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SHORT COMMUNICATION

Establishment of a procedure for bacterial spot inoculation and assessment in processing tomato field trials

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Abstract This study analyses two methods for establishing bacterial spot xanthomonad infection on processing tomatoes and three assessment methods to measure bacterial spot severity. These methods were evaluated at different crop stages under field conditions. The trials were conducted in 2010 and 2011. Three cultivars with known resistance to bacterial spot were used to validate the procedures. The plants were infected by spray inoculation with bacterial suspension, or by natural bacterial spot dissemination from previously inoculated plantlets of the susceptible cultivar Yuba, planted equidistant between the plant rows (indirect procedure). Disease severity was estimated by three methods according to the period of assessment: A) percentage of necrotic area of the third and fourth leaves of 12 plants per plot up to 30 days after transplanting; B) percentage of necrotic area of 24 leaflets per plot from 30 to approximately 60 days after transplanting, and C) a plot-based disease severity scale from 60 days after transplanting. Both inoculation procedures resulted in disease occurrence which was not as uniform as when plants were naturally infected by the inoculum source. Tomato cultivars were successfully differentiated in terms of quantitative resistance by the three assessment methods employed.

Keywords *Solanum lycopersicum* · *Xanthomonas perforans* · Epidemiology · Patometry

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Bacterial spot is one of the major diseases of processing tomatoes in midwestern Brazil, which is the main tomato producing region in the country (Quezado-Duval et al. 2013). The disease is caused by four species of the genus *Xanthomonas*: *X. euvesicatoria* Jones et al.; *X. gardneri* (*ex* Šutic) Jones et al.; *X. perforans* Jones et al.; and *X. vesicatoria* (*ex* Doidge) Vauterin et al. (Jones et al. 2004). However, *X. perforans* has been prevalent in processing tomato fields (Quezado-Duval et al. 2013).

Bacterial spot can be rapidly destructive under enduring foliar wetness and specific temperature ranges which vary depending on the xanthomonad species (Marcuzzo et al. 2009; Araújo et al. 2011). Studies on bacterial spot management are focused on the development of resistant cultivars and on the evaluation of pesticide efficiency (Quezado-Duval and Lopes 2010). Two factors are essential for the success of these studies in the field: an inoculation procedure to initiate the epidemic uniformly on the experimental plots, and a discriminative evaluation method to quantify the severity of the disease.

The inoculation procedure should mimic natural infection in order to achieve uniform inoculum pressure (Gitaitis et al. 1986). However, it should not lead to very severe disease levels so that the treatments tested cannot be differentiated. Disease assessment levels should be easy to use and lead to accurate and reproducible results (Madden et al. 2007).

Processing tomato cultivars possess a determinate growth habit, are appropriate for low-growing cultivation, and the plant is bush-shaped during a large part of the crop cycle.

The heterogeneity of plant architecture throughout its life cycle together with abundant vegetative growth complicate the assessment of the severity of leaf diseases such as bacterial spot. Field experiments are important for the validation of disease management techniques and for the determination of resistance levels of different plant cultivars. Therefore, this study was undertaken to evaluate two infection procedures in field trials and to establish methods of disease severity assessment throughout the crop cycle for processing tomatoes.

Two field trials were carried out in 2010 (February to April) and 2011 (March to May) at Embrapa Hortaliças (Brasília, DF, Brazil). They consisted of completely randomized block designs with two factors, cultivar and inoculation procedures, and three replications. Plots consisted of three rows of 20 plants spaced at 0.25 cm within the row, with a buffer row between them on each side spaced at 1.10 m. Seeds were treated with hot water (50 °C/20 min) to eliminate any bacterial seedborne disease. Seedlings were transplanted at 25 days after sowing.

