Hot water treatment as a means to eradicating *X. fragariae* from strawberry nursery stock

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Introduction

Angular leaf spot (ALS), caused by the bacterium *Xanthomonas fragariae*, is an important disease in California strawberry nursery production. Infected nursery stock is considered the primary means by which the pathogen is introduced in production fields. The European and Mediterranean Plant Protection Organization (EPPO) lists *X. fragariae* as an A2 quarantine pathogen (i.e., a pathogen absent from the majority of the strawberry-growing countries in Europe, but has the potential to establish there). Nurseries wishing to export plants to certain European countries must maintain strict phytosanitary standards. Specifically, planting material must be derived from mother plants certified free of *X. fragariae* and production sites should be documented free from ALS for the past 5 growing seasons. In situations where EPPO's production standards have not been met or were difficult to achieve, it would beneficial to employ a method capable of disinfesting or de-contaminating infected plants, and/or killing viable cells of *X. fragariae*. Hot-water or heat treatment is one such method. Heat treatment has been used to reduce or eliminate systemic bacterial infections in propagation material for a variety of crops, but little has been done to develop a procedure for strawberry.

Objective: Develop a heat treatment protocol for eradicating X. fragariae from strawberry nursery stock for use in California nursery production.

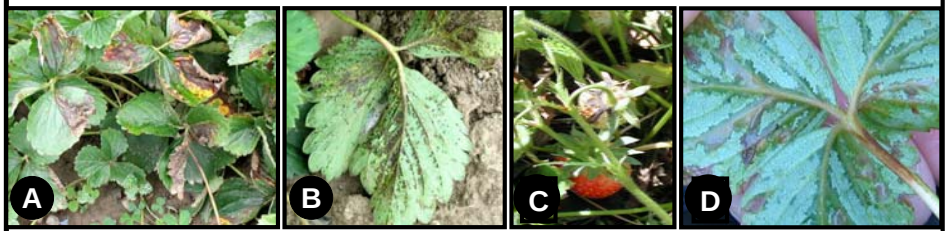

Fig. 1. Typical symptoms of angular leaf spot on the **A)** adaxial surface of a leaf, **B)** abaxial of the leaf, and **C)** on the calyx ("black cap"). **D)** Bacterial ooze on the underside of a leaf, typically encountered early in the morning in the presence of a heavy dew.

Materials and Methods

Bacteria Heat Treatment: Four representative strains of *X. fragariae* (Xf3, Xf6, Xf128, ATCC) were grown for ~5 days in sucrose peptone liquid culture. Working cultures of the bacteria were prepared by dilution in water and concentrations adjusted to 0.1 absorbance at 620 nm (~1×10^8 CFU/ml). One ml aliquots of each of the 4 isolates were dispensed in microcentrifuge tubes and the tubes submerged in water heated to 36, 40, 44, 48, 52, and 56 C for 0, 1, 2.5, 5, 10, 15, 30, 60, 120, 240, 360 and 480 min. An 8 μL aliquot was plated on sucrose peptone agar from each tube after treatment, and the plates were evaluated 5 days later for the presence of growth to determine if the bacteria survived the heat treatment. PCR was run on a selection of the resultant bacteria to confirm identity. The experiment was repeated 6 times for each strain and data report the proportion of instances where bacterial colonies were observed; i.e., the number of times out of 6 where growth was observed for the particular treatment.

Plant Heat Treatment: Cold-stored, bare-rooted plants of the cultivars Camarosa and Diamante were obtained from two California nurseries. Three plants of each variety/nursery combination were heat treated according to the following protocols: a) Plants were placed in metallic mesh cages and immersed directly into the water bath (industry standard, *direct dip*); b) Plants were sealed in a plastic bag and the bag immersed in the water bath (*bagged dry*); and c) Plants were wetted in warm water, sealed in a plastic bag, and then immersed in the water bath (*bagged wet*). Plants were treated at 44 C and 48 C for 0, 60, 120, 180, and 240 minutes. The selection of temperature and exposure times was based on the results of the bacterial heat treatment and preliminary work with plant heat treatment (data not shown). After treatment, the plants were potted in 10 cm diameter pots, and placed in a high-tunnel to observe growth. Survival of the heat treatment, the number of inflorescences, and the number of runners were recorded for each plant and compared to the non-heat-treated control plants. Data were analyzed in a generalized linear mixed model (GLMM) with the PROC MIXED procedure of SAS.

Results

The proportion of *X. fragariae* colonies surviving heat treatment is shown in **Fig 2.** Bacteria exposed to 56 and 52 C were killed completely after 15 and 60 min exposure, respectively. Bacterial populations exposed to 44 C for 240 min or 48 C for 120 min were reduced ~96%; these treatments were selected for plants. The proportion of 'Camarosa' and 'Diamante' plants surviving heat treatment is shown in **Fig 3.** The analysis indicated significant temperature, time, and treatment effects ($P<0.001$), but no statistical differences between nurseries or varieties. The wet- and dry-bagged treatments were superior at protecting plants from heat damage compared to direct dips. A surprising result was that the superior heat treatments significantly ($P<0.001$) reduced the average number of flowers per plant (**Fig 4**) and increased the average number of runners per plant for those plants that survived heat treatment (**Fig 5**).

