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BACTERIAL AND PHYTOPLASMA DISEASES

# Spatiotemporal distribution of tomato plants naturally infected with bacterial canker in greenhouses

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**Abstract** The distribution of plants with tomato bacterial canker within a greenhouse was analyzed using Morisita's binomial index of dispersion, $I_B$, to assess spatial distribution patterns. The distribution patterns of diseased plants were similar in four commercial greenhouses. The degree of clustering of added together diseased plants based on the $I_B$ index did not increase with time, but the statistical significance of the cluster distribution did increase, suggesting that new independent cluster points had formed during the investigation. Therefore, a scattered pattern of potentially or apparently diseased plants caused by primary inoculum from residual plants in the soil emerged with time.

**Keywords** Spatiotemporal distribution · Tomato bacterial canker · Morisita's $I_B$-index · Epidemiology

Bacterial canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis* (CMM) is one of the most important bacterial diseases of tomato and causes substantial economic losses worldwide (de León et al. 2011; Gleason et al. 1991, 1993; Jahr et al. 1999). Bacterial canker was first observed in Japan in 1958 (Narita and Baba 1958) and has been seen in field-grown tomatoes worldwide. In recent years, bacterial canker has emerged in commercial greenhouses in Okayama Prefecture, Japan.

CMM can survive on plant debris in soil for up to 2 or 3 years (Fatmi and Schaad 2002; Gleason et al. 1993). It enters the plant through wounds or natural openings including stomata (Carlton et al. 1992; Chang et al. 1991; Gleason et al. 1991, 1993; Grogan and Kimble 1967). The pathogen can also infest seeds and be transmitted to CMM-free areas via infested seeds (Biggerstaff et al. 2000; Gleason et al. 1993; Grogan and Kimble 1967). Secondary spread is caused by splashing water and contaminated equipment and workers' hands (Gitaitis 1991). Thus, tomato can be infected through several different infection routes, but it was unclear which route contributed the most to the explosive emergence in commercial greenhouses in Japan. We previously differentiated the CMM strains from eight locations in Japan into four haplotypes based on repetitive sequence-based polymerase chain reaction (rep-PCR) (Kawaguchi et al. 2010). Regardless of the year of isolation, location, or tomato cultivar, the strains in each greenhouse and location belonged to the same haplotype, suggesting that the strains originated from the previous greenhouse population (Kawaguchi et al. 2010).

Many observations have indicated that the number of diseased plants increase within a row of tomato plants in commercial greenhouses when buds and extra leaves are removed by hand or with nonsterilized scissors. In greenhouses in Japan, inoculum is not carried by splashing water because no heavy rain or wind enters from outside and water is delivered to plants in tubes under the plastic mulch. On the basis of Morisita's index of dispersion, $I_\delta$ (Morisita 1959, 1962), plants in greenhouses in which plants are disbudded and defoliated with scissors or by hand became diseased in the same direction of the manipulations; thus, CMM spread in an aggregated distribution in a quadrant along a row of plants, but the distribution of diseased plants indicated a random distribution in a quadrant along a furrow of plants, suggesting that disbudding and defoliation contribute highly to the secondary

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spread in commercial greenhouses (Kawaguchi et al. 2010).

As in our previous report (Kawaguchi et al. 2010), plants infected with CMM in the greenhouses were aggregated in a quadrant in the same direction as the disbudding and defoliation in each ridge. However, it is still unclear how these clusters of diseased plants formed over time during the season. Therefore, we investigated the spatiotemporal distribution of plants naturally infected with CMM in commercial greenhouses on three occasions after the first detection of the disease.

From 2005 to 2008 in four commercial greenhouses (A–D) with two rows of tomato plants per furrow in each greenhouse, we analyzed the spatial patterns of tomato bacterial canker spread in Okayama Prefecture, Japan. All farmers had purchased self-rooted or grafted transplants each year. Moreover, all farmers regularly disbudded and defoliated plants using nonsterilized scissors or hands.