The cultivars used were Ohio 8245, Heinz 9553, both with different levels of quantitative resistance to bacterial spot, and Yuba, as a susceptible reference, with 'Heinz 9553' being the intermediate among them (Pontes et al. 2012). In order to prevent or at least minimize the dissemination of disease between plots, plants of the genotype Hawaii 7981, which has a hypersensitive response and a high level of field resistance to the T3 strain of *X. perforans* (Scott et al. 1995), were used as buffer rows between plots, following the same spacing scheme described above. Plants were irrigated using a sprinkler system in a regime of 25 mm per week. Climate variables, such as temperature, humidity and rainfall, were recorded throughout the trials (Fig. 1).

Xanthomonas perforans race T3 isolate EH-2008-13, previously identified by Quezado-Duval et al. (2013), was used in the inoculations. Bacterial suspension was prepared in 10 mM $MgSO_4$, and its concentration was adjusted for a final concentration of 5×10^7 CFU/mL (1:10 dilution of $A_{600}=0.3$). One week after transplanting, the two methods of bacterial inoculation were applied, namely direct and indirect. The direct procedure consisted of spraying the plants in the field with bacterial suspension using a manual sprayer until runoff. For the indirect procedure, previously inoculated plants of the susceptible cultivar Yuba were used as inoculum source. These plants were inoculated 5 days before transplantation by dipping the leaves in a bacterial suspension (5×10^7 CFU/mL). Three plants were planted in line between the rows in the plot spaced at about 1.7 m from each other and 0.2 m apart from the central row of the plot. At the time of planting water-soaked symptoms were already noticed.

Due to changes in plant architecture throughout the crop cycle three methods for disease severity assessment were adopted. The first method (method A) was used up to approximately 30 days after transplanting, when the plants were still separated from each other and leaves could be easily located. Foliar disease severity was rated on 12 plants per plot as an estimated percentage of necrotic area of the third and fourth leaves of the plant. Thirty days after planting, as the plants had grown and individual plants could not be separated without damaging them, a second method of severity rating (method B) was adopted, which consisted of estimating the percentage of necrotic area of each of 24 symptomatic leaflets sampled at six equidistant points per plot. From 60 days after sowing, the disease severity rates were recorded based on a visual scale using a one to ten rating, established according to non-measured increases in disease severity in the plot (method C) (Quezado-Duval et al. 2011).

Analysis of variance (F, $p \leq 0.05$) was carried out for data from methods A and B, followed by Fisher's test (LSD, $p \leq 0.05$). The results obtained for method C were subjected to a non-parametric analysis according to Akritas et al. (1997). In this case, when a significant effect of the tested factors was observed, the non-parametric analysis suggested by Shah and Madden (2004) was performed. Median, rank, and relative treatment effects for this method of severity rating were calculated using SAS macros developed by Brunner et al. (2002). Statistical analyses were performed using the GLM, MIXED and RANK procedures of SAS v. 9.2 software (SAS Institute, Inc.).

In the experiment conducted in 2010, disease symptoms (water soaked and necrotic lesions) were observed four days after inoculation (DAI) in some of the plants of the cultivar Yuba inoculated directly, but no significant differences were observed among treatments (F, $p=0.10$; data not shown). At 7 DAI, disease symptoms were observed in all plots with direct inoculation. There was no significant difference between 'Ohio 8245' and 'Heinz 9553' for disease severity (0.24 and 0.49 % of necrotic foliage, respectively), which were different from 'Yuba' (0.86 %) (LSD, $p \leq 0.05$). At 10 DAI, symptoms were first observed on the plants of the indirect inoculation treatment and with lower severity than those of the direct inoculation (F, $p < 0.0001$). No differences among the cultivars were detected (F, $p=0.99$), a pattern that remained up to 18 DAI (Table 1), when method A was used for the last time. At 18 DAI, direct inoculation resulted in higher disease severity than indirect inoculation in 'Heinz 9553' (F, $p=0.05$) and 'Yuba' (F, $p < 0.0001$) (Table 1). At 32 DAI, by using method B of disease scoring, no significant interaction effect was detected (F, $p=0.50$). However, significant differences were observed among cultivars (F, $p \leq 0.01$) and between inoculation methods (F, $p=0.04$). Disease severity was higher using the direct method (41.36 %) than the indirect procedure (32.4 %) (Table 2). 'Ohio 8245' was the most resistant, followed by 'Heinz 9553' and 'Yuba' (Table 2). At 65 DAI, when method C was used for disease scoring, significant interaction was detected between the tested factors for disease severity (χ^2,

