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Automated Needle-free Injection Method for Delivery of Bacterial Suspensions into Citrus Leaf Tissues

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Abstract

A prototype needle-free device was evaluated for delivery of *Xanthomonas citri* subsp. *citri* bacteria into the leaves of cultivars susceptible and resistant to citrus canker. The device delivered a precisely controlled volume of bacterial suspension through infiltration of stomata by injection with pressurized gas. The device produced a uniform inoculation of bacteria into the leaves as measured by the volume of infiltration and diameter of the infiltrated area. No damage to the leaves was observed after inoculation with the automated device, even though a higher number of canker lesions developed compared to a hand-held needleless syringe injection method. The level of practice needed for operation of the automated device was minimal compared to considerable skill required to perform the hand-held injection. Results from inoculations with the automated device are in accord with the results with the hand-held syringe method that demonstrated kumquats are highly resistant to citrus canker while rough lemon and 'Hamlin' sweet orange are susceptible.

Introduction

Citrus canker, caused by *Xanthomonas citri* subsp. *citri*, (*Xcc*), is a serious citrus leaf and fruit spotting disease affecting susceptible citrus in the wet subtropics (Graham et al. 2004). Canker strain A of *Xcc* is pathogenic on all citrus species and produces disease reactions that range from highly susceptible to moderately resistant. In host range studies, leaf susceptibility to *Xcc* is measured by inoculation of immature tissues. Leaf mesophyll susceptibility to *Xcc* is quantified by inoculation of immature leaves by injection-infiltration of bacteria through the stomata (Gottwald and Graham 1992; Graham et al. 1992). The injection-infiltration of immature leaves is preferred over wound inoculation because this method permits the quantification of the number of lesions obtained from stomatal infections (Viloria et al. 2004; Francis et al. 2010). A major limitation of this inoculation method is that it depends on the ability of an operator to accurately inject bacterial inoculum into the leaf using a hand-held needleless syringe. A very accurate method for infiltration was achieved with a stomatal inoculation apparatus (SIA; Gottwald and Graham 1992). The SIA consisted of a small inoculation chamber attached to an intact leaf. Water and inoculum were introduced into an airstream to impact a 1-mm-diameter area of the leaf surface. SIA provided a reproducible non-injurious means to introduce *Xcc* into leaf tissues on the abaxial surface. The SIA was stationary on the laboratory bench and required mounting the leaf at a precise distance from the injector. Therefore, the SIA was not adaptable for use as a hand-held device for inoculation of plants in the greenhouse.

The objective of this study was to evaluate a hand-held device for delivery of bacterial inoculum at a precise volume and pressure against the abaxial leaf surface for inoculation of plants with *Xcc* to compare the susceptibility of leaves to citrus canker.

Materials and Methods

Bacteria and plant material

Xanthomonas citri subsp. *citri* strain X2002-0014 used in this study was isolated in 2002 from sweet orange (*Citrus sinensis* [L.] Osbeck) in Dade County, Florida. The culture was stored in glycerol under −80°C conditions in an ultra low freezer.

The cultivars chosen for comparison of inoculation methods were canker-susceptible rough lemon (RL; *C. jambhiri* Lush) and 'Hamlin' sweet orange (SWO; *C. sinensis*), and canker-resistant 'Meiwa' kumquat (MK; *Fortunella crassifolia* Swingle) and 'Nagami' kumquat (NK; *F. margarita* [Lour.] Swingle. Plants

were propagated in soil-less medium (The Scotts Co., Marysville, OH, USA) contained in 3.8-l pots and maintained in the greenhouse between 20 and 30°C. Plants were fertilized every 2 weeks with Peters 20-10-20 (0.5 g/l) and supplemented with Essential Minor Elements (5 g per pot; The Scotts Co.).

