Efficacy of Hot Water and Chlorine for Eradication of *Cladosporium variabile*, *Stemphylium botryosum*, and *Verticillium dahliae* from Spinach Seed

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ABSTRACT

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Cladosporium variabile, *Stemphylium botryosum*, and *Verticillium dahliae* are seedborne and seed-transmitted pathogens of spinach. Spinach seed treatments in 1.2% NaOCl for 10 to 60 min, or hot water (40, 45, 50, 55, and 60ºC) for 10 to 40 min, were evaluated for eradication of these fungi from seed. *C. variabile* and *V. dahliae* were largely eradicated by chlorine treatment for ≥10 min. Although chlorine treatment reduced the incidence of *S. botryosum*, this fungus was not eradicated after 60 min in chlorine. Seed germination was not affected adversely by chlorine treatment, even after 60 min. In contrast, germination was reduced significantly by hot water treatment at 50ºC for ≥30 min or 55 or 60ºC for ≥10 min. *C. variabile* was eradicated from seed treated in 40ºC water for 10 min. *V. dahliae* was eradicated from seed treated at 55ºC for ≥30 min or 60ºC for ≥10 min. *S. botryosum* was eradicated from a lightly infected seed lot (5% incidence) by hot water treatment at 55 or 60ºC for ≥10 min, but could not be eradicated from two heavily infected lots (>65% incidence), even at 60ºC for 40 min. Using precisely controlled parameters, chlorine or hot water seed treatments can be used to eradicate *C. variabile* and reduce the incidence of *S. botryosum* and *V. dahliae* in spinach seed without damaging germination.

Additional keywords: Cladosporium leaf spot, seed transmission, *Spinacia oleracea*, Stemphylium leaf spot, Verticillium wilt

Approximately 14,000 ha of spinach (*Spinacia oleracea* L.) are grown in the United States annually for fresh and processed (frozen and canned) markets (5). In addition, approximately 600 to 1,600 ha of spinach seed crops are grown annually in the cool, maritime region of western Oregon and western Washington, providing ≤50% of the U.S. supply and ≤20% of the world supply of spinach seed (14). Production spinach crops in the United States are seeded at populations ranging from 0.7 to 1.5 million seed/ha for processing crops, and 9.1 to 9.9 million seed/ha for baby leaf spinach crops (21,24,27). Therefore, seedborne pathogens are a concern to spinach growers because of the high risk of seed transmission at such dense seeding rates.

Cladosporium variabile (Cooke) G. A. De Vries, causal agent of Cladosporium leaf spot, was considered the major leaf spot pathogen of spinach seed crops in the Pacific Northwest, where this pathogen can cause significant losses when cool and moist conditions prevail (15,23). In 2001, du Toit and Derie (6) demonstrated that Stemphylium leaf spot, caused by *Stemphylium botryosum* Wallr., also causes a leaf spot in spinach seed crops in the Pacific Northwest. du Toit and Derie (7) also demonstrated that severity of Stemphylium and Cladosporium leaf spots is greater in the presence of spinach pollen, and that enhancement of disease in the presence of pollen is significantly greater for *S. botryosum* than *C. variabile*. The two pathogens can reduce seed quality (9,10) and yield (11). In addition, spinach leaf spots can reduce the quality of processing crops and may necessitate additional hand sorting for fresh-market crops (13,15,25).

Fuentes-Davila (15) reported *C. variabile* to be seedborne in spinach. Hernandez-Perez and du Toit (20; *unpublished*) detected *C. variabile* and *S. botryosum* in 48 and 100%, respectively, of 77 spinach seed lots produced in Denmark, the Netherlands, New Zealand, or the United States in 2000 to 2003. Although anecdotal evidence of seed transmission of *C. variabile* in spinach was reported in Denmark in 1952 (16), Fuentes-Davila (15) could not demonstrate seed transmission of *C. variabile* in greenhouse or growth chamber trials. Hernandez-Perez (18) recently demonstrated seed transmission of *C. variabile* and *S. botryosum* in greenhouse trials. Although seed transmission has not been proven under field conditions, Raid (31) indicated that the nature of outbreaks of Stemphylium leaf spot in baby leaf spinach crops in Florida suggested that infected seed may have been the source of inoculum. The prevalence of *S. botryosum* in spinach seed lots, combined with routine interstate and international distribution of spinach seed, might account for the recent first reports or observations of this pathogen in Arizona (*unpublished*), California (25), Florida (32), Maryland and Delaware (13), Oregon (M. L. Putnam and L. J. du Toit, *unpublished data*), and Washington (6).

Verticillium wilt has not been reported in fresh-market or processing spinach crops in the United States, even when spinach seed was planted into soils heavily infested with *V. dahliae* from previous crops (5,12). This may be explained by the fact that symptoms of Verticillium wilt on spinach appear to develop only after initiation of bolting (conversion from vegetative to reproductive growth; 12). Although Verticillium wilt has no economic impact on fresh-market or processing spinach crops, *V. dahliae* has been demonstrated to be prevalent in commercial seed lots and is highly systemic and readily seed transmitted in spinach (12,37,38). Therefore, *V. dahliae* is a concern to the spinach industry because of the risk of introducing the pathogen into fields from infested spinach seed, resulting in infection of subsequent crops susceptible to Verticillium wilt that may be grown in rotation with spinach (38). In addition, phytosanitary certification restrictions affect export of *V. dahliae*-infested spinach seed to some export markets (3).

Hot water and chlorine seed treatments have been reported to eradicate or significantly reduce the incidence of a number of seedborne fungi, without adversely affecting seed quality when carried out using precise treatment parameters (4,17,26,28–30,35,36). In a preliminary study, du Toit and Derie (8) demonstrated that the incidence of *S. botryosum* in a spinach seed lot was reduced from 54.8 to 23.3% when the seed was soaked in 1.2% NaOCl for 10 min, and to <20% for seed soaked in chlorine for 20, 30, or 40 min. In contrast, the incidence of *C. variabile* was reduced from 49.0 to 0.3% after chlorine treatment for 10 min, and was not detected in seed treated with chlorine for longer durations. To our knowledge, there are no other reports on the efficacy of hot water or chlo-

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rine for treatment of spinach seed. Although some seed companies currently utilize hot water, chlorine, or both for spinach seed (M. Lyons, Alf Christianson Seed Co., *personal communication*), the details of these seed treatment protocols are proprietary. Therefore, the objectives of this study were to determine (i) the efficacy of hot water seed treatments and chlorine seed treatments for eradication of *C. variabile*, *S. botryosum*, and *V. dahliae* from spinach seed and (ii) the effects of these seed treatments on germination of spinach seed. In addition, because the presence of nonpathogenic fungi on spinach seed may contribute to deterioration of the seed in storage (2), hot water and chlorine seed treatments were evaluated for their potential to clean seed lots infested with nonpathogenic fungi. Preliminary results have been presented (19).