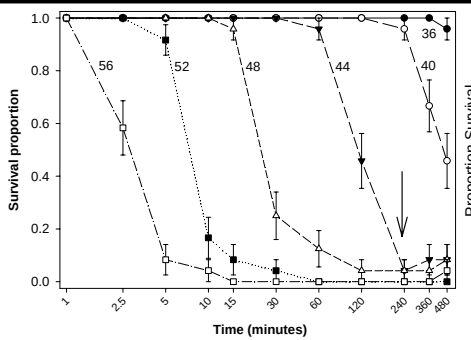

Fig. 2. The proportion of *X. fragariae* colonies surviving hot-water treatment. Each symbol represents the average of 4 bacterial strains over 6 experimental runs (24 obs.). Time is represented on a log scale to emphasize shorter duration treatments. The inset numbers represent the temperature treatment in Celsius. The 1 min treatment represents the 0 min control (because log(0) is undefined). The arrow identifies sufficient exposure time at 44 C.

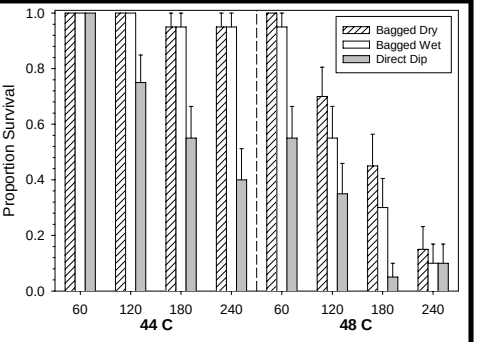

Fig. 3. The proportion of 'Camarosa' and 'Diamante' plants surviving heat treatment. Each bar represents the average of 18 plants (3 plants each over 6 experimental runs). Plants were treated at either 44 or 48 C for 60, 120, 180, or 240 min and were either dipped directly into the water (Direct Dip), wetted and sealed in a plastic bag (Bagged Wet), or were sealed dry in a plastic bag (Bagged Dry).

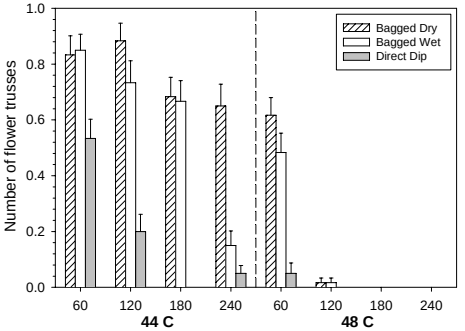

Fig. 4. The average number of flower trusses on 'Camarosa' and 'Diamante' plants exposed to different heat treatments (see **Fig 3** caption). The absence of flowering in treatments at higher temperature and longer exposure times is a result of plant death (compare w/ **Fig 3**). Each bar represents the average of 18 plants (3 plants each over 6 experimental runs).

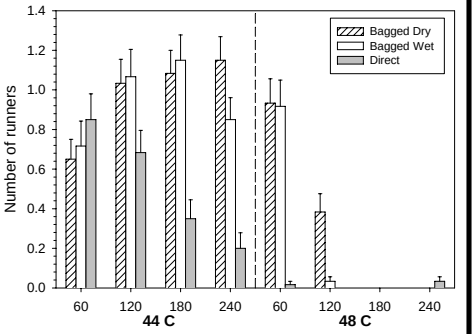

Fig. 5. The average number of runners on 'Camarosa' and 'Diamante' plants exposed to different heat treatments (see **Fig 3** caption). The absence of runnering in treatments at higher temperature and longer exposure is a result of plant death (compare w/ **Fig 3**). Each bar represents the average of 18 plants (3 plants each over 6 experimental runs).

Conclusions

An optimal heat treatment is one that maximizes kill of the pathogen while minimizing plant damage. In this study, a 240 min exposure at 44 C was optimal. Higher temperature and longer exposure times, although increasing the kill rate of bacteria, also increased the damage to treated plants. An unexpected result was that our optimal treatment reduced the average number of flowers, and increased the number of runners relative to untreated plants. This is a desirable result because of the significant effort nurseries undertake to deflower plants in the field, plus additional runners equates to a greater numbers of plants. The results here could lead to a more efficient way of eliminating *X. fragariae* from strawberry plants, but eventually may be used for other pathogens. Heat treatment is much more environmentally friendly than chemical alternatives, and it may prove to be cost, time and labor efficient as well.

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