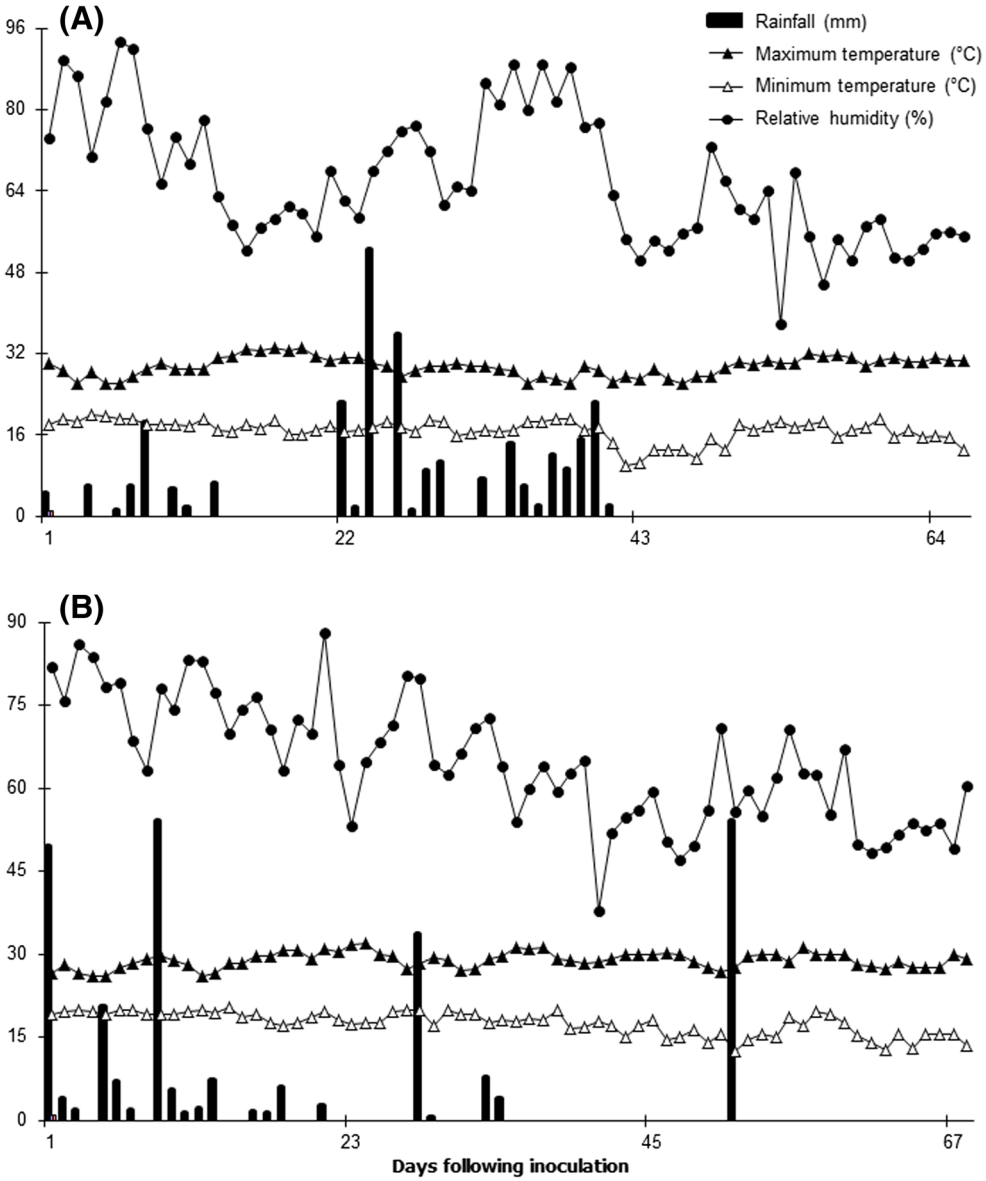

Fig. 1 Climate variables observed during the evaluation period of the severity of bacterial spot in tomato for the two experiments performed in 2010 **a** and 2011 **b**

