Crop Protection 30 (2011) 259–264

Contents lists available at ScienceDirect

Crop Protection

journal homepage: www.elsevier.com/locate/cropro

Infection and decontamination of citrus canker-inoculated leaf surfaces

C.H. Bock [a,*], P.E. Parker [b], A.Z. Cook [b], J.H. Graham [a], T.R. Gottwald [c]

[a] University of Florida-IFAS-CREC, 700 Experiment Station Rd., Lake Alfred, FL 33850, USA
[b] USDA-APHIS-PPQ, Moore Air Base, Edinburg, TX 78539, USA
[c] USDA-ARS-USHRL, 2001 S. Rock Rd., Ft. Pierce, FL 34945, USA

ARTICLE INFO

Article history:
Received 17 February 2010
Received in revised form
29 September 2010
Accepted 8 October 2010

Keywords:
Epidemiology
Infection
Citrus canker
Inoculum
Disease spread
Quaternary ammonium
Amines
Personnel sprayers

ABSTRACT

Citrus canker (*Xanthomonas citri* subsp. *citri, Xcc*) is now considered endemic in Florida and continues to spread. Various surfaces, including plant material, personnel and equipment can become contaminated. Decontamination is practiced in both disease-endemic and disease-free areas to reduce the risk of bacterial spread by man or machinery. We used grapefruit leaf surfaces to explore the efficacy of a commonly used personnel sprayer system applying a quaternary amine decontaminant. In three experiments, plants in flush (leaves $^3/_4$ expanded) were inoculated ($\sim 10^5$ CFU ml^{-1}). Immediately after inoculation, plants were passed through the sprayers 0, 1, 2, 3, or 6 times. Leaves were sampled at 0.5, 10 and 20 min after decontamination and tested for viable *Xcc* by dilution plating. There was a large and rapid decline in the quantity of live bacteria with one pass through the decontamination sprayer (>80% decrease in CFU ml^{-1}), and multiple sprays (2–6) resulted in up to 100% mortality of surface *Xcc*. Presumably more thorough coverage with multiple sprays killed remnant bacteria, although the first spray invariably caused the highest proportion of population mortality. The effect of the decontaminant spray was immediate (within 0.5 min only 3–11% of surface bacteria survived, and by 20 min <1–3% survived). Based on these results, use of a personnel sprayer with a quaternary amine compound is highly effective for reducing surface inoculum. A single spray kills a high proportion of the population, but multiple sprays increase mortality of *Xcc*. All the *Xcc*-inoculated plants subsequently developed symptoms of citrus canker. No significant difference in incidence or severity of grapefruit leaf infection was detected among decontamination treatments or compared to the untreated control. This finding indicates that infection occurred at, or very soon after, inoculation, and that *Xcc* was in protected sites inside the leaf before exposure to the decontaminant spray.

Published by Elsevier Ltd.

1. Introduction

Citrus canker (*Xanthomonas citri* subsp. *citri*, *Xcc*, (ex Hasse) Gabriel et al.) is endemic in Florida and is dispersed in rain splash often associated with wind (Gottwald and Graham, 1992; Pruvost et al., 2002; Bock et al., 2005 and Bock et al., 2010a; Gottwald and Irey, 2007). The pathogen can also be spread by people directly, and potentially on various surfaces including plant material, clothing, and various equipment or implements (Graham et al., 1989, 2000; Gottwald et al., 1992). Contamination of these surfaces might occur by brushing against wet canker-infected leaves after heavy dew or rain, and during orchard management such as pruning, or fruit harvest.