MATERIALS AND METHODS

Chlorine seed treatments. To determine efficacy of chlorine seed treatments for eradication of *C. variabile*, *S. botryosum*, and *V. dahliae* from spinach seed, four replications of five durations of seed treatment (0, 10, 20, 30, and 40 min) in 1.2% NaOCl were evaluated in fall 2004 using a randomized complete block (RCB) design. Spinach seed lot 03-409 used in this trial was harvested from a hybrid seed crop trial carried out at the Washington State University–Northwestern Washington Research & Extension Center (WSU-NWREC) in Mount Vernon, WA in 2003 (9). In October 2003, using the surface-sterilized freeze-blotter seed health assay described by du Toit et al. (12), the seed lot was demonstrated to be infected with *C. variabile*, *S. botryosum*, and *V. dahliae* (9). The seed lot was stored at 18 ± 3ºC and 60 ± 10% relative humidity.

For each replication of each treatment, a mesh tea strainer containing 220 seed was immersed in 100 ml of 1.2% NaOCl in a 250-ml glass beaker. The mouth of the beaker was covered with Parafilm (Pechiney Plastic Packaging, Menasha, WI) and the beaker was placed on an orbital shaker at 220 rpm for the appropriate duration at ambient temperature (24 ± 3ºC). The seed then were immediately triple-rinsed in the tea strainer using sterile deionized water, dried on sterile paper towel in a laminar flow hood for at least an hour, and placed in a sterile petri plate. For the control treatment (0 min) of each replication, 220 seed were triple-rinsed in sterile deionized water and dried. Each plate was sealed with Parafilm and stored in the dark at ambient temperature (24 ± 3ºC). The seed were stored for up to 4 weeks, with increasing duration of storage from the first to the fourth replications because of limited incubator space for completing seed health assays.

For each replication of each treatment, 100 treated seed were subjected to the freeze-blotter seed health assay following the method described by du Toit et al. (12), without surface sterilization. In summary, 20 seed were plated onto a sterile germination blotter moistened with sterile deionized water in each of five 10-cm-diameter petri dishes. Each dish was sealed with Parafilm. The seed were incubated in the dark at 24ºC for 24 h to imbibe, frozen at –20ºC for 24 h to prevent further germination, and placed in an incubator (Model I30BLL; Percival Scientific, Perry, IA) set at 24ºC for 12 to 14 days with a 12-h day-night cycle, with cool white fluorescent light and near-ultraviolet light by day. Approximately 4 to 5, 8 to 9, and 13 to 15 days after plating, the seed were examined microscopically (×8 to ×100 magnification) for development of *Alternaria* spp., *C. variabile*, other *Cladosporium* spp., *Fusarium* spp., *S. botryosum*, and *V. dahliae* as described by du Toit et al. (12) and Hernandez-Perez and du Toit (*unpublished*).

An additional 100 seed from each replication of each treatment were subjected to a germination seed assay based on the Association of Official Seed Analysts (AOSA) protocol (39). Fifty seed were placed onto each of two double layers of Anchor seed germination blotters (38# regular weight; Anchor Paper Co., St. Paul, MN), moistened with deionized water, and placed over a single sheet of wax paper. The seed on each blotter were covered with an additional moistened blotter. The blotters were rolled and stored upright in a plastic bag in a seed germinator (Stults Scientific Engineering Corp., Springfield, IL) at 15ºC with no light. Counts of germinated, abnormal, and rotten seed were carried out 4, 7, and 14 days later, following the criteria described by Yaklich (39).

The chlorine seed treatment trial was repeated 5 weeks later using seed lot 03-409. For the second trial, 50- and 60-min treatment durations in 1.2% NaOCl were added, for a total of seven durations of seed treatment arranged in an RCB design with four replications.

Hot water seed treatments. To determine the effect of hot water treatment on eradication of *C. variabile*, *S. botryosum*, and *V. dahliae* from spinach seed, a split-plot RCB design with four replications was established. Water temperature (40, 45, 50, 55, and 60ºC) was the whole-plot factor, and duration of treatment (10, 20, 30, and 40 min) at each temperature served as the subplot factor. For each replication of each hot water temperature, four mesh tea strainers, each containing 220 spinach seed of lot 03-409, were immersed in circulating deionized water heated to the appropriate temperature in a programmable water bath (Model 1203; Fisher Scientific International, Inc., Hampton, NH). After each duration of treatment, one tea strainer was removed from the bath and immediately rinsed in running deionized water for 5 min at ambient temperature. The seed were dried and stored for 2 to 5 days. The procedure was repeated at each temperature. The water bath was emptied between temperature treatments, disinfested with 70% ethyl alcohol, triple-rinsed with deionized water, and dried. For the control treatment (0 min) of each replication, 220 seed were rinsed for 5 min in deionized water, dried, and stored. Freeze-blotter seed health assays and germination assays were performed as described for the chlorine seed treatments.

The hot water seed treatment trial was completed two more times. The incidence of *S. botryosum* in seed lot 03-409 dropped almost fourfold during the 4 months between completion of the second chlorine trial and the first hot water trial. Therefore, seed lots 04-305 and 04-407, harvested from a spinach seed crop fungicide trial carried out at the WSU-NWREC in 2004 (10), were used in the second and third hot water trials, respectively. Freeze-blotter seed health assays demonstrated previously that lots 04-305 and 04-407 were infected with *C. variabile*, *S. botryosum*, and *V. dahliae* (10,12).