Untreated greenhouse A (6.0 × 40.0 m) in Maniwa City was planted with 360 tomato plants cv. Momotaro-8 on 23 May 2005 in three furrows and six rows spaced 60 cm apart with 60 cm between each plant; results were recorded on 1, 11 and 28 July 2005. The total percentage of plants with disease on each recording day was 5.3, 13.6, and 27.2 %, respectively. Greenhouse B (6.0 × 46.0 m) in Maniwa City was planted with 480 tomato plants (cv. Momotaro-8) on 13 May 2008 in three furrows and six rows spaced 60 cm apart with 60 cm between plants, and the results were recorded on to 1 and 29 August 2008 and 1 October 2008. The percentage of total plants with disease on each recording day was 0.6, 5.0, and 15.6, respectively. Greenhouse C (6.0 × 46.0 m) in Maniwa City was planted with 480 tomato plants (cv. Momotaro-8) on 13 May 2008 in three furrows and six rows spaced 60 cm apart and with 60 cm between plants. On 27 June 2008 and 1 and 29 August 2008, the percentage of plants with disease was 7.1, 20.6, and 38.5 %, respectively. Greenhouse D (6.0 × 40.0 m) in Tsuyama City was planted with 360 tomato plants (cv. Momotaro-York) on 5 March 2008 in three furrows and six rows spaced 60 cm apart with 60 cm between each plant. On 20 April 2008, 15 May 2008, and on 22 July 2008, the percentage of plants with disease was 2.5, 5.0, and 13.1 %, respectively. Greenhouses A, B, and C were unheated, whereas D was maintained at 10 °C.

Disease severity was indexed and mapped for each greenhouse in a quadrant pattern, which consisted of one row of six to eight plants. Plants with canker symptoms were counted. ImmunoStrips for CMM (Agdia, Elkhart, IN, USA) were used for a preliminary diagnosis. To confirm the identification, we isolated bacteria from about 10 plants in each greenhouse on potato semi-synthetic agar medium (PSA, 1 L of boiled extract of 300 g potato tubers, 0.5 g $Ca(NO_3)_2 \cdot 4H_2O$, 2 g $Na_2HPO_4 \cdot 12H_2O$, 5 g peptone, 20 g sucrose, 15 g agar, pH 6.8–7.0) and used the simplified isolation method with ImmunoStrips developed previously (Tanina and Kawaguchi 2011), then identification of suspect CCM was confirmed by standard PCR using the CMM-specific primer set CMM-5/CMM-6 as described (Dreier et al. 1995). There were no other diseases of tomato in any of the greenhouses.

Morisita's binomial index of dispersion, $I_B$, was calculated using the formulas:

$$I_\delta = n \sum_{i=1}^{n} x_i(x_i - 1)/[N(N-1)] \quad (1)$$

$$I_B = I_\delta(N - 1/n)/(N - 1), \quad (2)$$

where $n$ is the number of quadrants, $x_i$ is the number of diseased (wilted) tomato plants $i$ ($i = 1 \ldots n$), and $N$ is the total number of diseased tomato plants ($= \sum_{i=1}^{n} x_i$). The $I_\delta$ index is a measure of the dispersion of individuals in a population. The $I_B$ index is also a measure of dispersion of individuals in a population, especially in the case of a binominal distribution (Morisita 1962): $I_B < 1.0$ indicates a uniform distribution, $I_B = 1.0$ a random distribution, and $I_B > 1.0$ an aggregated distribution (Morisita 1962). According to the method of Morisita (1959, 1962), $I_B$ values constitute a statistically significant departure from a random distribution ($I_B = 1.0$) in comparison to $F_0$ value with the value of $F_{\infty}^{n-1}(\alpha)$:

$$F_0 = [I_B(N-1) + n - N]/(n-1). \quad (3)$$

When $I_B$ values did not significantly differ from 1.0, the distribution of diseased plants in the greenhouse was considered random.

Four commercial greenhouses planted with tomato plants were investigated in 2005 and 2008. The distribution of total diseased plants infected by CMM indicated an aggregated distribution in the four greenhouses during the investigation because $I_B$ values were significantly higher than 1.0 at $P < 0.05$ or 0.01, except for greenhouse B on 1 August 2008 (Figs. 1a, 2a, 3a, 4a). The degree of clustering of diseased plants based on the $I_B$ value did not increase with time, but the significance of the cluster distribution did increase (Figs. 1a, 2a, 3a, 4a). The number of new diseased plants found at each observation indicated an aggregated distribution in greenhouse A on 1 July 2005 and 11 July 2005 (Fig. 1b), in greenhouse B on 29 August 2008 and 1 October 2008 (Fig. 2b), in greenhouse C on 27 June 2008 and 1 August 2008 (Fig. 3b, 5), and in greenhouse D on 20 April 2008, 15 May 2008, and on 22 July 2008 (Fig. 4b).