Plant inoculation procedures

The pulse needle-free injection system (PNFIS) used in this study is based on the Pulse 50 Micro Dose Injection System (Pulse NeedleFree Systems, Inc., Lenexa, KS, USA) designed as an animal health injection system for transdermal needle-free injections at dosage volumes of 100–500 μl. The engineering modifications to the PNFIS for this study included reducing the dosage volume to provide dose-accurate injections at volumes as low as 10 μl (Fig. 1). This was achieved by modification of the injection force profile to eliminate the initial pressure spike required to penetrate livestock tissues and by prolonging the duration of injection from approximately 0.1–0.5 s to provide greater opportunity for infiltration of the mesophyll. The PNFIS inoculation head was modified to ensure uniform surface contact between the PNFIS nozzle face and the abaxial surface of the leaf.

Infiltrated volume was measured using a precision balance by weighing the leaves before and immediately after each infiltration. Comparison of the PNFIS with the hand-held syringe method (HHSM) previously described (Viloria et al. 2004) was conducted by

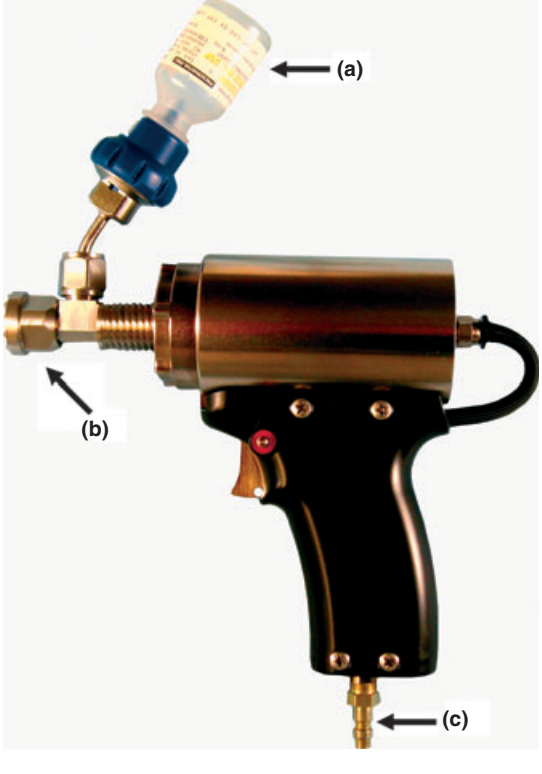

Fig. 1 Pulse 50 Micro Dose Injection System. (a) container for inoculum, (b) inoculation head and (c) connection to the compressed gas cylinder

injection of immature leaves (75% expanded) on shoots (20–25 cm long) produced by pruning vigorous greenhouse plants. Three injections per leaf were performed on each side of the leaf midrib for SWO and RL, and two injections were performed for kumquats with smaller leaves.

To produce bacterial inoculum, *Xcc* was grown overnight in nutrient broth, the suspension centrifuged at 10 000 ***g*** for 20 min and re-suspended in sterile saline phosphate buffer (PBS; 40 mM Na_2HPO_4 + 25 mM KH_2PO_4). The bacterial suspension was adjusted to 0.1 OD (at 620 nm), equivalent to 10^8 colony-forming units (cfu) per ml and diluted to 10^5 cfu per ml for inoculations. For the HHSM, a 1-cm^3 needleless tuberculin syringe was loaded with bacterial suspension and the syringe tip pressed against the abaxial surface of the leaf with the index finger of a latex-gloved hand supporting the leaf from behind. Approximately 2 μl of bacterial suspension was infiltrated into the leaf until the water-soaked area reached approximately 6 mm in diameter (Fig. 2a,b). For PNFIS, the inoculation head on the apparatus was pressed against the abaxial surface of the leaf with the index finger of a latex-gloved hand supporting the leaf from behind (Fig. 2c,d). A 10-μl volume of bacterial suspension was infiltrated into the leaf to produce a water-soaked area of approximately 10 mm in diameter. Leaves growing on two shoots of three plants of each cultivar were inoculated. Four leaves on one shoot were inoculated by the PNFIS and four leaves on the other shoot by the HHSM. There were a total of 72 inoculation sites for SWO and RL and 48 inoculation sites for MK and NK per inoculation method. After inoculation, shoots were covered with a plastic bag for 24 h and the plants held in the greenhouse at 27°C. At 12 days post-inoculation (12 dpi), the leaves were examined and the number of lesions that developed counted under a dissecting microscope at 6× magnification.

