Heat Treatment Effects on Strawberry Plant Survival and Angular Leaf Spot, Caused by *Xanthomonas fragariae*, in Nursery Production

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ABSTRACT

Turechek, W. W., and Peres, N. A. 2009. Heat treatment effects on strawberry plant survival and angular leaf spot, caused by *Xanthomonas fragariae*, in nursery production. Plant Dis. 93:299-308.

Angular leaf spot is an important disease in strawberry nursery production. The European and Mediterranean Plant Protection Organization (EPPO) lists *Xanthomonas fragariae* as an A2 quarantine pathogen. Therefore, nurseries wishing to export plants to European countries must maintain phytosanitary standards to exclude *X. fragariae*. To help nurseries achieve these standards, heat treatment for killing or reducing the number of viable bacterial cells in strawberry crown tissue was investigated. First, the sensitivity of bacteria to heat was determined by dispensing 1-ml aliquots of standardized cell suspensions in microcentrifuge tubes for each of four isolates of *X. fragariae*, including the type culture, and submerging the tubes in water at 36, 40, 44, 48, 52, and 56°C for 0, 1, 2.5, 5, 10, 15, 30, 60, 120, 240, 360, and 480 min. Bacteria were transferred to growth medium to determine the proportion surviving heat treatment. Two trials were conducted in a greenhouse to determine the sensitivity of bare-root plants to heat treatment. In the first trial, plants of cvs. Camarosa and Diamante from two different nurseries were heat treated as follows: (i) plants placed in metallic mesh cages and immersed directly into water (industry standard, direct dip); (ii) plants sealed in a plastic bag and the bag immersed in water (bagged dry); or (iii) plants wetted in warm water, sealed in a plastic bag, and then immersed in water (bagged wet). Plants were treated at 44 or 48°C for 0, 60, 120, 180, and 240 min. In the second trial, plants of cvs. Camarosa, Camino Real, Diamante, Oso Grande, Strawberry Festival, and Ventana from a single nursery were subjected to the same treatments. In both trials, plants were potted after treatment and rated for growth characteristics. Results showed that populations of bacteria exposed to 56 and 52°C were killed completely after 15 and 60 min of exposure, respectively; both treatments killed plants. Bacterial populations exposed to 44°C for 4 h or 48°C for 2 h were reduced by 10^5 or 10^6 CFU/ml. The same treatments minimally affected vegetative growth of plants bagged dry or wet, but flowering was adversely affected. These heat treatments were selected for testing of nursery stock of several cultivars in field trials established at two locations in successive years. The survival rate among cultivars was similar to that observed in greenhouse trials, and angular leaf spot developed appreciably only in non-heat-treated control plots. Heat treatment of strawberry nursery stock is feasible and can be used to supplement standard production practices for producing pathogen-free nursery stock.

Angular leaf spot (ALS), caused by the bacterium *Xanthomonas fragariae*, is one of the most damaging diseases in strawberry nursery production (17). The pathogen was first reported in Minnesota in 1962 and has since been found in several U.S. states as well as strawberry-growing regions in Europe, South America, Africa, Australia, and New Zealand (12,17). The disease affects the foliage, often attacks the calyx, and can move systemically within the vascular system of the plant to infect additional leaf tissue, crown tissue, and developing daughter plants (10,19). Plants with infected crowns are less productive and may die if infection is severe. Also, systemically infected plants likely produce the first infected leaves and serve as the primary source of inoculum in newly planted fields. Secondary cycles of infection result from bacteria exuded from foliar lesions and disseminated by splashing and wind-driven rain or irrigation water. Some commercial cultivars can tolerate foliar infections because the direct impact on yield is thought to be minimal. Infection of fruit calyces, however, leads to a symptom known as "black cap" that reduces the quality and marketability of the fruit. In some commercially important cultivars, however, severe foliar infection stunts growth and reduces the plant canopy, leaving the fruit susceptible to sun scald.

The pathogen is transmitted to production fields almost exclusively through infected nursery stock. This creates problems for U.S. nurseries that export plants to Europe, because 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 with the potential to become established there; 25), as well as to U.S. growers who expect to receive clean plants. Nurseries wishing to export plants to some European countries must maintain specific phytosanitary standards. 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 five growing seasons (25).

In situations where EPPO's ALS standards were not met, it would be beneficial to have available a postharvest procedure capable of killing *X. fragariae*. One possible method is hot-water or heat treatment (15,20,27,29). Heat treatment (HT) has been shown to be effective against systemic pathogens and has been used to reduce or eliminate systemic bacterial infections in propagation material in crops such as apple (3), cherry (6), and grape (11). In strawberry, hot-water treatment for 30 min at 37.8°C is currently being used by some nursery, government, and university breeding programs to eliminate cyclamen mite (4). Buchner (2) found strawberry plants to be intolerant to temperatures exceeding 51 to 52°C for 7 min. Less severe HT delayed growth for up to 2 to 3 weeks. These adverse effects make HT unsuitable for plants destined for fruit production fields but they have a lesser impact in nursery production and would likely be acceptable if the treatment could substantially reduce the pathogen or disease in the early stage of production. In the end, Buchner (2) recommended 5- to 7-min dips at 48 to 49°C for control of some insects and diseases in California nursery production; unfortunately, this treatment is insufficient for killing *X. fragariae* (9).

Where hot-water treatment is used (e.g., to eliminate cyclamen mite), the current

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Accepted for publication 29 November 2008.

doi:10.1094/PDIS-93-3-0299

practice is to place plants in a wire mesh cage and immerse them directly into the bath. However, in preliminary work, it was observed that the duration of HT tolerated by plants can be increased if plants are sealed in plastic bags prior to immersion (i.e., "dry" HT; 9). Plants exposed directly to hot water are more easily damaged by heat than those that are protected. Dry HT allows longer exposure to heat, which allows the heat to penetrate the vascular tissue and may be more effective at killing systemic *X. fragariae*. Additionally, the physical boundary of the bag reduces cross contamination between plant batches by insects or pathogens that may survive treatment.

The objectives of this research were to (i) determine the effect of temperature on survival of *X. fragariae*; (ii) determine the effect of temperature on survival, runner production, and flower production of several strawberry cultivars; and (iii) determine the effect of HT on reducing ALS and plant survival under field conditions. Results of this research are intended to provide the foundation for development of a HT protocol for eradicating *X. fragariae* from strawberry nursery stock. Preliminary findings were reported (9).

MATERIALS AND METHODS

In vitro HT. Cells of cultured *X. fragariae* were subjected to HT to determine minimum exposure times needed to substantially reduce cell viability at selected temperatures. Four strains of *X. fragariae* (Xf3, Xf6, Xf128, and ATCC 33239) were grown for approximately 5 days in sucrose-peptone liquid culture at room temperature (approximately 20°C; 7). Strains Xf3 and Xf6 originated from North Carolina and California, respectively, and were obtained originally from D. Ritchie (North Carolina State University, Raleigh). Strain Xf128 was collected in Quebec and maintained originally by P. Roberts (University of Florida, SWFREC, Immokalee). The type strain ATCC 33239 was obtained from the American Type Culture Collection (ATCC). Cultures of the bacteria were prepared by dilution in water and adjusting concentrations to 0.1 absorbance at 620 nm (approximately 1×10^8 CFU/ml; 8). After 1-ml aliquots of each of the four isolates were dispensed in microcentrifuge tubes, the tubes were submerged in water baths heated to 36, 40, 44, 48, 52, or 56°C for 0, 1, 2.5, 5, 10, 15, 30, 60, 120, 240, 360, or 480 min. Temperature was monitored continuously with mercury-filled thermometers. An 8-µl aliquot from each tube was transferred to sucrose peptone agar after treatment, the plates were maintained at room temperature (approximately 20°C), and colonies were counted 5 days later to determine the survival of the bacteria. Polymerase chain reaction (PCR) with specific primers 241, 245, or 295 was run on a selection of the resultant bacteria (about 10 per run) to confirm identity following either the original or modified (26) protocol of Pooler et al. (21). The experiment consisted of six replications for each of the four strains, with the individual replications being conducted over time. This experiment was conducted once.

