cloth dipped in bacteria suspension, first larger water soaked spots followed by spreading of necrotic areas over entire leaf surface causing yellowing and finally leaf dropping were observed. Such an effect was caused by epidermis injury and high infection pressure which was evoked by high inoculum concentration on area unit of inoculated leaf tissue. Macias (1997) reported high severity of disease with fairly even spot distribution over leaves surface using the "rubbing" method. The differences in the two studies might be due to various growth stage of inoculated plants. In our studies, seedlings at the fourth true leaf stage were subjected to the "rubbing" treatment, while Macias (1997) treated plants whose first inflorescence was blooming. Because these plants were older, no damage of leaves was observed. In conclusion, the "rubbing" method gave good distribution of spots after inoculation and consistent disease severity which may be successfully expressed using that method, however, low repeatability of the results shows its disadvantage for screening tomato for resistance to bacterial speck. Differentiation of cultigens in epidermic and parenchyma susceptibility to mechanical injury may also play a substantial role in variability of results. In addition, the method is also time consuming and in case of even small delay in disease evaluation plants could not be clearly classified as resistant or susceptible due to severe and undesired damage of the leaves.

Another method accomplished by dipping young seedlings in a suspension of *P. syringae* pv. *tomato* was found not useful for the distinction of resistant and susceptible genotypes. The very high disease severity was affected by both inoculum densities 10^7 and 10^8 cfu per 1 ml. Plants of susceptible cultigens A 100 and 'Kirys' were killed, and cultivar 'Ontario 7710', considered resistant, survived in a very

low percentage only. Emmatty et al. (1982) observed similar expression of disease on susceptible cultivars while 68% of 'Ontario 7710' seedlings showed cotyledonary lesions and plant stunting at the concentration of 10^8 cfu per 1 ml. This appearance of disease severity on both resistant and susceptible plants was probably a consequence of a large quantity of inoculum dosage and a higher susceptibility of plants at the early stage of growth (young seedlings).

Inoculation by spraying bacterial suspension on leaves detached from plants is recommended but only as a support method in assessment of resistant and susceptible genotypes. In case of repeating tests this method can confirm the results on single plants, e.g. within segregating families. This method is more laborious and requires higher experimental experience, but still gives high efficiency in determination of plant resistance level.

As our investigations showed, an inoculum density of 10^8 cfu per 1 ml proved to be optimum with the respect to the reproducibility of the results from all tests within different methods of inoculation. This concentration caused severe disease symptoms on plants of susceptible cultigens and some symptoms on resistant cultivar 'Ontario 7710'. Several authors have demonstrated that 'Ontario 7710' showed no symptoms of disease both at an inoculum concentration of 10^7 cfu per 1 ml (Pitblado and MacNeill 1983) and even at 10^8 cfu per 1 ml (Lawson and Summers 1984) race 0 *P. syringae* pv. *tomato*. Kozik and Sobiczewski (2000), Kozik (2002), and Milijasevic and Todorovic (2006) have reported that *P. syringae* pv. *tomato* induced some disease symptoms on 'Ontario 7710' after spray application with 10^8 cfu per 1 ml. In the present study, concentration of 10^7 cfu per 1 ml gave no symptoms on 'Ontario 7710' but in susceptible A 100 some percentage of "escapes" occurred as a result of too low density of inoculum.

Disease symptom intensity and development of a reliable assay are very important criteria for finding plant resistance. In previous experiments, scoring of plants after artificial inoculation was based on number of spots on a whole plant (Pilovsky and Zutra 1982, Saad and Abul Hassan 2000, Kozik and Sobiczewski 2000, Kozik 2002). In present investigations a good correlation between cultigen susceptibility and disease symptoms intensity on the first true leaves was found. It was shown that out of 12 susceptible cultigens tested, 11 had the third leaf most infected. Results suggest that difference in reaction to foliar infection caused by *P. syringae* pv. *tomato* after artificial inoculation techniques may be measured on the third true leaf instead scoring the whole plants.

The results generated from presented studies should be useful in developing an assay for *P. syringae* pv. *tomato* resistance in large populations of segregating progeny of tomato breeding programs.

Streszczenie

OKREŚLENIE PRZYDATNOŚCI METOD INOKULACJI DO OCENY ODPORNOŚCI ROŚLIN POMIDORA NA BAKTERYJNĄ CĘTKOWATOŚĆ (*PSEUDOMONAS SYRINGAE* PV. *TOMATO*)

Spośród czterech ocenianych metod inokulacji roślin pomidora zawiesiną bakterii *Pseudomonas syringae* pv. *tomato* najlepszą do oceny odporności na bakteryjną cętkowość okazała się metoda polegająca na opryskiwaniu liści roślin pomidora w fazie czterech–pięciu liści wodną zawiesiną bakterii o koncentracji 10^8 jtk na 1 ml. W przeciwieństwie do pozostałych (pocieranie górnej powierzchni liści na roślinie gazą moczoną w zawieszynie bakterii, opryskiwanie zawiesiną bakterii oddzielonych od roślin liści, zanurzanie liścieni siewek w zawieszynie bakterii na 1–2 s), metoda ta dawała równomierny rozkład plam na liściach konieczny do rozróżnienia osobników podatnych od odpornych. Określono też zależność między podatnością odmiany czy linii a stopniem nasilenia objawów chorobowych na pierwszych czterech liściach i wykazano, że u 11 spośród 12 badanych odmian podatnych najsilniej porażony był liść trzeci. Oznacza to, że ocena podatności materiału hodowlanego w trakcie testów infekcyjnych może być przeprowadzona na podstawie nasilenia choroby na trzecim liściu właściwym, zamiast określania porażenia całej rośliny.

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