Results

For calibration of the PNFIS, the volume was measured by comparing the weight of detached leaves before and after infiltration with water. Efficiency of infiltration was calculated according to the volume setting of 10 μl for the PNFIS, and the total amount infiltrated in the tissue was measured as the increase in weight of the leaf (% efficiency = 10 μl ÷ volume of uptake × 100). In the case of the HHSM, the infiltrated volume was measured by the increase in leaf weight after each infiltration. The efficiency of the HHSM method was not calculated because it is dependent on individual operator's skill in performing the injections.

To achieve a 10-mm-diameter infiltration area, the injection pressure was adjusted to 124 kPa to deliver approximately 10 μl per site. When abaxial surface of leaves of different cultivars was injected, SWO received 9.2 μl, representing a 92% efficiency of leaf infiltration (Table 1). For RL and NK, the volume infiltrated was 6.5 and 7.3 μl, respectively, for an average of 77%

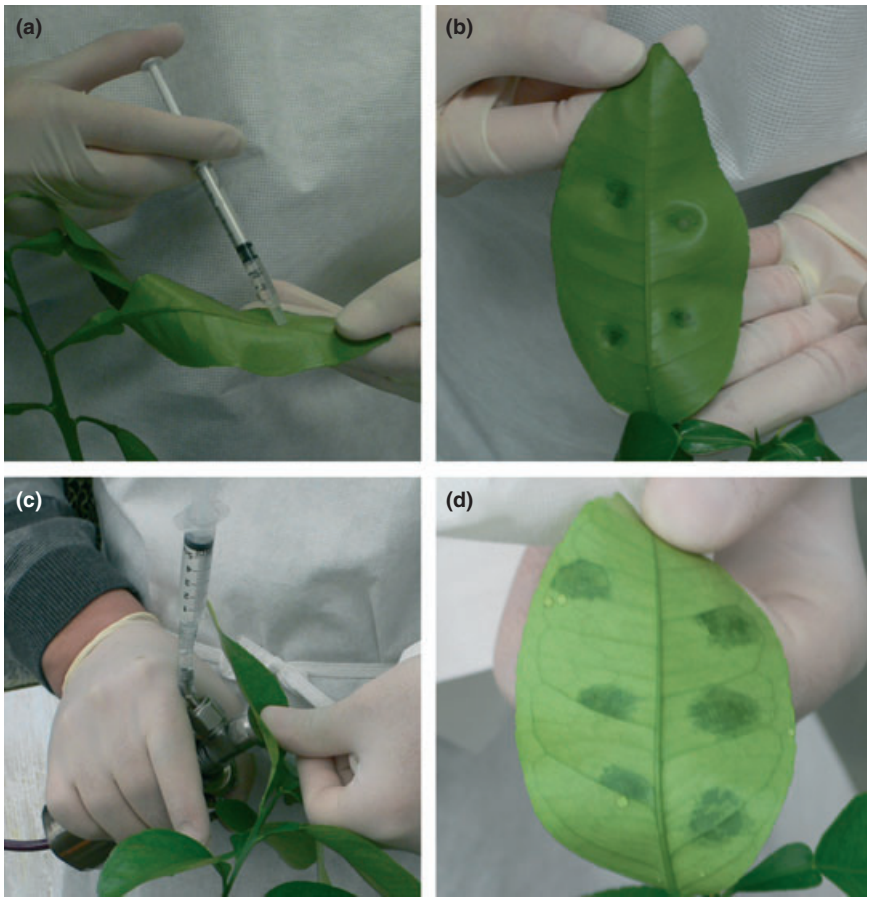

Fig. 2 Attached leaf inoculation. The hand-held syringe method showing (a) the syringe pressed against the abaxial side of the leaf and (b) the depression and irregularity of the infiltrated area; The pulse needle-free injection system showing (c) the position of the inoculation head against the abaxial side of the leaf and (d) the uniformity of infiltrated area