Table 1 Severity (%) of bacterial spot in tomato at 18 and 21 days after inoculation for the experiments performed in 2010 and 2011, respectively

	18 days (2010)		21 days (2011)	
Cultivar	Direct	Indirect	Direct	Indirect
Ohio 8245	5.96 A	4.01 A	33.67 A	29.51 A
Heinz 9553	10.75 A	5.35 A*	47.97 B	44.86 B
Yuba	28.53 B	8.63 A*	75.37 C	56.95 B*

Values are averages of severity observed in the third and fourth leaves of 12 plants from plots inoculated by spraying inoculum on the field (direct method) or planting inoculated seedlings among the plants of the plot (indirect method). Averages followed by the same letter in each column were not significantly different (LSD, $p \leq 0.05$)

* Significant difference between inoculation methods (F, $p \leq 0.05$)

Table 2 Severity (%) of bacterial spot in tomato at 32 and 35 days after inoculation in the 2010 and 2011 experiments, respectively

	32 days (2010)			35 days (2011)		
Cultivar	Direct	Indirect	Average	Direct	Indirect	Average
Ohio 8245	28.5	16.4	22.45 A[1]	19.56	16.29	17.92 A
Heinz 9553	39.85	37.4	38.62 B	37.82	32.04	34.93 B
Yuba	55.73	43.4	49.56 C	46.13	56.14	51.13 C
Average	41.36	32.4*		34.51	34.82	

Values are averages of disease severity on 24 leaflets collected randomly in plots inoculated by spraying inoculum on the field (direct method) or by planting inoculated seedlings among the plants of the plot (indirect method). Averages followed by different letters in each "average" column were significantly different (LSD, $p \leq 0.05$)

* Significant difference between inoculation methods (F, $p \leq 0.05$)

p=0.0001). No significant differences were detected among the methods for the three cultivars, as the confidence interval of the relative effect of the treatment "methods" overlaps for each of them (Fig. 2b). With direct inoculation no significant differences were observed between 'Heinz 9553' and 'Ohio 8245', but 'Yuba' was the most susceptible to the disease (Fig. 2a). With the indirect method, no significant differences were observed between 'Heinz 9553' and 'Yuba', but 'Ohio 8245' was the most resistant to the disease (Fig. 2a).

In the experiment conducted in 2011, significant interaction between factors was observed at 21 DAI (F, p=0.05), similar to the observations made in the previous year. Differences in cultivar resistance were observed only with the direct inoculation treatment (Table 1). However, with indirect inoculation, 'Heinz 9553' was not significantly different from 'Yuba' (LSD, $p \leq 0.05$) (Table 1). Significant difference between inoculation methods was only observed for 'Yuba' (F, p=0.04), with lower disease severity with the indirect method.