The distribution patterns of diseased plants within the four commercial greenhouses were all similarly aggregated during the investigation. In greenhouse B on 1 August 2008, the distribution of accumulated diseased plants did

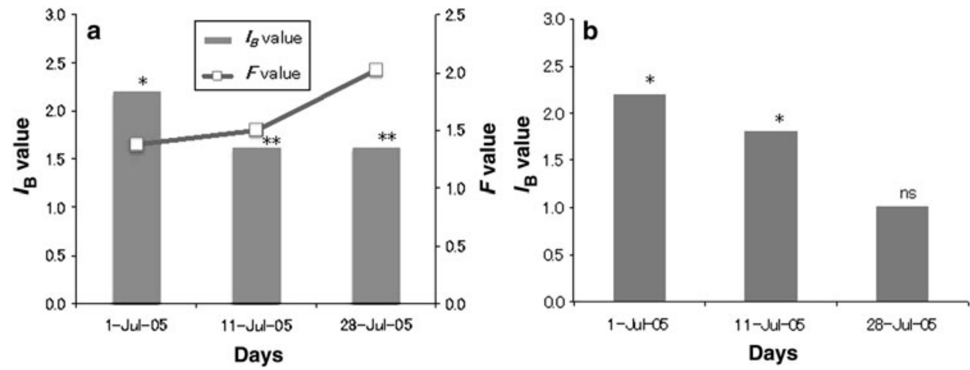


**Fig. 1** Morisita's $I_B$ and $F$ values for diseased tomato plants with bacterial canker in naturally infected greenhouse A in 2005. *Asterisks* indicate $I_B$ values differed significantly from 1.0 ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{ns}P > 0.05$) according to Morisita's $F$-test method (Morisita 1962). **a** Total number of diseased plants. **b** Number of diseased plants at each observation

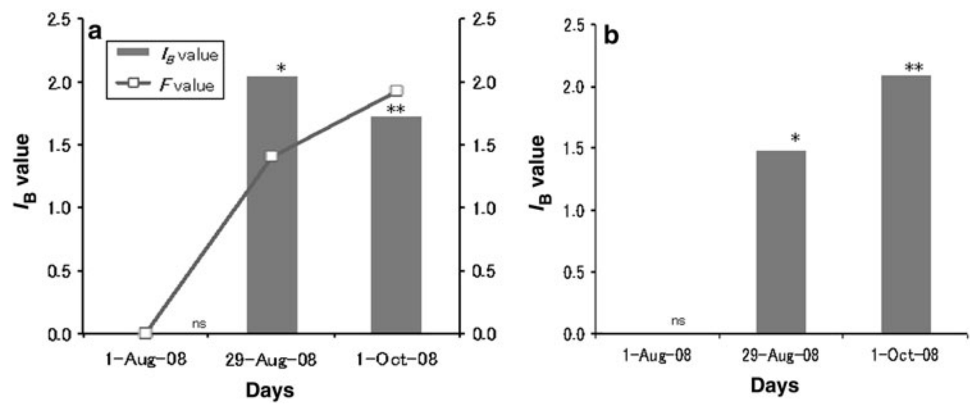


**Fig. 2** Morisita's $I_B$ and $F$ values for diseased tomato plants with bacterial canker in naturally infected greenhouse B in 2008. *Asterisks* indicate $I_B$ values significantly greater than 1.0 ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{ns}P > 0.05$) according to Morisita's $F$-test method (Morisita 1962). **a** Total number of diseased plants. **b** Number of diseased plants at each observation