The binary response survival (i.e., 0 = no bacteria detected and 1 = one or more bacteria detected in the 8-µl aliquot) was regressed against the independent variables temperature, exposure time, and bacterial strain. The variables temperature, exposure time, and their interaction were treated as continuous variables and bacterial strain and its interaction with exposure time and temperature as class variables. Wald's χ^2 was used to test the significance of the main effects. Outliers and high-leverage observations were identified with standard diagnostic plots and some of these observations were removed to improve the fit of the model under the condition that removal of the observations did not change the significance level of the main effects. The Hosmer and Lemeshow goodness-of-fit test and a pseudo R^2 statistic (R_L^2) were used to evaluate the fit of the final model to the data (18). The analysis was performed using the LOGISTIC procedure of SAS (ver. 9.1; SAS Inc., Cary, NC).

Plant HT. The following set of experiments was designed exclusively to test the effects of HT on plant growth parameters. These experiments were not designed to examine the effects of HT on the development of angular leaf spot.

Greenhouse trial I. Cold-stored, bareroot plants of cvs. Camarosa and Diamante were obtained from Lassen Canyon Nursery (Redding, CA) and Bonita Nursery (Stockton, CA). Plants were stored –4°C at the nursery and then at 4°C upon arrival in Beltsville, MD in mid-January. Plants were bundled in sets of three (experimental unit) by cultivar and nursery and were heat treated according to the following protocols: (i) plants placed in metallic mesh cages and immersed directly into the water bath (industry standard, direct dip); ii) plants were sealed in a 177-by-305 mm, 3-mil sterile blender plastic bag (Twirl 'Em Sampling Bags; Labplas, Quebec, Canada) and the bag immersed in the water bath (bagged dry); and iii) plants were wetted in warm water, sealed in the plastic bag, and then immersed in the water bath (bagged wet). Plants were treated at 44 or 48°C for 0, 60, 120, 180, or 240 min. The selection of temperatures and exposure times was based on the results of the bacterial HT trial described above and preliminary work with plant HT (9). After the prescribed treatment, the three plants were removed from the bag or cage, potted individually in 10-cm-diameter pots, and placed in a high tunnel to observe growth. Survival of the HT, the number of flower trusses, and the number of runners were recorded for each plant. The experiment consisted of five replications conducted over time, and HTs were performed on consecutive days for the two nurseries. HTs were performed on the following dates: 18 and 19 January 2006, 24 and 25 January 2006, 26 and 27 January 2006, 7 and 8 February 2006, and 9 and 10 February 2006 for replications 1 through 5, respectively.

Greenhouse trial II. Cold-stored, bareroot plants of cvs. Camarosa, Camino Real, Diamante, Oso Grande, Strawberry Festival, and Ventana were obtained from a single California nursery. Plants were bundled in sets of three and treated according to the three protocols defined above. After the prescribed treatment, three plants (experimental unit) of each cultivar were removed from the bag or cage, potted individually in 10-cm-diameter pots, and placed in a high tunnel to observe growth. Survival, the number of flower trusses, and the number of runners were recorded for each plant. The experiment consisted of three replications conducted over time and was performed on the following dates: 22 June 2006, 28 June 2006, and 5 July 2006 for replications 1 through 3, respectively.

Data analysis. Prior to data analysis, the number of plants surviving HT was transformed by applying the Haldane transformation $(0.5 + x)/(n + 1)$ to the raw data, where x was the number of plants surviving HT and n (usually 3) was the total number of plants exposed to a particular treatment combination for a given replicate, and then applying the arcsine transformation to the Haldane-corrected proportion. The number of runners and the number of inflorescences were transformed using $\ln(x + 1)$, where x is the variable undergoing transformation. The experimental design was considered a split-split plot with temperature serving as the whole-plot factor, treatment protocol as the split-plot factor, and cultivar, exposure time, and nursery (first trial only) serving as the split-split plot factors. The split-split plot factors were arranged in a factorial design. Replication and the interactions of replication with temperature (error a) and replication with temperature and treatment protocol (error b) were considered random effects (16). The variance-covariance structure for the random effects was specified as the default TYPE = VC (variance components), although the compound symmetry structure (TYPE = CS) was also evaluated as a possibility. The data were analyzed in a generalized linear mixed model (GLMM) using the SAS procedure GLIMMIX (ver. 9.1; SAS), specifying an identity link function and Gaussian (normal) error distribution. Standardized residual and normality plots were used to determine the adequacy of model fit. Pairwise treatment differences for the main effects were obtained using the LSMEANS statement and LINES option for main effects that had no significant interactions with other main effects or with the SLICEDIFF

option for main effects with significant interactions. The latter allows pairwise comparisons among levels of a main effect within levels of the interacting effect.

Field trials. The two most favorable HTs—determined subjectively from the greenhouse study results—were selected for the field trial: 44°C for 4 h bagged dry and 48°C for 2 h bagged dry. Field trials were established at two locations in successive years and cultivars were selected based on availability at the nursery. We relied on natural levels of infection in the field, and the assumption that all plants of all cultivars were equally infected by the pathogen, prior to assigning plants to treatments. The field trials were designed primarily to test the effects of HT on controlling ALS, and less so for examining plant parameters.

The first planting was established at the USDA-ARS Beltsville Agricultural Research Center in Beltsville, MD. Prior to planting, the entire field was treated with glyphosate and cultivated to reduce weed pressure, and 1.2-m-wide plastic-mulched raised beds were prepared. The planting was designed to accommodate a randomized block consisting of four blocks of 18 plots. The plots within blocks were 4.8 m long spaced 1.2 m apart to accommodate 24 plants planted in two staggered rows of 12, with plants spaced 0.4 m apart within rows. The distance between blocks was 3.6 m on all sides. Cold-stored, bare-root plants of cvs. Camarosa, Camino Real, Diamante, Oso Grande, Strawberry Festival, and Ventana were donated by Lassen Canyon Nursery and stored locally at 4°C for approximately 1 week prior to conducting the HT experiment on 21 and 22 August 2006. For each treatment–replication combination, 24 plants of each cultivar were sealed in separate plastic 177-by-305-mm, 3-mil sterile blender plastic bags (Twirl 'Em Sampling Bags; Labplas) and then the individual bags were sealed in a second plastic bag of the same dimensions and thickness according to the time period in which they were to be removed from the water bath. The double-bagging ensured that the plants would remain dry for the duration of their treatment and would allow quick removal of all the cultivars at once, thus minimizing potential cooling of the bath water due to removal of the bath cover. After treatment, the plants were planted and were irrigated 2 to 4 h per day by overhead sprinklers for 10 to 12 days to aid in establishment and to provide favorable conditions for the development of ALS. Plants were rated for ALS on 18 September (replication 1), 19 September (replications 2 and 3), and 21 September (replication 4) by examining each leaf on all plants for the presence of angular leaf spot symptoms. Plant survival was also recorded on each date. The number of runners on each plant was counted on 27 October.