Table 1
Comparison of lesion number and volume of water uptake after infiltration with pulse needle-free injection system (PNFIS) and the hand-held syringe method (HHSM)

Inoculation method	Variable	Cultivar			
		SWO	RL	MK	NK
PNFIS	Number of lesions[a]	174.1 ± 10.6a[c]	181.4 ± 9.5a	1.5 ± 0.4a	4.6 ± 0.8a
	Volume[b]	9.2 ± 0.5	6.5 ± 0.3	7.3 ± 0.7	n/a
HHSM	Number of lesions	98.4 ± 4.5b	111.9 ± 4.8b	1.9 ± 0.5a	1.6 ± 0.2a
	Volume[b]	2.7 ± 0.2	1.3 ± 0.1	3.0 ± 0.2	2.6 ± 0.3

[a]Canker lesions per inoculation site 12 days after injection infiltration with 10^5 *Xcc* per ml. Three plants and four leaves per plant were inoculated by each injection method. Mean of 72 replicate inoculations (6 inoculation sites per leaf) for 'Hamlin' sweet orange (SWO) and rough lemon (RL) and 48 replications (four inoculation sites per leaf) for 'Meiwa' kumquat (MK) and 'Nagami' kumquat (NK). Means followed by the same letter are not significantly different at $P \leq 0.05$ according to Student–Newman–Keuls test.
[b]Volume in μl infiltrated per inoculation site. Volume was estimated by infiltration of detached leaves with distilled water and measurement of the increase in weight of the leaf.
[c]Number of lesions ±1 standard error. Different letters in each column denote significant differences at $P < 0.05$ according to paired *t*-test.

efficiency of leaf infiltration (Table 1). The diameter of the infiltrated area was highly reproducible (Fig. 2d) and the accuracy of infiltration determined as standard error of the mean was ±1 μl.

The volume of water infiltrated by the HHSM ranged from 3.0 to 1.3 μl with higher volumes of infiltration for KN, KM and SWO (3.0–2.6 μl) compared to RL (1.3 μl). When employing the HHSM, it was difficult to precisely reproduce an infiltrated area of 6 mm diameter (Fig. 2b). Excessive pressure produced a larger infiltrated area delimited by the leaf veins. To achieve a reproducible infiltrated area using the HHSM required operator skill and an optimal stage of leaf maturity for infiltration.

The ratio of infiltrated volume between PNFIS and HHSM (PNFIS ÷ HHSM) ranged from 5.0 (RL) to 2.4 (KN). Therefore, the PNFIS delivered 2.5- and 5-fold greater volume than the HHSM. Leaves of the four cultivars were mock-inoculated with buffer to evaluate mechanical damage caused during the inoculation process with both systems. Tissue damage was observed by light microscopy after the HHSM