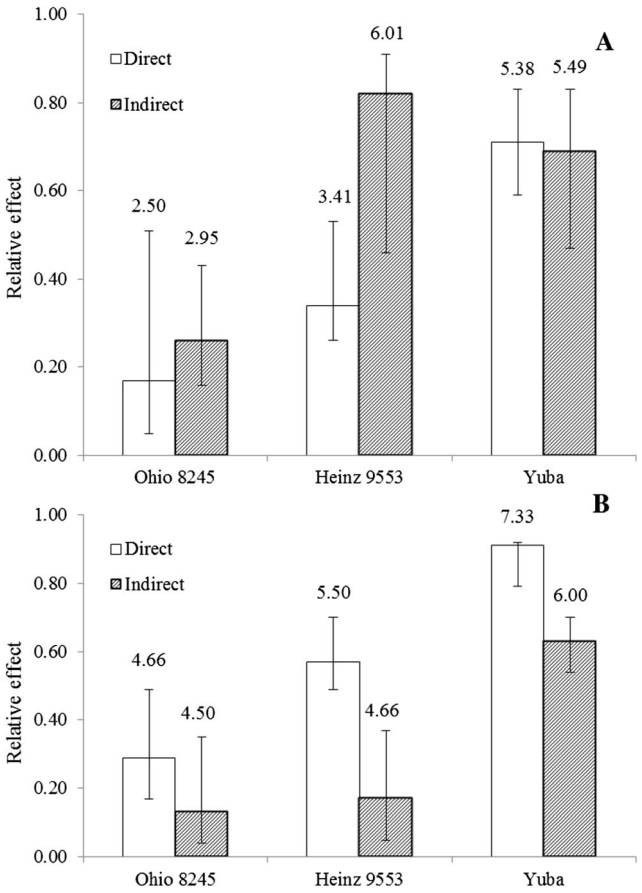

Fig. 2 Relative treatment effects for bacterial spot severity observed at 65 and 68 days after inoculation in 2010 **a** and 2011 **b**, respectively. Vertical bars represent the lower and upper limit of confidence interval (95 %) for relative treatment effect. Whenever there is an overlap between the confidence interval, there is no significant difference. Values above the bars were obtained using the evaluated grade scale with grades from 1 to 10

At 35 DAI, significant differences in disease severity levels were observed with method B of disease scoring (F, $p \leq 0.01$), similar to observations made earlier at 32 DAI in 2010 (Table 2). The factor "inoculation method", and interaction between this factor and "cultivar" were not significant (F, p=0.93 and 0.26, respectively). Significant interaction was detected between the two factors at 68 DAI (χ^2, p=0.01) for disease severity. Differently from observations made in 2010 at 65 DAI, the methods were different according to the cultivar, with no overlaps of the confidence interval of the relative effect of the treatment "methods" for 'Heinz 9553' and 'Yuba'. With direct inoculation, significant differences in disease severity levels were observed among the cultivars (Fig. 2b). However, this result was not observed in the field for the indirect procedure of infection because 'Ohio 8245' and 'Heinz 9553' were not significantly different from each other (p=0.05). Significant differences were detected between the two inoculation procedures for 'Heinz 9553' (χ^2, p=0.0003) and 'Yuba' (χ^2, p=0.01) (Fig. 2b).

In the present study, the manner by which the field plots were infected in the indirect inoculation procedure resulted in severely diseased plants. This simulated an infection from diseased volunteer plants, seedlings or alternative hosts. The role of these plants as inoculum sources for bacterial spot has been studied (Jones et al. 1984). In processing tomatoes fields in Brazil, volunteer plants constitute an important bacterial spot inoculum source (Quezado-Duval and Lopes 2010). These plants are originated from seeds of the fruit residues left in the field after tomato mechanical harvesting, which can be infected by the bacterial spot pathogens. Moreover, the ability of bacterial spot xanthomonad to infect tomato seeds as pointed by Jones et al. (1984) is probably linked to the appearance of symptoms on the volunteer plants which can be transmitted to the crop under favorable environmental conditions.

In both years, intense rainfall was recorded during the first days following inoculation (Fig. 1). The rain frequency decreased over the growing season. However, with the development of the crop, the canopy closes, thus forming a microclimate favorable to the disease, which resulted in the widespread occurrence of the disease in plants of both treatments. Although sprinkler irrigation was used to facilitate the dispersal of the pathogen, the disease occurred faster and appeared more uniformly distributed when plants were directly inoculated than when planting infected seedlings.

It is postulated that the use of infected plants as inoculum sources in field trials, a common practice in pathogen dispersal studies (Gregory 1968; Madden et al. 2007), could result in less disease pressure which facilitates the recognition of quantitative features of a chemical product or variety in test. However, occurrence of water splash and wind for aerosol formation which is responsible for bacterial dispersal from plant to

plant (McInnes et al. 1988) probably occurs in an erratic way (Gitaitis et al. 1986), which, on the other hand, could lead to misinterpretation of the results. This hypothesis might support the fact that at 65 DAI in 2010, the disease severity was higher for the moderately resistant 'Heinz 9553' than for 'Yuba' (Fig. 2a).