The experiment was repeated in 2007 at the University of Florida's Gulf Coast Research and Education Center in Wimauma, FL. Fresh-dug, bare-root runner plants of cvs. Camarosa, Camino Real, Strawberry Festival, and Ventana were shipped to the location and stored at 4°C for approximately 1 week prior to conducting the HT experiment. The plants were donated by Lassen Canyon Nursery except for Strawberry Festival, which was donated by Lareault Nursery (Lavaltrie, Quebec, Canada). HTs were performed on 1 November 2007 as described above, with the inclusion of an additional dry HT applied to a second batch of Strawberry Festival plants. These plants were bagged as above but placed in heated-air chambers (Percival Growth Chamber model E-30B; Percival Scientific, Perry, IA) for exposure to the HT rather than being submerged in a hot-water bath. The heat-treated plants were transplanted into methyl bromide/chloropicrin (98:2)-fumigated soil in plastic-mulched raised beds. Transplants were irrigated for several hours per day by overhead sprinklers for 10 to 12 days to aid establishment, then irrigated and fertilized through drip tape as needed for the duration of the study. Research plots were arranged similarly to those described above, except the plots contained 20 plants instead of 24. Plants were rated for ALS on 21 November 2007 and 20 December 2007 by examining each leaf on each plant for the presence or absence of ALS symptoms. Plant survival was also recorded on each date. Runners were counted on 12 March 2008.

Data analysis. The proportion of plants developing ALS was transformed by first applying the Haldane transformation to the raw data (i.e., $[0.5 + x][(n + 1])$ and then applying the arcsine transformation to the Haldane-corrected proportion. The number of runners was transformed using the logarithmic transformation. The transformed response was analyzed in a GLMM treating cultivar and HT as crossed factors and block as a random effect, specifying an identity link function and Gaussian (normal) error distribution, using the SAS Procedure PROC GLMMIX. Treatment differences were obtained using the LSMEANS statement with the PDIFF option.

RESULTS

In vitro HT. The proportion of 1-ml aliquots in which at least one *X. fragariae* colony survived HT is shown in Figure 1. Bacteria exposed to 56 and 52°C were killed after 15- and 60-min exposures, respectively. Mortality of bacteria exposed to 44°C for 240 min or 48°C for 120 min was approximately 96% (i.e., 1 of 24 aliquots had a few colonies surviving HT); these treatments were selected for trials with plants. Generally, only a few colonies (<10 per plate) survived these treatments. Therefore, if 10 colonies represented the maximum number of bacteria found in an aliquot, a 10^5 to 10^6 reduction of viable cells from the original aliquot was achieved. Logistic regression analysis indicated significant effects on bacterial survival due to exposure time and temperature and its interaction ($P < 0.0001$; Table 1). No significant differences were found among bacterial strains or replications. The final model fit to the data excluded 12 (of

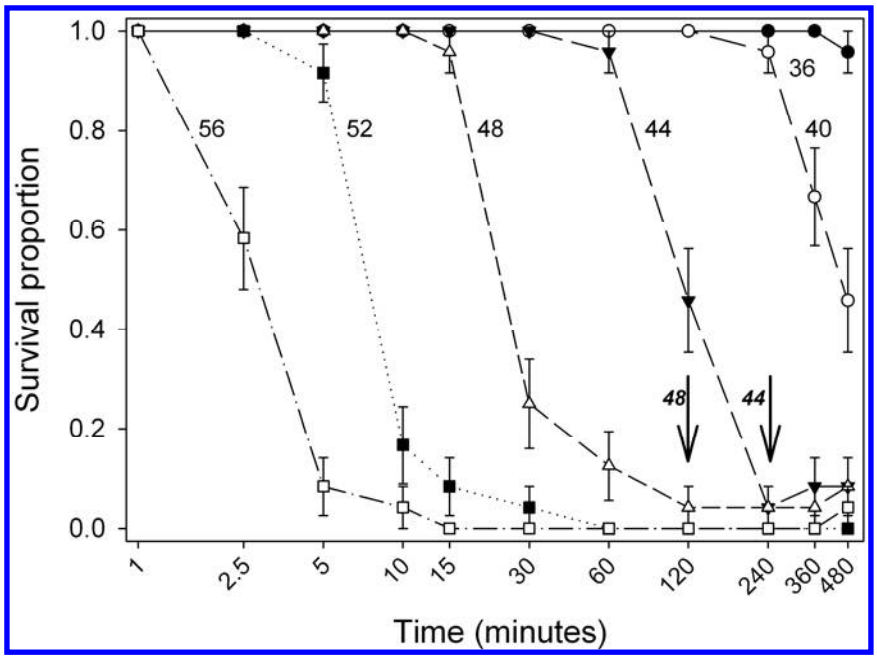

Fig. 1. Observed proportion of 1-ml aliquots in which at least 1 CFU of *Xanthomonas fragariae* was detected as a function of the temperature and of time of exposure to the heat treatment. Bars represent the standard errors for the mean survival of six replicates of three strains, and arrows indicate heat treatments selected for additional testing. Time is shown on a log scale to separate early time points.

Table 1. Logistic regression analysis of *Xanthomonas fragariae* survival as a function of the heat treatment variables temperature, exposure time, bacterial strain, and their interactions for six replications[y]

Variable	df	Wald χ^2	$P > \chi^2$	Odds ratio[z]
Rep	5	5.27	0.3842	…
Strain	3	4.62	0.2019	…
Time	1	109.78	<0.0001	1.715
Temperature	1	127.91	<0.0001	0.566
Strain × time	3	0.27	0.9647	…
Strain × temperature	3	3.90	0.2728	…
Time × temperature	1	113.20	<0.0001	0.986

[y] Odds ratios are shown for significant effects. Wald statistic = $W_k = (b_k/\text{standard error of } b_k)^2$, where b_k is the kth parameter estimate. $W_k = \chi^2$ with 1 df.

[z] The odds ratio is a measure of effect size. For a continuous variable x, it is defined as the ratio of the odds of survival at $x + 1$ to the odds of survival at x. An odds ratio greater than 1 indicates an increase in odds of survival for a 1 unit increase in x, whereas an odds ratio less than 1 indicates a decrease in odds of survival for a 1 unit increase in x.