Data analyses. Using PROC GLM of SAS (version 8.2; SAS Institute, Cary, NC), analysis of variance, means comparisons using Fisher's protected least significance difference (LSD; $P < 0.05$) or Friedman's nonparametric rank test, correlation coefficients, and polynomial regression analysis (34) were calculated for the dependent variables measured in the seed health and germination assays. To generate homogeneous variances and normal residuals for parametric analyses of results for the chlorine trials (34), each dependent variable was calculated as a percentage of the control treatment of each replication. In addition, incidence (percent) of *S. botryosum* and incidence of *Fusarium* spp. detected in trial 2 were subjected to square root transformation to generate homogeneous variances. Friedman's nonparametric rank test was used to compare means for the incidences of *C. variabile* and *Fusarium* spp. detected in trial 1, and the incidence of *C. variabile* and seed germination after 7 days in trial 2, because transformations did not generate homogeneous variances or normal residuals (34).

For the hot water trials, each dependent variable was analyzed using Friedman's nonparametric rank test because of non-normal data, except for the following variables: abnormal germination, rotten seed, and incidence of other *Cladosporium* spp. detected in trial 1, and total germination in trial 2. Each variable was calculated as a percentage of the control treatment within each replication, as described above, and the following transformations were used to generate homogeneous variances or normal residuals: square root transformation for percent rotten seed in trial 1 and percent total germination in trial 2, and arcsin

transformation for the incidence of other *Cladosporium* spp. detected in trial 1.

RESULTS

Chlorine seed treatments. Treatment of spinach seed with 1.2% NaOCl did not have a significant effect on seed quality in either of the chlorine trials, whether measured as percent germinated, abnormal, or rotten seed (Table 1; Fig. 1A and B). However, chlorine seed treatment significantly reduced the incidence of *C. variabile* detected in both trials (Table 1), regardless of the duration of treatment (Fig. 1C and 1D). In trial 1, the incidence of *C. variabile* was reduced from 34.5% for nontreated seed to ≤1.0% for seed treated in 1.2% NaOCl for 10, 20, 30, or 40 min (Fig. 1C). Similarly, in trial 2, the incidence of *C. variabile* was reduced from 28.3% for nontreated seed to ≤0.5% for seed treated with chlorine (Fig. 1D). There was no significant difference in incidence of *C. variabile* on seed treated with 1.2% NaOCl for 10, 20, 30, 40, 50, or 60 min (Fig. 1D). The relationship between percent *C. variabile* detected on the seed (Y) and duration of seed treatment in 1.2% NaOCl (X) was best described by the following polynomial regressions: $Y = 34.50 - 6.73X + 0.45X^2 - 0.10X^3$ ($R^2 = 0.97$, coefficient of variation [CV] = 38.58%) for trial 1, and $Y = 27.86 - 4.50X + 0.23X^2$ ($R^2 = 0.81$, CV = 121.58%) for trial 2.

The mean incidence of *S. botryosum* was reduced from 16.8% for nontreated seed to 9.5% for seed treated with chlorine for 10 min, and to <7.0% for seed treated with chlorine for 20, 30, or 40 min in trial 1 (Fig. 1C). However, there was no significant difference in incidence of *S. botryosum* detected on seed treated with chlorine for 10, 20, 30, or 40 min in this trial (Table 1; Fig. 1C). In trial 2, carried out 5 weeks later, the mean incidence of *S. botryosum* had dropped to 7.8% for nontreated seed of lot 03-409, which was not significantly different from the incidence of *S. botryosum* detected on seed treated with chlorine, regardless of the duration of treatment (Fig. 1D). The relationship between percent *S. botryosum* observed on the seed (Y) and duration of seed treatment in 1.2% NaOCl (X) was best shown by the following regressions: $Y = 15.06 - 0.69X + 0.01X^2$ ($R^2 = 0.42$, CV = 31.55%) for trial 1, and $Y = 8.24 - 0.08X$ ($R^2 = 0.32$, CV = 43.41%) for trial 2.

The incidence of *V. dahliae* observed on the seed was reduced from 33.8% for nontreated seed to <4.0% for seed treated with chlorine in trial 1 (Fig. 1C), and from 47.0 to <3.0% in trial 2 (Fig. 1D). However, as for *C. variabile*, there was no significant difference in incidence of *V. dahliae* among the durations of chlorine seed

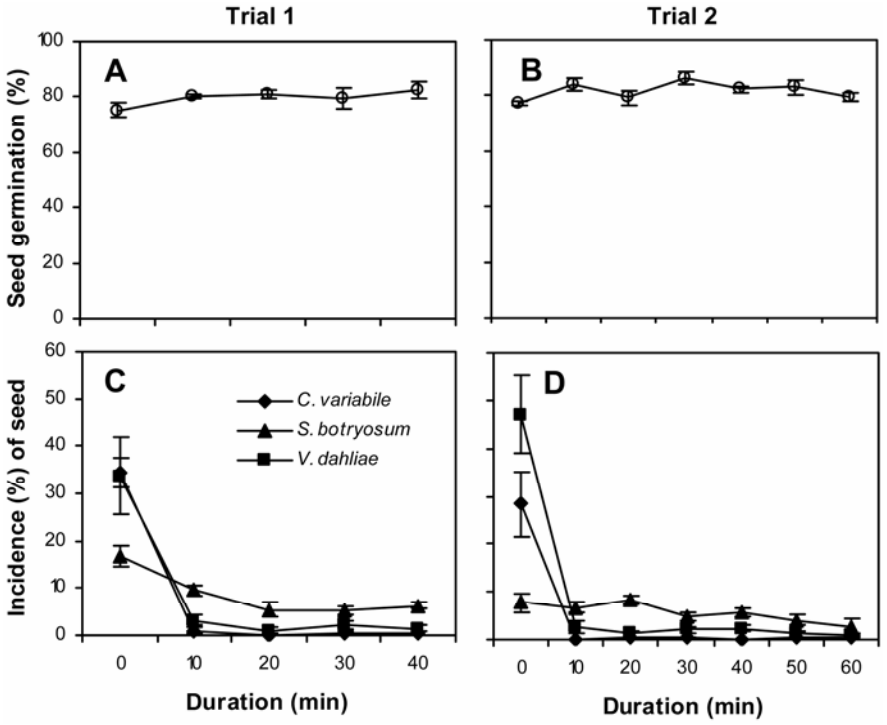

Fig. 1. Efficacy of 0, 10, 20, 30, and 40 min of chlorine (1.2% NaOCl) seed treatment on germination of spinach seed **(A** and **B)** and eradication of *Cladosporium variabile*, *Stemphylium botryosum*, and *Verticillium dahliae* from the seed **(C** and **D)** in each of two trials (A and C = trial 1, B and D = trial 2). Each data point is the mean of four replications in a randomized complete block design, and each bar is the standard error of the mean.