Once dispersed and on a susceptible leaf surface, various factors are thought to play a role in infection including direct access of the pathogen into stomata through the force of wind-blown rain splash (Gottwald and Graham, 1992; Graham et al., 1992), injury (Christiano et al., 2007; Bock et al., 2010b), and temperature and leaf wetness duration (Dalla Pria et al., 2006; Christiano et al., 2009). Infection with *Xcc* is thought to occur when bacteria gain access to the leaf through stomata, and infecting host cells proximal to the endophytic population (Koizumi, 1976a, Koizumi, 1976b, Koizumi, 1977; Gottwald and Graham, 1992; Graham et al., 1992). Thus access to the stomata might occur immediately upon arrival of the bacteria on the leaf surface when they are driven into the stomata by rain splash (Gottwald and Graham, 1992) or over a period of several hours (Dalla Pria et al., 2006; Christiano et al., 2009), perhaps from a population of *Xcc* existing in the phyllosphere (Rigano et al., 2007). Bacteria are common inhabitants of leaf surfaces and often form epiphytic biofilms (Beattie and Lindow, 1995; Lindow and Brandl, 2003; Monier and Lindow, 2005).

* Corresponding author. Present address: USDA-ARS-SEFTNRL, 21 Dunbar Road, Byron, GA 31008, USA. Tel.: +1 478 956 6421; fax: +1 478 956 2929.
E-mail address: clive.bock@ars.usda.gov (C.H. Bock).

0261-2194/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.cropro.2010.10.001

Epiphytic biofilms of bacteria that are also endophytes are thought to be involved in survival, and possibly infection (Monier and Lindow, 2003, 2005; Jacques et al., 2005; Rigano et al., 2007; Lindow, 2009).

Surface decontamination removes a proportion of bacteria associated with the phyllosphere – those on the surfaces that are exposed. Bacteria in protected sites such as the sub-stomatal cavity or in the mesophyll are not exposed to the disinfecting agent (Wilson et al., 1999; Niemira, 2007; Pruvost et al., 2009), or where bacteria have had time to form a biofilm (Beattie and Lindow, 1995; Rigano et al., 2007). Surface sterilization with various agents has been used to study development of epi- and endophytic populations of plant pathogenic and non-pathogenic bacteria (Wilson et al., 1999; Pruvost et al., 2009), as well as to study human pathogens (USDA-FDA, 2001; Solomon et al., 2002; Niemira, 2007), and surface decontamination procedures have been shown to reduce bacteria populations on citrus (Brown and Schubert, 1987; Gottwald et al., 2009). A decontaminant treatment that kills surface bacteria of *Xcc* could provide insight into the earliest timing of infection in relation to arrival of inoculum on the leaf surface, while providing data to demonstrate the efficacy of surface spray application.

Xcc has been shown to be ephemeral on surfaces, surviving up to 72 h (Graham et al., 1989, 2000). Nevertheless, to minimize the risk of spreading the pathogen via contaminated personnel or equipment, individuals and their implements are commonly surface-disinfected before leaving a grove or facility where the presence of canker is known or suspected. Quaternary amines are recommended for this purpose (Schubert and Sun, 2003; Roberts et al., 2004). On commercial groves and in government and state facilities these disinfectants are applied with hand-held sprayers to hands and feet, and also via walk-through sprayers. Walk-through sprayers consist of spray nozzles on two vertical tubes that produce a mist of disinfectant that coats the individual and any items (for example, carts, notebooks, bags) taken through the sprayer system. The assumption is that spray coverage is sufficient to cause high mortality, or complete kill of *Xcc*. Vehicles and machinery are decontaminated with similar, but often more potent disinfectants (Roberts et al., 2004) using a hoop, or arch structure with sprayer nozzles positioned to produce a cloud of disinfectant through which the equipment is passed.

Quaternary amines are very effective at killing microbial contaminants and many kinds of bacteria (McDonnell and Russell, 1999; Gamage, 2003), but they rapidly decompose as they come in contact with material in the environment. While the efficacy of some personnel decontaminants like quaternary amines, and various application systems have been investigated in the medical, veterinary and food sciences (Jeffrey, 1997; McDonnell and Russell, 1999; Amass et al., 2000; Sutton et al., 2002; Gamage, 2003; Goeres et al., 2004; Sawant et al., 2008), they do not appear to have received much attention in relation to decontamination or management of plant pathogens (Letal, 1977; Teviotdale, 1991; Chase, 1992; Draper et al., 2003; Rugh and Rosenberger, 2005). In situations where containment or eradication is important, it would be useful to have data to support the efficacy of application methods and decontaminants used to reduce the risk of pathogen spread. It is of particular importance to establish the effectiveness of various delivery systems for disinfesting surfaces of complex topography, such as citrus leaves.