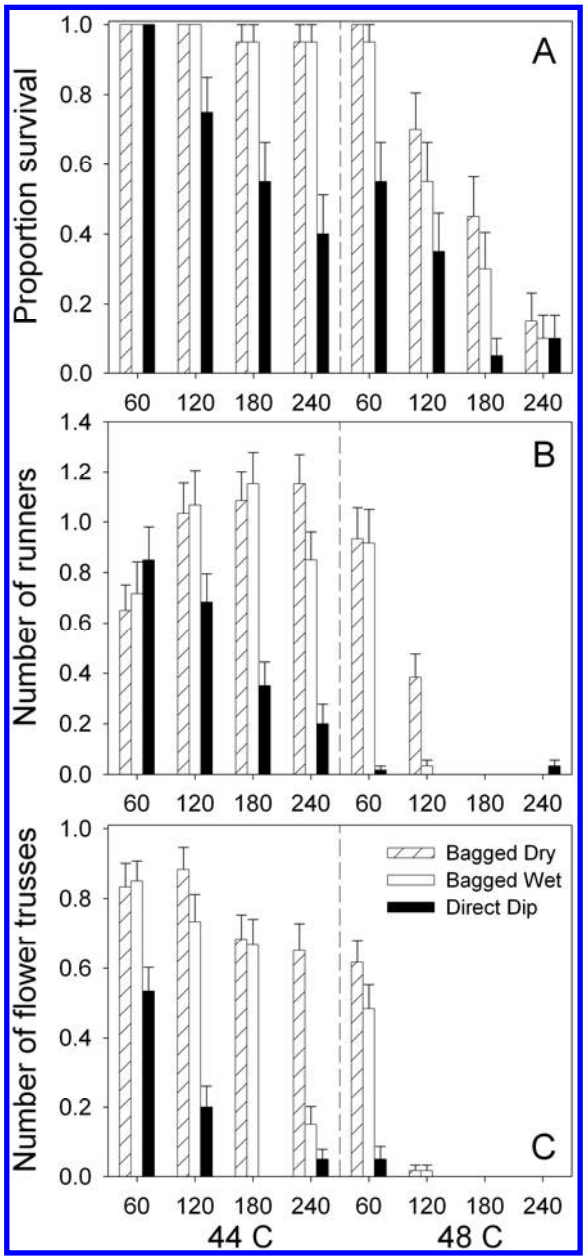

Fig. 2. A, Proportion of plant survival; **B,** average number of runners per plant; and **C,** average number of flower trusses per plant for plants exposed to hot water treatment of 44 or 48°C for 60, 120, 180, or 240 min in which plants were either submerged directly in to the water bath (Direct Dip) or sealed in waterproof plastic bags dry (Bagged Dry) or wet (Bagged Wet) prior to being submerged into the bath. Data are from greenhouse trial I and the bars represent the average of three plants for each of two cultivars over five treatment replications (30 observations).

1,584) observations due to poor fit as identified through standard diagnostic analysis. With the exclusion of the 12 points, the model fit of the data was excellent according to the Hosmer and Lemeshow goodness-of-fit test ($P = 0.7907$) and $R_L^2 = 0.66$.

Plant HT. *Greenhouse trial I.* The proportion of Camarosa and Diamante plants surviving HT is shown in Figure 2A. The analysis indicated that there were significant temperature, exposure time, and treatment protocol effects ($P < 0.001$) on survival but no statistical differences attributable to the main effects of nursery and cultivar (Table 2). However, the interaction between the nursery and cultivar was significant due the difference in survival of Diamante between the two nurseries ($P = 0.0016$; *data not shown*). The wet- and dry-bagged treatments protected plants from heat damage compared with direct dipping (no bag). The least square means generated from the significant three-way interaction between temperature, treatment protocol, and exposure time are shown in Table 3. Nearly all of the direct-dipped treatments had significantly lower proportions of survival than the bagging treatments. Some HTs, for those plants that survived it, led to an increase in the average number of runners per plant (Fig. 2B; Table 2) and to a significant ($P < 0.001$) reduction in the average number of flower trusses per plant (Fig. 2C; Table 2). There were also significant differences in runner and flower production due to nursery but, as with survival, this was attributed to Diamante from nursery 1.

Greenhouse trial II. The proportion of plants surviving HT is shown by cultivar in Figure 3. The analysis indicated significant effects on survival due to the three-way interaction of temperature, exposure time, and treatment protocol ($P < 0.001$), as well as to temperature, treatment protocol, and cultivar ($P < 0.001$), but no statistical differences attributable any other higher-order interaction (Table 2). The least square means generated from the significant three-way interaction between temperature, exposure time, and treatment protocol are shown in Table 3. As seen above, the bagging treatments provided plants with better protection from heat damage than plants that were exposed to the direct-dipping treatment. Also, the effect of temperature and exposure time on plant survival was similar to that observed in the first greenhouse trial. Although evaluating the effect of cultivar within its interaction with temperature and treatment protocol is too cumbersome to display, evaluating cultivar differences within the simple main effect represents the individual cultivar performances within the bagging treatment by temperature grouping. Camarosa and Ventana had significantly lower survival rates than the other four cultivars, whereas Strawberry Festival had a significantly higher rate of survival than all other culti-

vars except for Diamante. Diamante, Camino Real, and Oso Grande had statistically equivalent survival rates. Runnering and flowering were affected by cultivar, as would be expected based on their horticultural traits, but only runner production was affected by the interaction of cultivar with temperature and treatment protocol (Table 2). Flowering and runner production were affected by the three-way interaction of temperature, treatment protocol, and exposure time (Table 2). Unlike the first experiment, there was no peak in runner production. The effect was a reduction in flowering and runner production with higher temperatures and longer exposure periods (Fig. 4A and B).

Field trials. *Maryland.* ALS developed only in the control plots of Strawberry Festival, Camarosa, and Oso Grande (Fig. 5A and C). For Oso Grande, both HTs reduced the incidence of ALS to zero, but plants treated at 48°C for 2 h had a lower rate of survival relative to the control (Table 4). Strawberry Festival treated at 48°C for 2 h had significantly less ALS than the corresponding control, and the survival rate was minimally affected by any HT. Camarosa treated at 44°C for 4 h, despite having no ALS, was not significantly different from the control (Table 4). Camarosa plants treated at 44°C were severely stunted or killed relative to the control and the HT at 48°C. Camino Real treated at 44°C for 4 h had very low incidence of ALS, but the control plot had none. Survival rates among the other cultivars tended to be lower at 44 than 48°C and affected cvs. Camarosa and Ventana most significantly. For any single cultivar, the mean number of runners per plant was highest in the control plots, followed by the 48°C and then the 44°C treatment plots (Table 4). For the 48°C treatment, the number of runners was statistically equivalent to the control plants for all cultivars except Camarosa and Ventana (Table 4).

Florida. ALS developed in all control plots and at much higher incidences than in Maryland (Fig. 5B). Also, disease developed at relatively low incidences in some of the treated plots. During the first rating, disease was found on a single plant of Camarosa treated at 48°C and single plants of Ventana treated at 44 and 48°C.