Table 1. Analyses of variance for germination and seed health assays of spinach seed treated with chlorine (1.2% NaOCl) for durations ranging from 0 to 40 min (trial 1) or 60 min (trial 2)[a]

	Trial 1				Trial 2			
			MS Prob > *F*				MS Prob > *F*	
Dependent variable (%)[b]	R^2	CV (%)	Replication	Duration	R^2	CV (%)	Replication	Duration
Germination assay								
Germination at 4 days	0.359	–67.56	0.7512	0.3416	0.610	–102.13	0.0040	0.7450
Germination at 7 days	0.385	–81.72	0.5167	0.4114	0.140	57.58	1.0000	0.8100
Total germination	0.722	–84.30	0.0081	0.8270	0.446	–89.03	0.3300	0.2020
Abnormal germination	0.538	–196.19	0.1613	0.3248	0.748	–148.52	0.0002	0.3260
Rotten seed	0.690	–2,293.19	0.0327	0.1702	0.611	–227.54	0.0058	0.4653
Seed health assay (seed infected)								
Cladosporium variabile	0.671	32.63	1.0000	0.0063	0.632	2.04	1.0000	0.0030
Stemphylium botryosum	0.675	26.34	0.0557	0.1215	0.603	11.11	0.0129	0.0941
Verticillium spp.	0.635	4.07	0.0805	0.1700	0.295	2.96	0.6640	0.4960
Other *Cladosporium* spp.	0.694	3.09	0.1233	0.0390	0.696	4.50	0.0010	0.2910
Fusarium spp.	0.843	19.84	1.0000	<0.0001	0.454	36.44	0.0700	0.5890
Alternaria spp.	0.579	15.42	0.4666	0.0760	0.467	14.52	0.1270	0.3180

[a] Each trial consisted of a randomized complete block design with four replications of each duration of treatment in 1.2% NaOCl, as described in the text; MS Prob = mean square probability, R^2 = coefficient of determination, and CV = coefficient of variation.

[b] For each replication of each treatment, 100 seed were subjected to a blotter germination assay (39) and 100 seed were subjected to a freeze-blotter seed health assay (12). To facilitate parametric statistical analyses, the dependent variable was calculated as a percentage of the control treatment. Results for *S. botryosum* and *Fusarium* spp. in trial 2 were square root transformed to generate homogeneous variances. Friedman's nonparametric analyses were used for *C. variabile* and *Fusarium* spp. in trial 1, and *C. variabile* and germination at 7 days in trial 2, because transformations did not generate homogeneous variances.

treatment in either trial (Table 1; Fig. 1C and D). The relationship between percent *V. dahliae* observed on the seed (*Y*) and duration of seed treatment in 1.2% NaOCl (*X*) was best described by the following regressions: $Y = 33.47 - 4.62X + 0.20X^2$ ($R^2 = 0.95$, CV = 39.09%) for trial 1, and $Y = 46.53 - 7.10X + 0.36\ X^2 + 0.01\ X^3$ ($R^2 = 0.88$, CV = 76.90%) for trial 2.

The mean incidence of *Cladosporium* spp. other than *C. variabile* observed on the spinach seed was reduced significantly in both trials as a result of chlorine treatment, from 76.5% for nontreated seed to <11.0% in trial 1, and from 44.8 to <7.0% in trial 2 (*data not shown*). Significant differences among the durations of chlorine treatment were detected only in trial 1 (Table 1), in which the incidence of other *Cladosporium* spp. decreased incrementally from 10.3 to 5.3% with increasing duration of chlorine treatment from 10 to 40 min (*data not shown*). Similar results were observed for the mean incidence of *Fusarium* spp. detected in each trial (Table 1), although a low incidence of *Fusarium* spp. was present in the nontreated seed in each trial (mean of 3.0 and 1.3% in trials 1 and 2, respectively). The mean incidence of *Alternaria* spp. observed on the seed was significantly lower for all durations of chlorine seed treatment compared with the control treatment (58.8 and 57.3% in trials 1 and 2, respectively; *data not shown*). However, in both trials, there were no significant differences in the mean incidence of *Alternaria* spp. among the durations of chlorine treatment (Table 1). In the first chlorine trial, a significant correlation was detected between percent rotten seed in the germination assay and percentage of each of the following fungi enumerated in the seed health assay: $r = 0.49$ at $P = 0.0497$ for *V. dahliae*, $r = 0.53$ at $P = 0.0344$ for other *Cladosporium* spp., and $r = 0.63$ at $P = 0.0095$ for *Fusarium* spp.

Hot water seed treatments. For each of the variables measured in the seed germination assays following hot water seed treatment, a significant ($0.01 < P \leq 0.05$) or highly significant ($P \leq 0.01$) interaction was detected between the hot water temperatures and the duration of treatment, except for percent rotten seed in trial 1 (Table 2). Of the four dependent variables analyzed using parametric analyses for the hot water trials (Table 2), the whole-plot factor of temperature was significant for three variables: percent abnormal germination and percent other *Cladosporium* spp. detected in trial 1, and percent total germination in trial 2. The subplot factor of duration of treatment was significant for percent other *Cladosporium* spp. detected in trial 1 and percent total germination in trial 2.