To investigate the efficacy of a personnel sprayer delivery system and a common quaternary disinfectant, we used healthy, injury-free, susceptible grapefruit leaf surfaces inoculated with *Xcc* and immediately exposed them to a personnel decontaminant, and measured surface populations of *Xcc* after treatment, and also assessed subsequent disease development on these plants. Citrus leaves were chosen over other surfaces as there exists a substantial literature on surface decontamination of leaf surfaces for comparative purposes (Monier and Lindow, 2003, 2005; Jacques et al., 2005; Lindow, 2009; Niemira, 2007; Rigano et al., 2007; Pruvost et al., 2009;). The objectives were to ascertain the efficacy of the personnel sprayer delivery system for killing surface bacteria of *Xcc*, and to gain some insight into whether bacteria gained access to protected sites at the time of inoculation.

2. Materials and methods

2.1. Experiment plan and procedure

Three replicate experiments examined surface decontamination (experiments I, II and III). In all three experiments, inoculum of *Xcc* was grown on a semi-selective media composed of Nutrient Agar (NA) amended with kasugamycin (16 mg L^{-1}), cephalexin (35 mg L^{-1}) and chlorothalanil (12 mg L^{-1} tetrachloroisophthalonitrile). Colonies were maintained in an incubator at 27 °C for five days. Inoculum (10^5 bacteria ml^{-1}) was prepared in sterile distilled water (Table 1).

In all experiments, Duncan grapefruit plants (*Citrus x paradisi* Macfad.) in flush (leaves $^3/_4$ expanded) were sprayed to run-off with inoculum of *Xcc* prepared as above. Immediately after inoculation (±30 s) the plants were exposed to a decontaminant disinfectant spray (Canker Guard®, Flo-Tec, Largo, FL; active ingredients are a proprietary blend of two quaternary amines, alkyl-dimethyl-benzyl-ammonium chloride and alkyl dimethyl ethylbenzyl ammonium chloride). The decontamination spray was applied by passing the plants at a slow walk (approx 0.7 m s^{-1}) through a standard personnel decontamination spray system (Fig. 1) ensuring good droplet coverage of the plants with each pass. Three sprayers (Conejet X1, Spraying Systems Co., Wheaton, IL) arranged vertically on either side provided the spray (at heights of 60, 100 and 140 cm, with 76 cm horizontal distance between pairs of nozzles). Three replicate plants were passed through the decontaminant sprayer 1, 2, 3 or 6 times at a height of 120 cm. An additional set of plants was not passed through the sprayer, but were inoculated. These plants served as the positive control. Subsequent to passing the plants through the sprayer, a single leaf of approximately the same size was sampled from a plant receiving each spray treatment at 0 (no decontaminant spray), 0.5 min (immediately after the decontaminant spray), 10 and 20 min after the decontamination process. Each leaf was placed in a 50 ml tube containing 40 ml sterile distilled water and vortexed for 5 s (*Xcc* bacteria are naturally dispersed in rainwater, which has few dissolved impurities and therefore a low osmotic potential akin to distilled water). Bacteria recovered in the leaf wash were dilution plated on the semi-selective medium as described above. After incubation for 5 days at 27 °C, the colony forming units (CFU) were counted and bacteria ml^{-1} calculated. In two experiments (II and III) the plants were transferred to a greenhouse immediately after exposure to the decontaminant spray (greenhouse venting set to 27 °C, heating set to 20 °C). Inoculum and disinfectant were

Table 1
Dates and concentration of *Xcc* inoculum in sterile distilled water used for each experiment. Plants were sprayed to run-off with inoculum using a hand-held sprayer. Inoculum concentration was confirmed by dilution plating on KCB nutrient agar.

Expt. no.	Date	Inoculum conc. (*Xcc* bacteria ml^{-1})
I	19 Dec 2007	1.7×10^5
II	22 May 2008	1.5×10^5
III	23 Aug 2008	8.6×10^5