Table 3. Least square means of the proportion of strawberry plants surviving hot water treatments of 44 or 48°C for treatment periods of 60, 120, 180, or 240 min in which plants were submerged directly into the water bath (direct dip) or were sealed in waterproof plastic bags dry (bagged dry) or wet (bagged wet) prior to being submerged into the bath for the treatments

			Least square mean for greenhouse trials[z]	
Treatment	**Temp (°C)**	**Duration (min)**	**Trial I**	**Trial II**
Bagged wet	44	60	1.000 a	0.970 ab
Bagged wet	44	120	0.987 ab	0.970 ab
Bagged dry	44	60	0.987 ab	1.000 a
Bagged dry	44	120	0.987 ab	1.000 a
Bagged dry	48	60	0.987 ab	0.985 ab
Bagged dry	44	180	0.946 ab	0.956 ab
Direct dip	44	60	0.934 ab	0.783 bcd
Bagged dry	44	240	0.934 ab	0.906 abc
Bagged wet	44	180	0.918 ab	0.857 abcd
Bagged wet	48	60	0.870 abc	0.909 abc
Bagged wet	44	240	0.839 bc	0.701 cde
Direct dip	44	120	0.684 cd	0.497 efg
Bagged dry	48	120	0.610 de	0.909 abc
Direct dip	44	180	0.427 ef	0.394 fgh
Direct dip	48	60	0.404 f	0.181 hi
Bagged wet	48	120	0.335 f	0.628 def
Direct dip	44	240	0.313 f	0.296 h
Bagged dry	48	180	0.297 fg	0.835 abcd
Bagged wet	48	180	0.125 gh	0.237 h
Direct dip	48	120	0.102 h	0.028 j
Direct dip	48	240	0.080 h	0.000 j
Bagged dry	48	240	0.041 h	0.375 gh
Bagged wet	48	240	0.030 h	0.058 ij
Direct dip	48	180	0.013 h	0.000 j

[z] Least square means are derived from the three-way interaction of temperature, exposure time, and treatment protocol from the analysis of greenhouse trials. Values under greenhouse trial I represent the means of two cultivars (Camarosa and Diamante) obtained from two nurseries, and values under greenhouse trial II represent the means of six cultivars (Camarosa, Camino Real, Diamante, Oso Grande, Strawberry Festival, and Ventana) obtained from a single nursery. The means shown are the back transformation of the arcsine square-root, Haldane-corrected proportion. Means followed by the same letter are not significantly different from each other according to the PDIFF option of SAS PROC GLIMMIX ($P < 0.05$).

Table 2. Mixed model analysis of the effects of the experimental factors temperature (T), exposure time (Ti), treatment protocol (Tr), cultivar (C), nursery (N; greenhouse trial I only), and selected interactions on plant survival and runner and flower production for heat treatments applied to strawberry nursery plants for two greenhouse trials

		Survival			Runners			Flowers		
Trial, factors	**Num[z]**	**Den**	***F***	***P***	**Den**	***F***	***P***	**Den**	***F***	***P***
Greenhouse trial I										
T	1	4	200.5	<0.0001	24	121.4	<0.0001	20	68.72	<0.0001
Tr	2	16	62.15	<0.0001	24	0.97	0.3944	20	1.79	0.1929
T × Tr	2	16	3.03	0.0762	24	0.66	0.5275	20	1.34	0.285
Ti	3	360	68.11	<0.0001	360	20.06	<0.0001	360	90.5	<0.0001
T × Ti	3	360	13.12	<0.0001	360	26.21	<0.0001	360	15.38	<0.0001
C	1	360	0.00	0.9628	360	0.84	0.3604	360	0.01	0.9185
N	1	360	3.58	0.0591	360	8.87	0.0031	360	6.03	0.0145
N × C	1	360	6.69	0.0101	360	4.41	0.0364	360	1.39	0.2386
T × Tr × Ti	6	360	8.48	<0.0001	360	2.79	0.0114	360	1.95	0.0718
Greenhouse trial II										
T	1	12	45.99	<0.0001	12	24.73	0.0003	12	41.45	<0.0001
Tr	2	12	46.41	<0.0001	12	32.35	0.0001	12	50.7	<0.0001
T × Tr	2	12	2.29	0.1438	12	2.66	0.1104	12	1.59	0.2444
C	5	276	19.99	<0.0001	276	87.29	<0.0001	276	15.32	<0.0001
Ti	3	276	63.06	<0.0001	276	45.9	<0.0001	276	43.07	<0.0001
C × Ti	12	276	0.74	0.7376	276	1.99	0.0161	276	1.16	0.2995
T × Tr × C	10	276	4.17	<0.0001	276	4.71	<0.0001	276	1.48	0.1478
T × Tr × Ti	6	276	6.87	<0.0001	276	6.95	<0.0001	276	9.58	<0.0001

[z] *F* tests were performed on fixed effects with numerator (Num) and denominator degrees of freedom (Den) shown in the table. The denominator degrees of freedom were calculated using the kenwardroger method and rounded to nearest whole number for presentation (14).

At the time of the second rating, ALS was found on a second plant of Ventana treated at 48°C and on two plants in a dry Strawberry Festival plot treated at 48°C. The least squares means (back-transformed) for the two ratings are shown in Table 5. For both ratings, all HTs had significantly lower incidences of ALS than their corresponding control treatment (Table 5). Survival was somewhat lower in this trial than in the Maryland trial. In general, plants treated at 48°C for 2 h did better than plants treated at 44°C for 4 h (Fig. 5D); this was particularly evident with Ventana. Also, survival for Strawberry Festival plants exposed to the additional dry treatment was substantially better than the corresponding wet treatment. Runner counts are not reported because of low or no production in all plots (including the controls). It is not absolutely clear why runners were not produced; however, a damaging freeze event on the evenings of 2 and 3 January 2008 is the suspected cause.

DISCUSSION

Our findings of significantly reduced ALS among heat-treated plants compared with nontreated controls for four different strawberry cultivars (Camarosa, Camino Real, Strawberry Festival, and Ventana) planted in the field suggest that HT may be a promising option for managing ALS in the nursery. However, there are several considerations, limitations, and consequences of using these treatments. First, because cultivars respond differently to heat, HT may not be suitable for all cultivars. Second, under greenhouse conditions, flowering and bud break were affected on most cultivars to varying degrees; therefore, it is likely not a treatment that could be used on plants destined for fruit production fields. Third, it is uncertain how plants would be heat treated in a commercial setting where thousands of plants need to be treated at once to keep up with the demand in a typical nursery. Fourth, although the reduction in ALS is substantial, some bacteria will survive; therefore, additional management may be necessary when environmental conditions are highly favorable for disease. On a positive note, HT can be applied to almost all cultivars and can be