Table 2. Analyses of variance for germination and seed health assays of spinach seed treated with hot water[a]

Trial, assay, and dependent variable (%)[b]	R^2	CV (%)	Mean square probability > *F*				
			Temp	Rep	Rep–Temp	Dur	Temp–Dur
Trial 1							
Germination assay							
Germination at 4 days	0.80	27.56	–	1.0000	–	–	<0.0001
Total germination	0.77	30.54	–	1.0000	–	–	<0.0001
Abnormal germination	0.82	311.60	0.0492	<0.0001	0.1314	0.4530	<0.0001
Rotten seed	0.80	13.46	0.1761	<0.0001	0.1724	0.4321	0.7500
Seed health assay							
Stemphylium botryosum	0.57	37.57	–	1.0000	–	–	<0.0001
Cladosporium variabile	0.82	246.14	–	0.3992	–	–	<0.0001
V. dahliae	0.94	13.38	–	1.0000	–	–	<0.0001
Other *Cladosporium* spp.	0.69	5.89	0.0143	<0.0001	0.1001	0.0026	0.0009
Fusarium spp.	0.70	28.21	–	1.0000	–	–	<0.0001
Alternaria spp.	0.88	21.80	–	1.0000	–	–	<0.0001
Trial 2							
Germination assay							
Germination at 4 days	0.85	25.10	–	1.0000	–	–	<0.0001
Total germination	0.89	14.81	<0.0001	<0.0001	0.1831	<0.0001	0.0200
Abnormal germination	0.71	35.02	–	1.0000	–	–	<0.0001
Rotten seed	0.37	5.53	–	1.0000	–	–	<0.0001
Seed health assay							
S. botryosum	0.91	37.57	–	1.0000	–	–	<0.0001
C. variabile	0.61	14.60	–	1.0000	–	–	<0.0001
V. dahliae	0.91	18.43	–	1.0000	–	–	<0.0001
Other *Cladosporium* spp.	0.63	36.41	–	1.0000	–	–	<0.0001
Fusarium spp.	0.96	13.45	–	1.0000	–	–	<0.0001
Alternaria spp.	0.96	12.32	–	1.0000	–	–	<0.0001
Trial 3							
Germination assay							
Germination at 4 days	0.83	26.47	–	1.0000	–	–	<0.0001
Total germination	0.77	31.25	–	1.0000	–	–	<0.0001
Abnormal germination	0.56	42.92	–	1.0000	–	–	<0.0001
Rotten seed	0.24	56.42	–	1.0000	–	–	<0.0001
Seed health assay							
S. botryosum	0.90	20.63	–	1.0000	–	–	<0.0001
C. variabile	0.75	10.41	–	1.0000	–	–	<0.0001
V. dahliae	0.90	19.79	–	1.0000	–	–	<0.0001
Other *Cladosporium* spp.	0.66	32.89	–	1.0000	–	–	<0.0001
Fusarium spp.	0.97	11.76	–	1.0000	–	–	<0.0001
Alternaria spp.	0.95	14.05	–	1.0000	–	–	<0.0001

[a] Each trial consisted of a split-plot randomized complete block design with four replications. Temp = water temperatures of 40, 45, 50, 55, or 60ºC (whole plots); Rep = replication; Dur = durations of 0, 10, 20, 30, or 40 min of treatment (subplots); R^2 = coefficient of determination, and CV = coefficient of variation.

[b] Seed lots 03-409, 04-305, and 04-407 were used in trials 1, 2, and 3, respectively. For each replication of each treatment combination, 100 seed were subjected to a blotter germination assay (39), and 100 seed were subjected to a freeze-blotter seed health assay (12). Except for abnormal germination, rotten seed, and other *Cladosporium* spp. in trial 1, and total germination in trial 2, dependent variables were analyzed using Friedman's nonparametric rank test because of nonnormal data. Variables subjected to parametric analyses were calculated as a percentage of the control treatment. Square root transformations were used for rotten seed in trial 1 and total germination in trial 2, and arcsin transformation for other *Cladosporium* spp. in trial 1.

In trial 1, seed germination after 4 days was significantly lower for seed treated at 50ºC for 40 min, 55ºC for ≥20 min, or 60ºC for any duration compared with nontreated seed (*data not shown*). Total germination measured after 14 days was significantly lower than that of the nontreated seed (mean of 80.3%) for seed treated at 55ºC for 30 or 40 min or at 60ºC for any duration, but not for seed treated at 40, 45, or 50ºC for up to 40 min (Fig. 2A). Germination was prevented completely when the seed was soaked at 60ºC for 30 or 40 min (Fig. 2A). Germination of nontreated seed was much lower for seed lots 04-305 (48.0%) and 04-407 (40.8%) used in trials 2 and 3, respectively, compared with lot 03-409 (80.3%) used in trial 1 (Fig. 2A, B, and C). In trials 2 and 3, seed germination after 4 days was reduced significantly by treatment at 50ºC for only 20 min compared with 40 min in trial 1, and at all durations of treatment at 55 and 60ºC (*data not shown*). Results similar to those of trial 1 were observed for total germination measured in hot water trials 2 and 3 (Fig. 2B and C, respectively), except that germination also was reduced significantly by treatment in 55ºC water for ≥20 min in trial 2 and ≥10 min in trial 3, and at 50ºC for ≥30 min in trial 3.

In hot water trial 1, the percent rotten seed observed in the germination assay was not affected significantly by any of the hot water treatments when compared with the nontreated seed (mean of 0.8%; *data not shown*). The percent abnormal seed was reduced significantly from 3.5% for nontreated seed to 0.0% for seed treated at 60ºC for 30 to 40 min in trial 1, because none of the seed germinated following these treatments. Similar results for percent rotten seed and abnormal seed were detected in trials 2 and 3, except that the