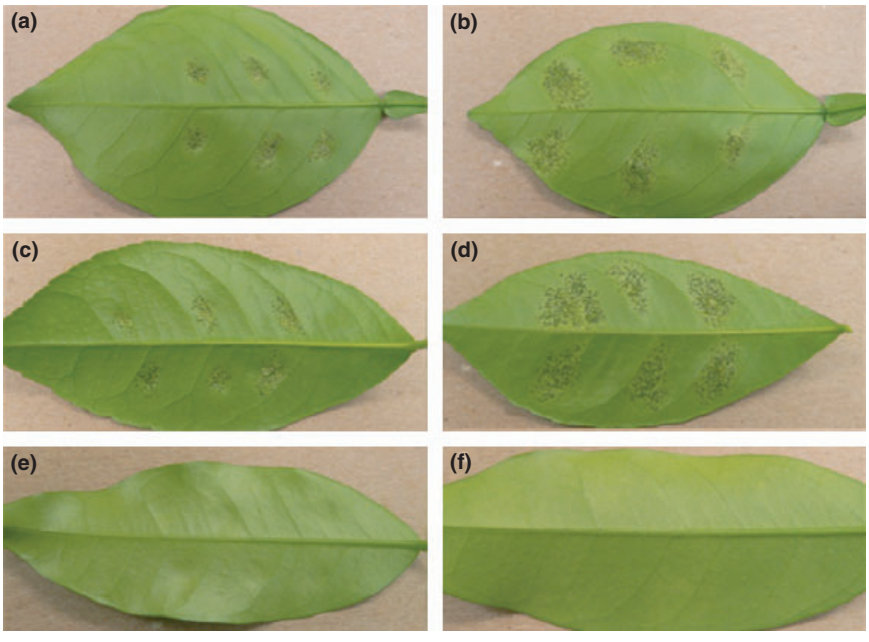

Fig. 3 Comparison of lesions on 'Hamlin' sweet orange, rough lemon and 'Meiwa' kumquat 12 days after inoculation with hand-held syringe method (a, c and e) and pulse needle-free Inoculation System (b, d and f). No symptoms were observed in resistant 'Meiwa' kumquat cultivar inoculated by either method (e and f), lower number of lesions and smaller inoculation areas were obtained in rough lemon (c) and 'Hamlin' sweet orange (a) by the HHSM, compared with the high number of lesions obtained with the PNFIS in susceptible rough lemon (d) and 'Hamlin' sweet orange (b)

infiltration but not the PNFIS. In the case of HHSM, there is contact between the leaf surface and a syringe tip (an opening of 4 mm diameter with a plastic border of 1 mm) that can result in injury to the cuticle when the syringe is pressed into immature leaf tissue to infiltrate the bacteria. In contrast, PNFIS nozzle was designed as a flat circular surface of 1 cm diameter with a central hole of 2 mm where the pressured gas delivers the inoculum. This type of nozzle reduces the damage to the leaf surface. Comparison of lesions on attached leaves of SWO, RL and KM at 12 dpi inoculated with the HHSM (Fig. 3a,c,e) and the PNFIS (Fig. 3b,d,f) demonstrated the uniform development of lesions on leaves inoculated with the PNFIS.

Discussion

Results obtained with the PNFIS are in accordance with those obtained previously using the HHSM regarding reaction of resistant vs. susceptible cultivars (Francis et al. 2010). The number of canker lesions per inoculation site for susceptible cultivars SWO and RL was significantly higher after inoculation by the PFNIS than by the HHSM, but not so for KN and KM (Table 1). As expected, high numbers of lesions were observed for susceptible cultivars (RL and SWO), and very few lesions developed for resistant cultivars (KN and KM) using either inoculation method. Kumquats produce an HR with rapid death of mesophyll cells within the inoculation site (Francis et al. 2010) and *Xcc* infections are unable to develop which explains the small number and size of lesions for kumquats using either method of inoculation. These results confirmed that the highest resistance to canker occurs in kumquats (*Fortunella* spp.), along with a few other citrus relatives (Gottwald et al. 1993; Viloria et al. 2004; Francis et al. 2010).

The PNFIS delivered a consistent volume of inoculum into leaves of hosts assayed, and canker lesions were distributed uniformly within the infiltrated area. The number of lesions obtained with the PNFIS was higher than that obtained with the HHSM because a higher volume of inoculum was delivered into the leaf. Depending on cultivar susceptibility, for the PNFIS, the inoculum concentration may need to be adjusted to a lower level to more easily count the number of lesions in each inoculation site.

The mechanized PNFIS injection process may reduce the variation in infiltration that is associated with traditional syringe inoculation methods. Although injection processing times were not measured in this study, the PNFIS should significantly reduce the time required to conduct inoculations. Another problem with manual injection that will be overcome with PNFIS is that once the syringe plunger is pressed against the leaf surface, most of the released volume of inoculum is lost. This requires refilling the syringe with bacterial inoculum for infiltration of three to four leaves.

Inoculation with the PNFIS was more reliable and reproducible than with the HHSM. The PNFIS represents a promising tool for resistance screening purposes where a more definitive inoculation method for *Xcc* is desired for rapid and accurate quantification of different genotypes originating from breeding programs. Although the PNFIS may increase plant inoculation costs, the use of the PNFIS could be justified by the improved speed and accuracy versus conventional methods. Further research should focus on the compatibility of this inoculation method for other plant species.

Acknowledgements

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