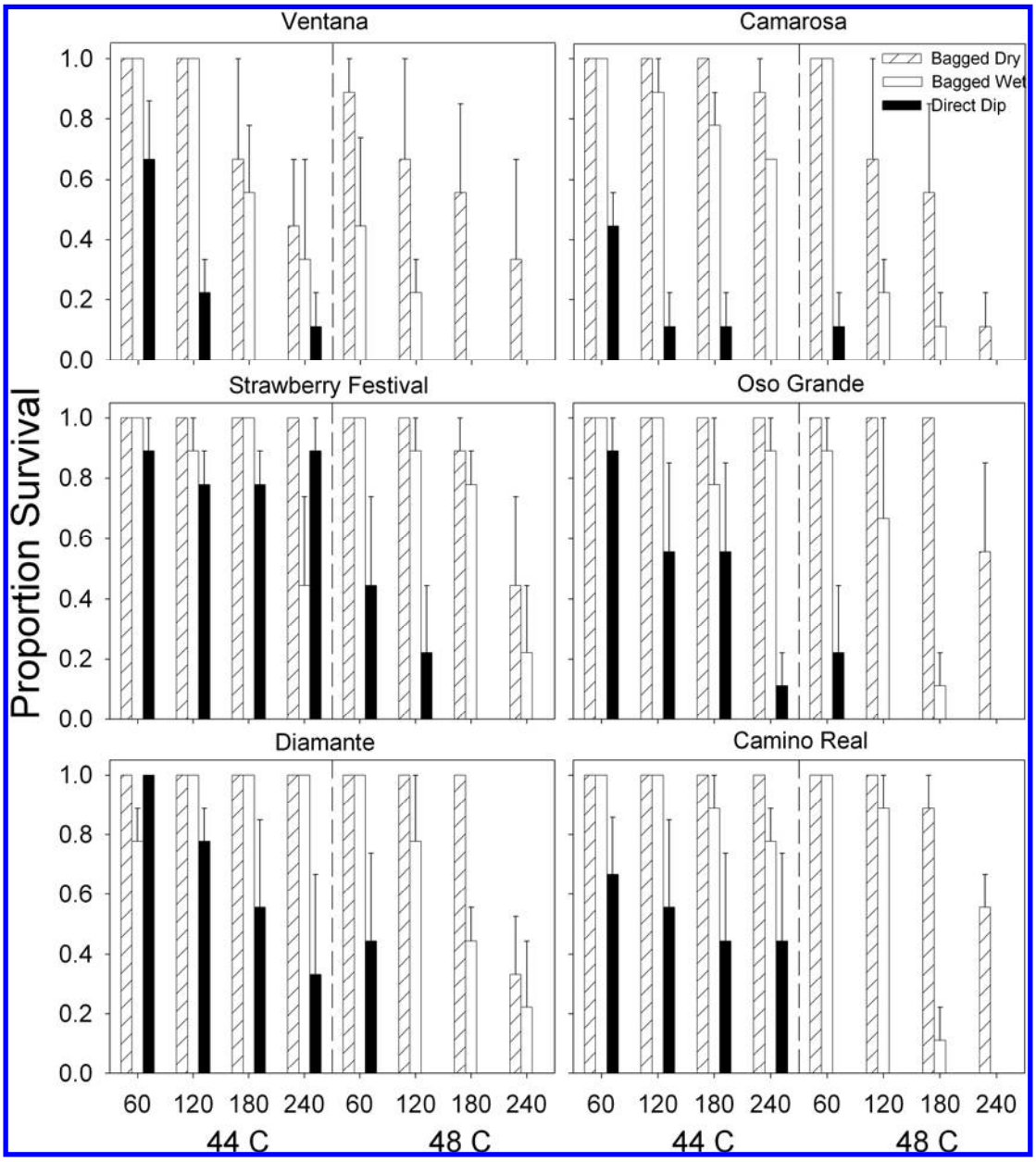

Fig. 3. Proportion of plants in greenhouse trial II surviving exposure to hot water treatment of 44 or 48°C for 60, 120, 180, or 240 min in which plants were either submerged directly in to the water bath (Direct Dip) or sealed in waterproof plastic bags dry (Bagged Dry) or wet (Bagged Wet) prior to being submerged into the bath for their respective treatment. For each cultivar, bars represent the average of three plants in three treatment replications (nine observations).

used to disinfest important planting stock (but with some loss). Not all cultivars are equally damaged; thus, evaluations of flowering should be conducted for each cultivar to determine whether HT could be applied to plants destined for fruit production fields. HT is likely to be effective against other pests as well as on ALS but further research is needed to determine the extent to which this is true.

In our in vitro studies, we found that the number of living cells of *X. fragariae* exposed to HTs of 48°C for 2 h or 44°C for 4 h could be reduced by a minimum of 10^5 or 10^6 CFU/ml. In most instances, HT of bacterial suspensions resulted in no colony growth (presumed dead) or the production of only a few colonies when plated on SPA. The few bacteria that survived HT produced atypical colonies: usually lacking extracellular polysaccharide but still testing positive via PCR (21,26), and perhaps not pathogenic, but this was not tested. These observations did not differ among the four strains used in this study (Table 1), although the type strain was dissimilar in appearance and growth rate compared with the other strains. The relevant question is whether this reduction is sufficient to prevent epidemic development. This was not answered directly in this study and is a challenging question because, unless the tolerance for infection or the pathogen is zero (necessary for some quarantine pests), the threshold for disease development in the field and whether or not it will have an economic impact is variable and related to a number of factors, including inoculum load, environmental conditions, the susceptibility of the host, location, the timing of epidemic development relative to crop phenology, and so on. Thresholds have been established for some seedtransmitted bacterial pathogens (e.g., *X. campestris* pv. *carota* and *X. campestris* pv. *campestris*), in which hot-water treatment is standard practice for reducing, but not necessarily eradicating, seedborne infection (23,24,28). In these instances, the use of hot-water treatment is not intended to eradicate the pathogen but as another tool for disease management where some finite level of disease is acceptable. This is the likely role for the HTs developed in this study for strawberry.

The field trial in Maryland had a low incidence and severity of ALS relative to the Florida trial. This could be the result of several factors. The first, and the most plausible explanation, is that the plants used in the Florida trial may have had a higher level of initial (systemic) infection than those used in the Maryland trial. This is not easily testable because quantifying the level of systemic infection of *X. fragariae* requires destructive sampling, and a sampling protocol has yet to be developed that will provide an estimate of pathogen prevalence in a population based on some minimum number of samples. A second explanation is that environmental conditions for the development of disease may have been more suitable in Florida than Maryland. This is unlikely, given that disease can develop over a wide range of temperatures and irrigation water was supplied in more-than-sufficient quantities in both trials. Third, differences in the preparation of the planting sites (e.g., fumigated versus not fumigated) or in the vegetation surrounding the planting sites could explain or induce differences in endemic or resident populations of the pathogen in soil, plant debris, or alternate hosts. This, too, is unlikely because the bacteria is known not to survive freely in soil (13), plant debris (in Florida) is thoroughly destroyed with desiccant herbicide between plantings (22), and the host range of *X. fragariae* is limited to strawberry (17,25). A fourth possibility is that fresh-dug plants (used in the Florida trial), in general, may have greater numbers of active or viable bacteria than plants that have been cold stored (used in Maryland). Observations from grower's fields suggest that this also is an unlikely explanation because ALS is quite common in fields planted with cold-stored plants.

The inherent differences between fresh-dug plants and cold-stored or "frigo plants", however, warrants further discussion. The duration of cold storage—either at subfreezing temperatures at the nursery (for up to several months) or locally in coolers set at 4°C in the days, or sometimes weeks, prior to planting—may impact the ability of a plant to tolerate HT. In our trials, some cultivars were less tolerant of heat as pre-heat-treatment storage time