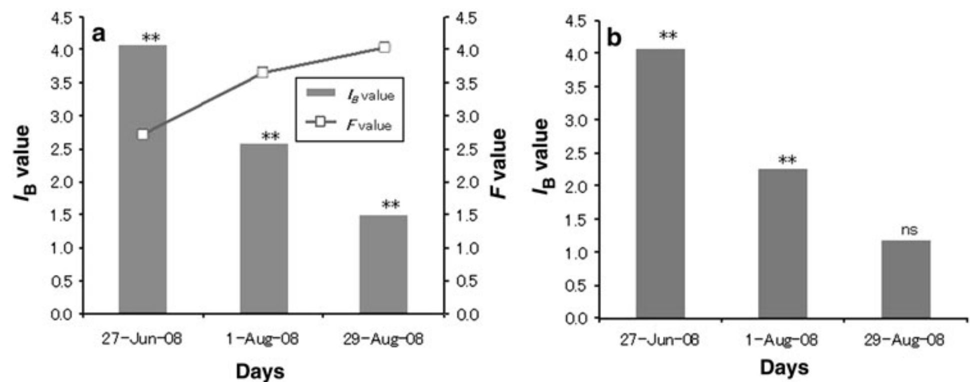


**Fig. 3** Morisita's $I_B$ and $F$ values for diseased tomato plants with bacterial canker in naturally infected greenhouse C in 2008. *Asterisks* indicate $I_B$ values significantly greater than 1.0 ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{ns}P > 0.05$) according to Morisita's $F$-test method (Morisita 1962). **a** Total number of diseased plants. **b** Number of diseased plants at each observation

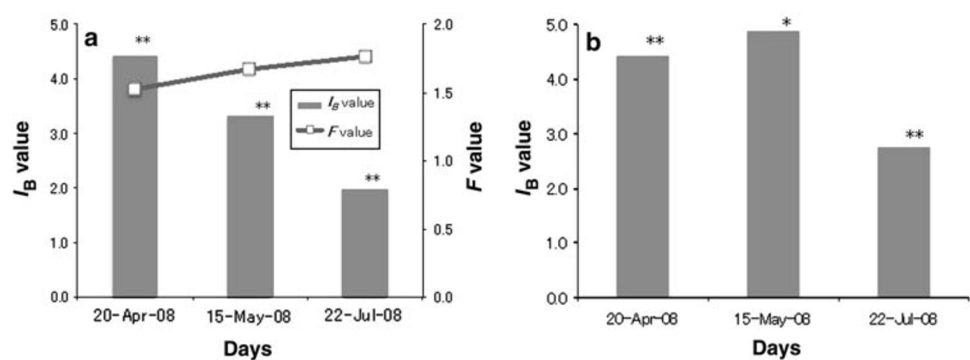


**Fig. 4** Morisita's $I_B$ and $F$ values for diseased tomato plants with bacterial canker in naturally infected greenhouse D in 2008. *Asterisks* indicate $I_B$ values significantly greater than 1.0 ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{ns}P > 0.05$) according to Morisita's $F$-test method (Morisita 1962). **a** Total number of diseased plants. **b** Number of diseased plants at each observation