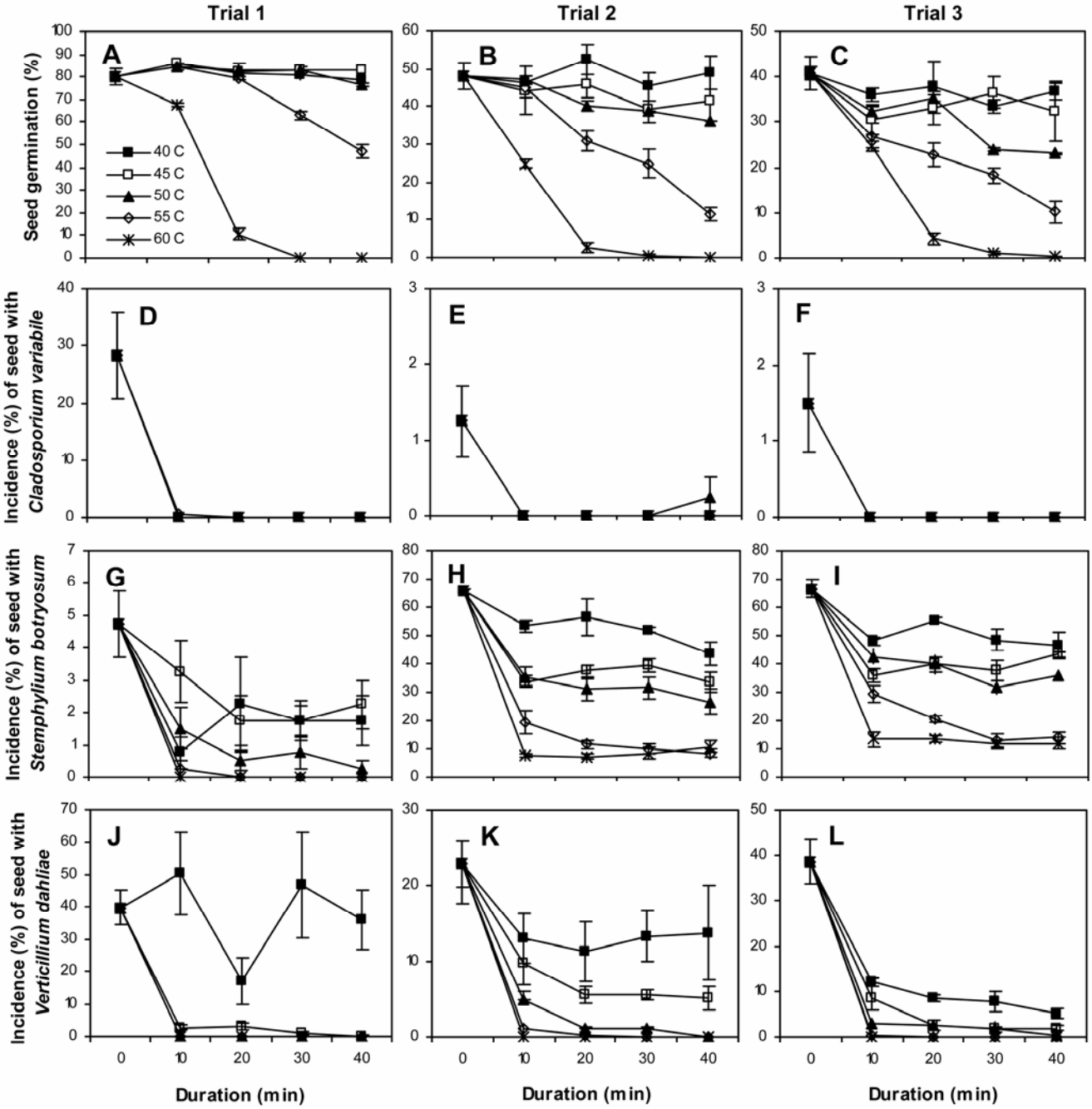

Fig. 2. Efficacy of 0, 10, 20, 30, and 40 min of hot-water treatment at 40, 45, 50, 55, and 60ºC on germination of spinach seed **(A** to **C)** and eradication of *Cladosporium variabile* **(D** to **F)**, *Stemphylium botryosum* **(G** to **I)**, and *Verticillium dahliae* **(J** to **L)** from the seed. Seed lot 03-409 was used in trial 1 (A, D, G, and J), lot 04-305 was used in trial 2 (B, E, H, and K), and lot 04-407 was used in trial 3 (C, F, I, and L). Each data point is the mean of four replications in a split-plot randomized complete block design, and each bar is the standard error of the mean.

percent rotten seed was reduced significantly for seed treated at 60ºC for 40 min in trial 2, and at 60ºC for ≥20 min in trial 3, because none of the seed germinated following these temperature-duration treatments (*data not shown*).

For each of the dependent variables recorded in the freeze-blotter seed health assays, a highly significant interaction was detected between water temperature and the duration of treatment at each temperature (Table 2). In all three trials, *C. variabile* was eradicated from the seed by all hot water treatments (Fig. 2D, E, and F), except at 50ºC for 40 min in trial 2, when *C. variabile* was detected at an incidence of 0.3% (Fig. 2E). The mean incidence of *S. botryosum* in the nontreated seed was only 4.8% in trial 1 (Fig. 2G). Consistent and significant reductions in the mean incidence of this pathogen were detected on seed treated at 50ºC or higher for any duration in trial 1. In this trial, *S. botryosum* was eradicated from seed treated at 55ºC for ≥20 min or 60ºC for ≥10 min (Fig. 2G). In trials 2 and 3, *S. botryosum* was observed on 65.8 and 66.8% of the nontreated seed, respectively (Fig. 2H and I, respectively). In both trials, significant reductions in incidence of *S. botryosum* were detected on seed heated to 40ºC for 40 min, or at higher temperatures for any duration (Fig. 2H and I). However, *S. botryosum* was not eradicated from spinach seed in either trial, even at 60ºC for 40 min, at which temperature and duration the incidence of *S. botryosum* was reduced to 10.8% in trial 2 (Fig. 2H) and 12.0% in trial 3 (Fig. 2I). The mean incidence of *V. dahliae* in nontreated seed was 39.8, 23.0, and 38.8% in trials 1, 2, and 3, respectively (Fig. 2J, K, and L). Significant reductions in the incidence of *V. dahliae* were observed for seed treated at ≥45ºC in all three trials, and at 40ºC in trial 3 (Fig. 2L). *V. dahliae* was eradicated from spinach seed treated at ≥50ºC in trial 1 (Fig. 2J); 50ºC for 40 min, 55ºC for ≥30 min, or 60ºC for any duration in trial 2 (Fig. 2K); and 55ºC for ≥20 min or 60ºC for any duration in trial 3 (Fig. 2L).

The relationship between incidence of seedborne *C. variabile* (*Y*), water temperature (*X*), and duration of treatment at that temperature (*Z*), was best described by the following regressions: $Y = 26.34 - 3.98Z + 0.17Z^2$ ($R^2 = 0.76$, CV = 76.90%) in trial 1, $Y = 1.16 - 0.17Z + 0.01Z^2$ ($R^2 = 0.57$, CV = 319.12%) in trial 2, and $Y = 1.40 - 0.21Z + 0.01Z^2$ ($R^2 = 0.59$, CV = 370.67%) in trial 3. Similarly, the regressions for *S. botryosum* were: $Y = 6.72 - 0.11X - 0.02Z$ ($R^2 = 0.47$, CV = 108.73%) in trial 1, $Y = 106.15 - 2.10X + 4.32Z - 0.18Z^2$ ($R^2 = 0.86$, CV = 23.77%) in trial 2, and $Y = 100.76 - 1.86X + 4.32Z - 0.20Z^2$ ($R^2 = 0.85$, CV = 19.56%) in trial 3. The best-fitting regressions for seedborne *V. dahliae* were: $Y = 3329.97 - 190.25X + 3.60X^2 - 0.02X^3$ ($R^2 = 0.64$, CV = 144.50%) in trial 1, $Y = 34.71 - 0.61X + 0.26Z - 0.02Z^2$ ($R^2 = 0.65$, CV = 87.64%) in trial 2, and $Y = 71.68 - 2.06X + 0.01X^2 - 0.43Z - 0.01Z^2 + 0.01XZ$ ($R^2 = 0.90$, CV = 61.82%) in trial 3.