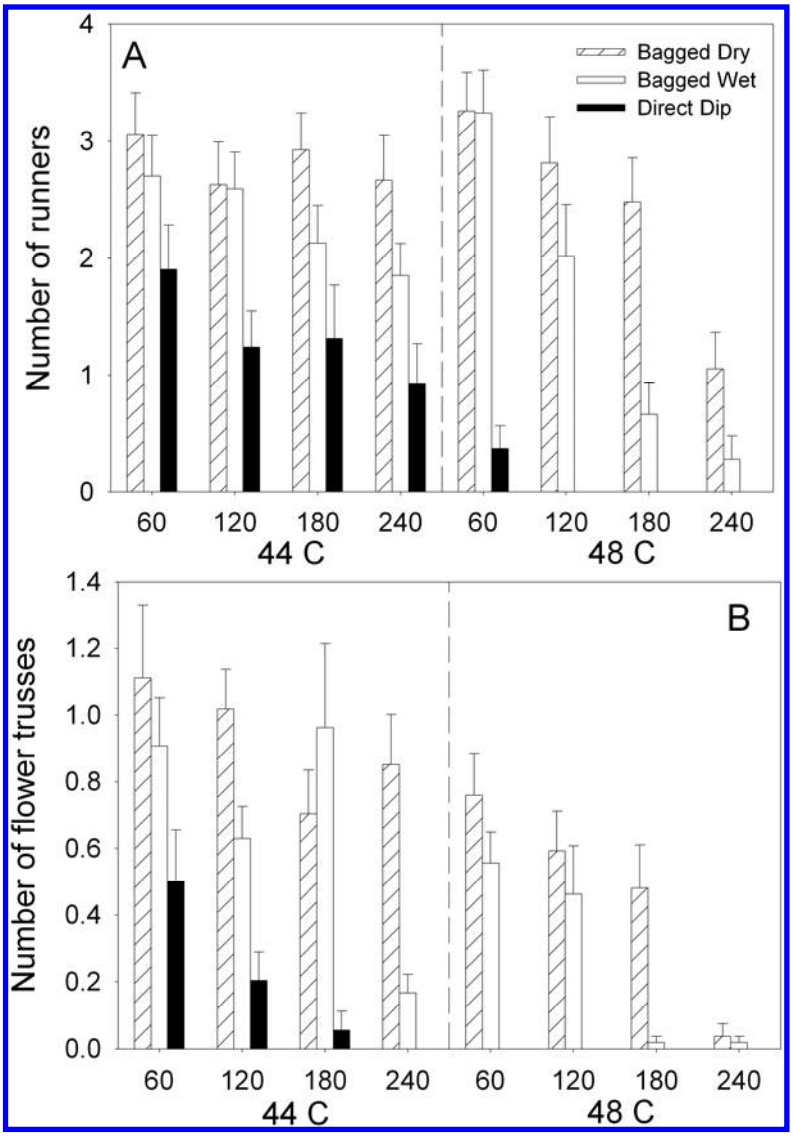

Fig. 4. A, Average number of runners per plant and **B,** average number of flower trusses per plant for plants exposed to hot water treatment of 44 or 48°C for 60, 120, 180, or 240 min in which plants were either submerged directly in to the water bath (Direct Dip) or sealed in waterproof plastic bags dry (Bagged Dry) or wet (Bagged Wet) prior to being submerged into the bath for their respective treatment. Data are from greenhouse trial II and the bars represent the average of three plants for each of six cultivars (Ventana, Camarosa, Strawberry Festival, Oso Grande, Diamante, and Camino Real) over three treatment replications (54 observations).

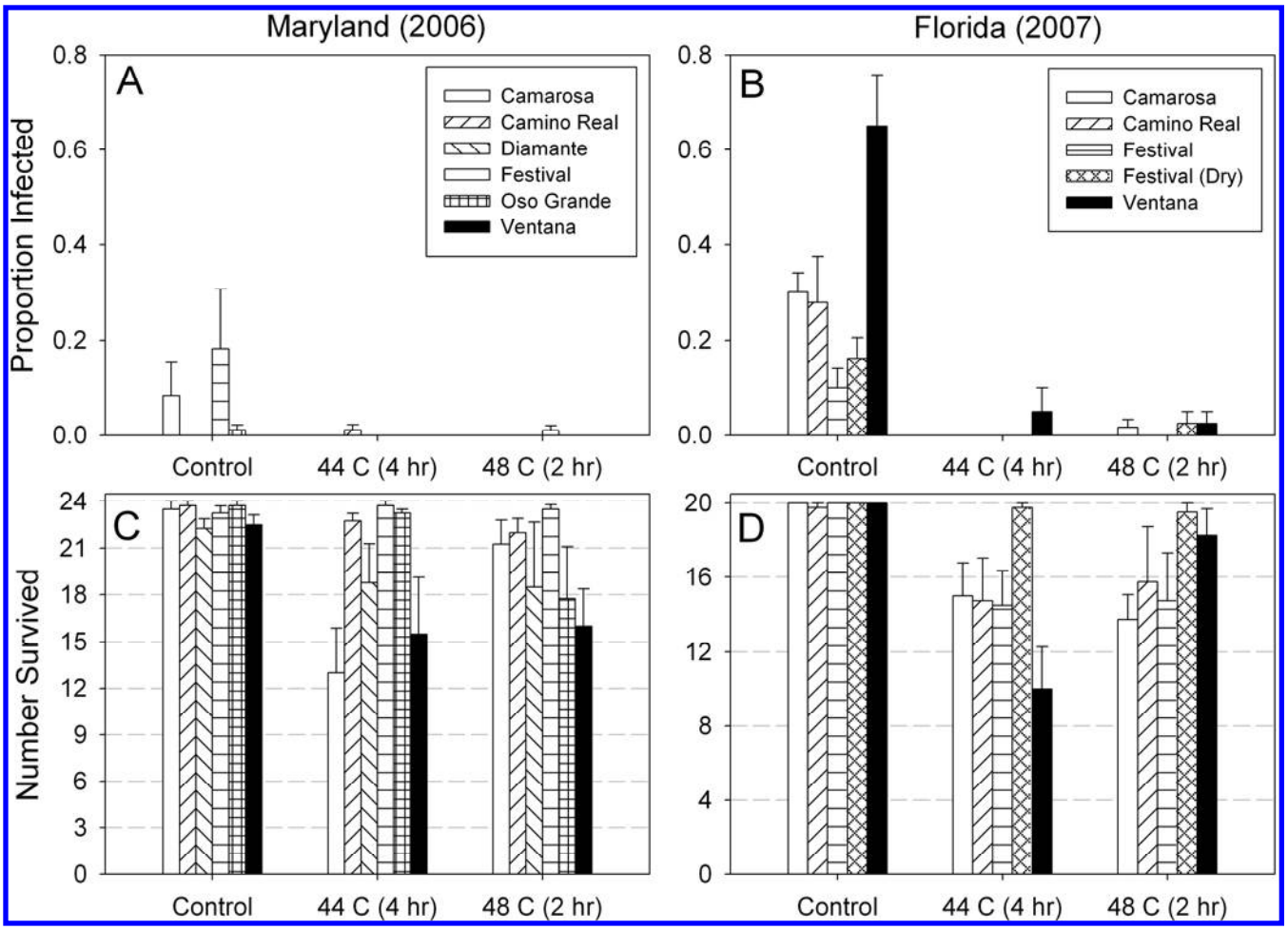

Fig. 5. Proportion of plants showing symptoms of angular leaf spot and the mean number of plants surviving heat treatment in field experiments conducted in **A** and **C,** Beltsville, MD in 2006 and **B** and **D,** Wimauma, FL in 2007 (2nd rating). Except for the Festival (Dry) treatment (B and D), plants were sealed in waterproof plastic bags dry (bagged dry) prior to being submerged into the bath for their respective treatment. The Festival (Dry) treatment was treated by placing bagged plants in heated growth chambers.