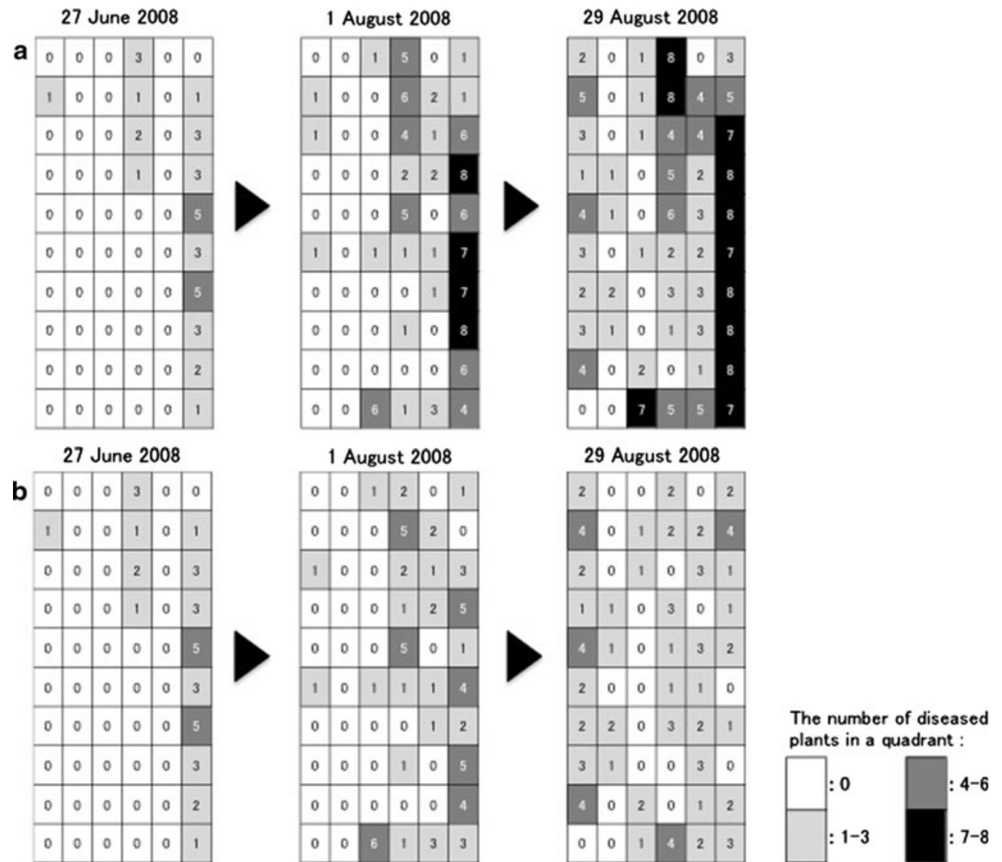


**Fig. 5** Plan of greenhouse C in 2008 divided into 60 quadrants of eight tomato plants each. Numbers of diseased plants that correspond to each degree of shading also are shown. **a** Total number of diseased plants. **b** Number of diseased plants at each observation

not qualify as an aggregated distribution because too few plants were diseased (0.6 %).

In general, when the number of diseased plants and the Morisita index increase with time, new infections cluster around a point, and each cluster point expands (Ohsaki et al. 2009). On the other hand, when the Morisita index does not increase but the *F* value, as a signifier of the cluster distribution, does increase with time, newly diseased plants form new independent points of clustering (Ohsaki et al. 2009). In this study, the degree of clustering of the total diseased plants based on the $I_B$ value did not increase with time, but the significance of the cluster distribution did increase. Moreover, the distribution of diseased plants at each observation indicated an aggregated distribution at two of the three observations in greenhouses A, B, and C, and at all three times in greenhouse D. Thus, these results suggest that new independent points of clustering had formed during the investigation.

Similar results were reported for Fusarium head blight of wheat (Ohsaki et al. 2009); populations of the pathogen formed new independent clusters during the investigation, suggesting that ascospores from the primary inoculum started new infections over long periods in fields and that conidia have a limited role in secondary infection in the disease cycle (Ohsaki et al. 2009). In the case of tomato bacterial canker, however, CMM enters a production area primarily through infected seed (Grogan and Kimble 1967), latently infected tomato transplants (Gleason et al. 1993), and as we previously reported for greenhouse-grown tomato plants in Okayama, Japan, primary inoculum can originate each year from residual plant debris in the soil (Kawaguchi et al. 2010). We also reported that disbudding and defoliation contributed highly to secondary outbreaks of bacterial canker in commercial greenhouses (Kawaguchi et al. 2010).

Our present statistical analysis showed that independent points of clustering formed during the investigation, indicating that the primary inoculum emerged at scattered points and that scattered clusters of diseased plants resulted from secondary inoculum over long periods in greenhouses. We speculate that the primary inoculum is residual plant debris in the soil and that secondary inoculum is spread by contaminated equipment and hands during disbudding and defoliation. Although CMM does not survive well in soil, it can survive on plant debris for several years (Fatmi and Schaad 2002; Gleason et al. 1993). Thus, farmers are advised to discard infected tomato plants and tomato debris and to disinfest the soil by soil-sterilization methods because CMM from the primary inoculum can cause new infections over long periods. Moreover, we

recommend that farmers sterilize scissors and gloves by dipping them in a disinfectant to prevent the secondary spread during disbudding and defoliation. Such a dip in 0.2 % calcium hypochlorite reduces CMM infection by about 80 % (Kawaguchi 2012).

In conclusion, CMM from the primary inoculum initiates new infections, and the scattered clusters of diseased plants are started by secondary inoculum on contaminated scissors and hands during disbudding and defoliation over long periods in greenhouses in Japan. This study shows that statistical analysis of spatiotemporal distribution provides clues to infer the source and mode of spread of the bacteria causing tomato bacterial canker.

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