The mean incidence of *Cladosporium* spp. other than *C. variabile* observed on the seed was reduced significantly from that of the nontreated seed by treatment at 40ºC for ≥10 min, similar to the effects of hot water treatment on *C. variabile* (*data not shown*). However, a low incidence (0.3 to 2.0%) of these other *Cladosporium* spp. was still detected on seed treated at 60ºC in each trial. The mean incidence of *Fusarium* spp. was reduced significantly from that of the nontreated seed by hot water treatment at ≥45ºC in trial 1, and at 40ºC for ≥20 min in trial 2 or ≥30 min in trial 3. However, the mean incidence of *Fusarium* spp. on nontreated seed was low in trial 1 (2.5%) compared with trials 2 and 3 (52.8 and 60.8%, respectively; *data not shown*). The mean incidence of *Alternaria* spp. was reduced significantly by treating seed at 40ºC for ≥20 min in trials 1 and 3, and ≥30 min in trial 2. *Alternaria* spp. were largely eradicated from seed treated at 55 or 60ºC (*data not shown*).

Significant correlations were detected between the percent rotten seed and the incidence of each of the fungi observed on the seed in trial 3: $r = 0.88$ at $P = 0.0206$ for *C. variabile*, $r = 0.94$ at $P = 0.0047$ for *S. botryosum*, $r = 0.95$ at $P = 0.0034$ for *V. dahliae*, $r = 0.91$ at $P = 0.0126$ for other *Cladosporium* spp., $r = 0.94$ at $P = 0.0046$ for *Fusarium* spp., and $r = 0.94$ at $P = 0.0053$ for *Alternaria* spp.

DISCUSSION

Treatment of spinach seed in 1.2% NaOCl or in hot water significantly reduced the incidences of *C. variabile*, *S. botryosum*, and *V. dahliae* detected on the seed. Seed germination was not adversely affected by chlorine treatment, even after 60 min in 1.2% NaCOl. However, seed germination was adversely affected by soaking spinach seed in 50ºC water for ≥30 min, or 55 or 60ºC water for ≥10 min, demonstrating the need to control water temperature precisely during hot water seed treatments to avoid damaging spinach seed.

C. variabile and *V. dahliae* were largely eradicated from spinach seed by chlorine treatment for as little as 10 min. In contrast, *S. botryosum* was not eradicated from spinach seed by chlorine treatment, even after 60 min in 1.2% NaOCl. There was no significant difference in the incidence of the fungus detected on seed treated with chlorine for durations ranging from 10 to 60 min, although the incidence of seedborne *S. botryosum* was reduced by 43 to 67% of that detected on the nontreated seed. Similar results were obtained by du Toit and Derie (8) and du Toit et al. (12) for spinach seed infected with *C. variabile* (49.0%), *S. botryosum* (54.8%), and *V. dahliae* (8.3%). *C. variabile* was eradicated following 20 to 40 min in 1.2% NaOCl (8). The incidence of *S. botryosum* was reduced by chlorine treatment, with the greatest reduction (67% of that on the nontreated seed) observed on seed treated for 40 min, after which *S. botryosum* was still observed on 18% of the seed (8). Similarly, *V. dahliae* was observed in <1.0% of the seed following chlorine treatment for 20 to 40 min (12).

Hot water seed treatment was very effective at eradicating *C. variabile* from spinach seed in this study. The fungus could not be detected on seed treated in 40ºC water for only 10 min, with no adverse effect on germination. *V. dahliae* was less sensitive to hot water treatment than *C. variabile*, necessitating temperatures of ≥50ºC for eradication of this pathogen from the seed. Nonetheless, seed treatment at 45ºC may be valuable for reducing the incidence of seedborne *V. dahliae*. For example, the incidence of seedborne *V. dahliae* was reduced to <10% on seed treated at 45ºC for 20 min or longer in this study, the threshold set by a phytosanitary certification requirement for export of spinach seed to Mexico (3). *S. botryosum* was eradicated from a spinach seed lot with a low incidence of infection (5%), by treating the seed in 55ºC water for ≥20 min or 60ºC for ≥10 min. However, except for the 20 min treatment at 55ºC, germination was reduced significantly by these temperature–duration combinations. Furthermore, hot water treatment did not eradicate *S. botryosum* from either of two heavily infected seed lots (>65% incidence), even at 60ºC for 40 min.

The relative efficacy of hot water treatments against seedborne *C. variabile*, *S. botryosum*, and *V. dahliae* may reflect the degree of internal infection of spinach seed by the pathogens. Using transmission electron microscopy, Hernandez-Perez (18) observed intracellular hyphae of *C. variabile* and *S. botryosum* in the pericarps of spinach seed. Component seed assays, in which the pericarp and embryo of each seed were separated and tested individually by freeze-blotter seed health assay, demonstrated that both *C. variabile* and *S. botryosum* can infect the pericarps and embryos of spinach seed (18). However, the component seed assays also demonstrated that infection of spinach seed by *S. botryosum* was more internal than that of *C. variabile*, because a higher percentage of the embryos were infected with *S. botryosum* than *C. variabile* for each of the seed lots evaluated (18). A component seed assay carried out 3 months after completion of the third hot water trial in this study showed that *S. botryosum* was present in 39 and 54% of the embryos and pericarps, respectively, of lot 04-407 (*data not shown*). In comparison, *C. variabile* could no longer be detected in any of the seed.

The more deep-seated nature of infection of spinach seed by *S. botryosum* than *C. variabile* may make *C. variabile* more vulnerable than *S. botryosum* to the fungicidal effects of chlorine and heat during seed treatment.