On the other hand, inoculum spraying can be criticized for being an artificial way of simulating the occurrence of the disease, and on this way to be so aggressive as to prevent treatment discrimination in field trials. Louws et al. (2001) evaluated the efficiency of a resistance inducer for the control of bacterial spot in the field using direct and indirect bacterial infection, both with satisfactory results. However, these authors considered direct inoculation more adequate because they were able to establish the precise timing of inoculum deposition on the plants. In contrast, the dispersal of the bacteria from diseased plants to healthy plants occurs over an extended period of time in indirect inoculation. In our study, even quantitative resistance could be differentiated with the direct method, especially at 32 DAI and 35 DAI in 2010 and 2011, respectively (Table 2). No differences were detected between the two procedures at later assessment times (Fig. 2). Thus, it seems that the assessment time also plays a role in effectively discriminating differences among severity levels.

Ji et al. (2006) used a similar method for fresh-market (indeterminate growth) tomato plants. They attributed grades of bacterial spot in percentages to leaves located between the second and fourth internode of plants from each plot for 3 weeks following inoculation. However, in the case of determinate growth tomatoes in this study, from a certain point in their development, locating and separating the branches is too labor intensive and time consuming to be practical.

Disease evaluation rated as a percentage of foliar necrosis on sampled leaflets proved to be a viable alternative for quantification of bacterial spot in processing tomatoes when plants have not yet set down, in our study and in others (Louws et al. 2001; Byrne et al. 2005; Ji et al. 2006). In addition, besides allowing the detection of differences among quantitatively resistance cultivars, they were observed in both assessment experiments performed, regardless of the inoculation procedure applied. Moreover, the collection of leaflets allows the identification of the etiological agent of leaf spot with greater precision, as a bacterial efflux test and isolation of the pathogen can be done. In field trials such diagnostic procedures are recommended as bacterial spot can be easily confounded with other tomato plant diseases (Al-Dahmani et al. 2003).

With the closing of the canopy, which usually occurs between days 50 and 60 after transplantation, it becomes difficult for the evaluators to move between plants without damaging them, which could compromise the yield data. This makes the collection of leaflets at later stages of the crop development impracticable. Descriptive scales have been used in previous studies of bacterial spot for both indeterminate and determinate tomato plants (McInnes et al. 1988; Louws et al. 2001; Al-Dahmani et al. 2003). Illustrated scale methods are practical and less time consuming, and can be useful, but can be less discriminative when used at a specific point in the growing season.

The use of more than one disease assessment method is an option for monitoring bacterial spot in tomato over the growing season (Gitaitis et al. 1986). This strategy has been previously used for disease progression studies also on fresh-market indeterminate tomato plants (Flaherty et al. 2000). In evaluating plant management treatments, it is thus possible, for example, to determine the moment when treatments start or cease to be effective due to expression of resistance components or to the aggressive nature of a disease under very favorable environmental conditions, respectively. Therefore, the use of a data set over time will be more likely to be successful for the selection of treatments resulting in higher production.

The use of direct inoculation together with evaluations over time, validated in this study, could be recommended to standardize and facilitate the procedures for evaluations of treatments and practices for bacterial spot management on processing tomatoes.

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References

Akritas MG, Arnold SF, Brunner E (1997) Nonparametric hypothesis and rank statistics for unbalanced factorial designs. J Am Stat Assoc 92: 258–265

Al-Dahmani JH, Abbasi PA, Miller SA, Hoitink HAJ (2003) Suppression of bacterial spot of tomato with foliar sprays of compost extracts under greenhouse and field conditions. Plant Dis 87:913–919

Araújo ER, Pereira RC, Ferreira MASV, Café-Filho AC, Moita AW, Quezado-Duval AM (2011) Effect of temperature on pathogenicity components of tomato bacterial spot and competition between *Xanthomonas perforans* and *X. gardneri*. Acta Horticult 914:39–42