Table 4. Least square means (LSM) of the proportion of plants with symptoms of angular leaf spot, mean number of runners per surviving plant, and the mean number of plants surviving heat treatment in a field experiment conducted in Beltsville, MD in 2006

Cultivar	Temp (°C)[w]	Duration (min)[w]	LSM[x]	Runners[y]	Survival[z]
Festival	UTC	UTC	**0.131 a**	11.2 a	23.25
Festival	48	120	**0.006 bc**	9.9 ab	23.50
Camarosa	UTC	UTC	**0.053 b**	9.7 ab	23.50
Ventana	UTC	UTC	0.000 c	9.4 bc	22.50
Oso Grande	UTC	UTC	**0.006 bc**	9.1 bc	23.75
Festival	44	240	0.000 c	8.6 bc	23.75
Camino Real	UTC	UTC	0.000 c	8.6 bcd	23.75
Oso Grande	48	120	0.000 bc	7.7 cde	17.75
Camino Real	48	120	0.000 c	7.3 de	22.00
Oso Grande	44	240	0.000 c	6.9 ef	23.25
Camino Real	44	240	**0.006 bc**	6.4 ef	22.75
Camarosa	48	120	0.000 c	6.3 efg	21.25
Diamante	UTC	UTC	0.000 c	5.9 fgh	22.25
Diamante	48	120	0.000 bc	4.9 ghi	18.50
Ventana	44	240	0.000 bc	4.9 hi	15.50
Ventana	48	120	0.000 bc	4.7 i	16.00
Camarosa	44	240	0.000 bc	4.3 i	13.00
Diamante	44	240	0.000 c	4.3 i	18.75

[w] For each cultivar, treatments were 44°C for 4 h bagged dry, 48°C for 2 h bagged dry, or an untreated control (UTC).

[x] LSM are derived from the interaction of cultivar–treatment, and were back transformed using mean survival in place of *n* when back transforming the Haldane-corrected proportion.. Means followed by the same letter are not significantly different from each other according to the PDIFF option of SAS PROC GLIMMIX ($P < 0.05$). Values in bold indicate the plots in which angular leaf spot developed.

[y] Mean number of runners per surviving plant for a given treatment. The maximum number of plants that the average could be calculated from is 96.

[z] Mean number of plants per plot (maximum = 24) surviving heat treatment.

increased (*data not shown*). Also, some cultivars had a variable response to HT that might be attributable to the duration or type of cold storage. This was particularly evident with Ventana. In greenhouse trials and in the first field trial, cold-stored Ventana was very intolerant to heat (Table 4) whereas, in the second field trial, fresh-dug Ventana treated at 48°C for 2 h was one of the best-performing cultivars (Table 5). In contrast, Strawberry Festival was very tolerant to HT independent of cold storage. Strawberry Festival was bred at the University of Florida and, thus, may have the ability to tolerate heat as part of its genetic disposition. If the ability to tolerate heat is a heritable trait, breeding cultivars resistant to heat would be desirable because it would permit HT to be used as standard treatment for disease management.

Results of the second field trial, in which heated air was tested, suggested that exposing plants to heated air may be a better alternative than submerging bagged plants in heated water. It is not fully understood why heated air was less damaging to plants than sealing plants in plastic bags and submerging them in hot water. A likely explanation is that the lack of oxygen and

Table 5. Mean number of plants surviving heat treatment and least square means (LSM) of the proportion of plants showing symptoms of angular leaf spot in a field experiment conducted in Wimauma, FL in 2007

			Early rating (21 November 2007)		Late rating (20 December 2007)	
Cultivar	**Temp (°C)[x]**	**Duration (min)[x]**	**Survival[y]**	**LSM[z]**	**Survival[y]**	**LSM[z]**
Ventana	UTC	UTC	20.00	0.475 a	20.00	0.692 a
Camino Real	UTC	UTC	19.75	0.169 b	19.75	0.268 bc
Camarosa	UTC	UTC	20.00	0.157 bc	20.00	0.302 b
Festival	UTC	UTC	20.00	0.072 bcd	20.00	0.083 de
Festival (dry)	UTC	UTC	20.00	0.068 cde	20.00	0.153 cd
Ventana	44	240	11.00	0.019 def	10.00	0.024 def
Camarosa	48	120	14.25	0.006 def	13.75	0.006 ef
Ventana	48	120	18.00	0.005 def	18.00	0.013 ef
Festival	44	240	15.00	0.000 ef	14.50	0.000 ef
Festival	48	120	15.25	0.000 f	14.75	0.000 ef
Camino Real	48	120	16.00	0.000 f	15.75	0.000 ef
Camino Real	44	240	15.50	0.000 f	14.75	0.000 ef
Camarosa	44	240	16.50	0.000 f	15.00	0.000 f
Festival (dry)	48	120	19.50	0.000 f	19.50	0.012 ef
Festival (dry)	44	240	19.75	0.000 f	19.75	0.000 f

[x] For each cultivar, treatments were 44°C for 4 h bagged dry, 48°C for 2 h bagged dry, or an untreated control (UTC).
[y] Mean number of plants per plot (maximum = 20) surviving heat treatment.
[z] LSM are derived from the interaction of cultivar–treatment, and were back transformed using mean survival in place of *n* when back transforming the Haldane-corrected proportion. Means followed by the same letter are not significantly different from each other according to the PDIFF option of SAS PROC GLIMMIX ($P < 0.05$).

oxygen exchange within the submerged bag had a deleterious affect on respiration, inducing fermentation and the diversion of carbon in glycolysis to acetaldehyde, ethanol, and lactate (1). The use of heated air warrants further study based on these preliminary results because survival may be increased across some of the more sensitive cultivars. Second, it may be easier to manipulate and move large numbers of plants in and out of heated-air chambers than water baths. Lastly, mistakes or accidents that rupture bags are likely to be lethal for plants submerged in water whereas, with heated air, plants will suffer less damage.

Heat had an adverse affect on flowering, although it appeared that some cultivars (e.g., Strawberry Festival) were less affected than others. In the first greenhouse trial, runner production was clearly enhanced, probably due to an increase in vegetative growth resulting from killing of flower buds (5). However, runner production was reduced in all cultivars (not always significantly) in the second greenhouse trial and the field trial in Maryland. The level of reduction, however, seems marginal and would likely not affect the utility of HT as a disease management tool. Heat also had an adverse affect on the timing of bud break (*data not shown*) but this effect has been documented previously (2). This is not a trivial result because HT could be detrimental to plants destined for fruit production fields. In nursery production, however, the goal is to multiply plants. In a typical nursery, plants are deflowered to promote vegetative growth and runner production and, hence, daughter plant production. HT may facilitate this goal and could reduce labor costs associated with this practice.

In summary, the research presented provides the information necessary to begin implementing HT on strawberry nursery stock. The treatment is not foolproof and requires careful attention depending upon the cultivar selected for treatment. However, for those cultivars where HT is an option, ALS can be reduced throughout the propagation cycle and its repeated use will lower pathogen populations overall. The reduction will likely be sufficient to reduce epidemics in production fields but further research is needed to determine other measures that may be needed on the part of the grower. Future experiments should focus on applying HT to large quantities of plants, determining the effect of HT on horticultural traits of more cultivars, determining the impact of reducing *X. fragariae* on epidemic development in subsequent crops, and a cost-benefit analysis to determine the economics of using HT in a commercial setting.

ACKNOWLEDGMENTS

We thank J. Crane, P. Edmonds, K. Herder, G. Hess, E. Pfeufer, L. Rios, T. Seijo, and C. Yeh for technical assistance and thoughtful inputs; and E. Ponce and Lassen Canyon Nursery (Redding, CA), and R. Whitaker and Bonita Nursery (Stockton, CA) for their donation of plants and discussions about the practicality of heat treatment in California. Financial support was provided in part by the North American Strawberry Growers Association, and by USDA-ARS CRIS projects 1275-21220-181-00D, 1275-21000-159-00D, and 6618-22000-026-00D.

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