The highly systemic nature of infection of spinach plants by *V. dahliae* suggests internal infection of spinach seed by *V. dahliae* (12,37,38). This might explain the greater tolerance of *V. dahliae* to hot water seed treatment compared with *C. variabile*, but does not explain the greater tolerance of *S. botryosum* to hot water treatment compared with *V. dahliae*, or the greater sensitivity of both *C. variabile* and *V. dahliae* to chlorine seed treatment compared with *S. botryosum*. Van der Spek (37) provided limited evidence of infection of spinach seed by *V. dahliae* by a vascular link from the mother plants. In contrast, Snyder and Wilhem (33) reported that seedborne infection by this fungus resulted from external contamination of spinach flowers. The specific mode or modes of infection of spinach seed by *V. dahliae* may affect the degree of internal infection. Research is needed to clarify the specific locations of infection of spinach seed by *V. dahliae* (e.g., using component seed assays and electron microscopy). In addition, research on the relative heat tolerance and chlorine tolerance of *C. variabile*, *S. botryosum*, and *V. dahliae* may help clarify the results observed in this study.

Competitive interactions among fungi present on spinach seed after hot water or chlorine treatment may influence which fungi develop on the seed and can be detected using a dissecting microscope. In an attempt to counteract this effect when using the freeze-blotter seed health assay in this study, the seed were examined at 4- to 5-day intervals for up to 2 weeks after plating, thereby maximizing the opportunity to detect slow-growing, less-competitive fungi present on the seed. *Cladosporium* spp. other than *C. variabile* were observed on the spinach seed lots evaluated in this study. These fungi displayed chlorine and hot water sensitivity similar to that of *C. variabile*, because either 10 min of chlorine treatment or ≥10 min in water heated to ≥45ºC significantly reduced the incidence of these fungi. The incidence of seedborne *Fusarium* spp. also was reduced significantly by chlorine treatment, as was the incidence of *Alternaria* spp., which proved slightly less sensitive to chlorine seed treatment than *Fusarium* spp. *Fusarium* and *Alternaria* spp. were mostly eradicated from spinach seed by treatment in 55 or 60ºC water. Significant reductions in incidence of these genera also were detected by treatment in 45 or 50ºC water, with increasing efficacy the longer the duration of treatment. Although the pathogenicity of these fungi on spinach was not evaluated in this study, their presence on the seed may contribute to deterioration of spinach seed in storage (2). Therefore, chlorine or hot water seed treatments could be used to clean seed heavily infested with such saprophytic fungi.

Results of the hot water seed treatments evaluated in this study corroborate several other studies on the efficacy of hot water for control of a number of seedborne pathogens of vegetables. Nega et al. (29) found that hot water treatment at 50ºC for 20 to 30 min, or at 53ºC for 10 to 30 min controlled *Alternaria dauci*, *A. radicina*, *A. alternata*, and *A. brassicicola* on carrot, cabbage, celery, parsley, and lamb's lettuce seed. Aveling et al. (4) treated onion seed at 50ºC for 20 min to control *A. porri* and *S. vesicarium*. Various studies on hot water treatment of carrot seed illustrate the importance of evaluating a range of temperatures and durations of treatment for control of different seedborne pathogens. Pryor et al. (30) demonstrated that carrot seed treated in water or 1.0% NaOCl heated to 50ºC for 20 min eradicated *A. radicina* with minimal reduction in germination. Strandberg (35) reduced the incidence of *A. dauci* by treating carrot seed in 50ºC water for 12 min. Strandberg and White (36) subsequently demonstrated that the duration of survival of *A. dauci* in carrot seed could be reduced more effectively by hot water treatment at 55ºC for 20 min, but longer durations of treatment at this temperature reduced emergence severely. Similarly, Hermansen et al. (17) showed that placing carrot seed in 54ºC water for 20 min eradicated *A. dauci* without affecting germination, emergence, or yield adversely.

Based on the results of this study, if a spinach seed lot infected with *C. variabile*, *S. botryosum*, and *Verticillium* spp. is treated in 1.2% NaOCl for ≥20 min, *C. variabile* and *V. dahliae* probably will be eradicated from the seed, and the incidence of *S. botryosum* may be reduced significantly with no adverse effect on seed germination. Results of the hot water seed treatment trials suggest that a spinach seed lot infested with *C. variabile*, *S. botryosum*, and *V. dahliae* could be treated in 50ºC water for 20 min to eradicate *C. variabile*, reduce the incidence of *S. botryosum* and *V. dahliae*, and largely eradicate *Fusarium, Alternaria*, and other *Cladosporium* spp. with no adverse effect on seed germination. Research is needed to determine the effect of hot water or chlorine seed treatments on other important seedborne pathogens of spinach (e.g., *Cucumber mosaic virus* [40] and *Peronospora farinosa* f. sp. *spinaciae* [22]). In addition, specific temperatures and durations of hot water treatment need to be evaluated on a broader range of spinach cultivars to determine the optimum conditions necessary to avoid injuring the seed while maximizing control of seedborne pathogens. The three seed lots evaluated in this study differed slightly in response to hot water treatments, with reduction in germination observed at lower temperatures and shorter durations of hot water treatment for the two seed lots with poor seed germination. Similarly, Strandberg and White (36) observed differential responses in seed performance of 25 carrot cultivars subjected to hot water seed treatment. They suggested that different seed lots of the same cultivar may differ in seed performance following hot water seed treatment. Similarly, Hermansen et al. (17) reported that small carrot seed were more sensitive to hot water than larger seed. Strandberg and White (36) demonstrated that emergence from hot water-treated carrot seed was reduced following 6 weeks of seed storage at 70 to 80% relative humidity, but not at 20 to 60% relative humidity. Therefore, research is needed to determine the long-term effects of hot water and chlorine seed treatments on shelf-life of spinach seed.

Hot water seed treatments could be used in conventional and organic spinach production. Although seed treatment with chlorine or hot water did not eradicate *S. botryosum* from spinach seed without affecting germination, it may be possible to combine chlorine or hot water seed treatments with fungicide or biological seed treatments to eradicate the pathogen or prevent seed transmission. Research is needed to evaluate the efficacy of hot chlorine solutions and other methods of seed treatment (e.g., hot air, aerated steam, and radiation; 1,26,30) on seedborne pathogens of spinach, particularly recalcitrant internal infections by pathogens such as *S. botryosum*.

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