Brunner E, Domhof S, Langer F (2002) Nonparametric analysis of longitudinal data in factorial experiments. Wiley, New York

Byrne JM, Dianese AC, Ji P, Campbell HL, Cuppels DA, Louws FJ, Miller SA, Jones JB, Wilson M (2005) Biological control of bacterial spot of tomato under field conditions at several locations in North America. Biol Control 32:408–418

Flaherty JE, Jones JB, Harbaugh BK, Somodil GC, Jackson LE (2000) Control of bacterial spot on tomato in the greenhouse and field with h-mutant bacteriophages. HortSci 35:882–884

Gitaitis RD, Jones JB, McCarter SM (1986) Evaluation of chemical control of bacterial diseases of tomato. In: Hickey KD (ed) Methods for evaluating pesticides for control of plant pathogens. APS Press, St. Paul, pp 205–209

Gregory PH (1968) Interpreting plant disease dispersal gradients. Annu Rev Phytopathol 6:189–212

Ji P, Campbell HL, Kloepper JW, Jones JB, Suslow TV, Wilson M (2006) Integrated biological control of bacterial speck and spot of tomato under field conditions using foliar biological control agents and plant growth-promoting rhizobacteria. Biol Control 36:358–367

Jones JB, Stall RE, Jones JP, Phoronezny KL (1984) Survival of *Xanthomonas campestris* pv. *vesicatoria* in Florida. Phytopathology 74:858–858

Jones JB, Lacy GH, Bouzar H, Stall RE, Schaad NW (2004) Reclassification of the xanthomonads associated with bacterial spot disease of tomato and pepper. Syst Appl Microbiol 27:755–762

Louws FJ, Wilson M, Campbell HL, Cuppels DA, Jones JB, Shoemaker PB, Sahin F, Miller SA (2001) Field control of bacterial spot and bacterial speck of tomato using a plant activator. Plant Dis 85:481–488

Madden LV, Hughes G, van den Bosch F (2007) The study of plant disease epidemics. APS Press, St. Paul

Marcuzzo LL, Becker WF, Fernandes JMC (2009) Alguns aspectos epidemiológicos da mancha bacteriana (*Xanthomonas* spp.) do tomateiro na região de Caçador/SC. Summa Phytopathol 35:132–135

McInnes TB, Gotaotos RD, McCarter SM, Jawprslo CA, Phatak SC (1988) Airborne dispersal of bacteria in tomato and pepper transplant fields. Plant Dis 72:575–579

Pontes NC, Moita AW, Quezado-Duval AM (2012) Resistance stability of 'Ohio 8245' and 'Heinz 9553' to tomato bacterial spot. Hortic Bras 30:99–105

Quezado-Duval AM, Lopes CA (2010) Mancha bacteriana: uma atualização para o sistema de produção integrada de tomate indústria. Embrapa Hortaliças, Circ Tec 84:1–24

Quezado-Duval AM, Pontes NC, Nascimento AR, Moita AW (2011) Metodologia de avaliação da severidade da mancha bacteriana em tomateiro para processamento industrial. Embrapa Hortaliças, Bol Pesq Desenvol 73:1–24

Quezado-Duval AM, Inoue-Nagata AK, Reis A, Pinheiro JB, Lopes CA, Araújo ER, Fontenelle MR, Costa JR, Guimarães CMN, Rossato M, Becker WF, Costa H, Ferreira MASV, Destéfano SAL (2013) Levantamento de doenças e mosca-branca em tomateiro em regiões produtoras no Brasil. Embrapa Hortaliças, Bol Pesq Desenvol 100:1–36

Scott JW, Jones JB, Somodi GC (1995) Screening tomato accessions for resistance to *Xanthomonas campestris* pv. *vesicatoria*, raceT3. HortSci 30:579–581

Shah DA, Madden LV (2004) Nonparametric analysis of ordinal data in designed factorial experiments. Phytopathology 94: 33–43