Biological Suppression of Seedborne *Fusarium* spp. During Cold Stratification of Douglas Fir Seeds

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ABSTRACT

Hoefnagels, M. H., and Linderman, R. G. 1999. Biological suppression of seedborne *Fusarium* spp. during cold stratification of Douglas fir seeds. Plant Dis. 83:845-852.

Fusarium spp. are important soil- and seedborne pathogens of Douglas fir (*Pseudotsuga menziesii*) in conifer seedling nurseries. We investigated the effects of culture media and cold stratification on isolation of *Fusarium* spp. from three Douglas fir seedlots and tested whether the numbers and species mixtures found on stratified seeds could cause disease. Nearly all cold-stratified seeds plated on three semiselective culture media yielded *Fusarium* spp., including *F. avenaceum, F. culmorum, F. lateritium, F. moniliforme, F. poae, F. proliferatum, F. sambucinum, F. solani,* and *F. tricinctum.* Species composition did not differ significantly among the media. Isolation of *Fusarium* spp. from seeds plated on Komada's medium (pH 6.8) at various stages of imbibition and cold stratification progressively increased from 10 to 22% to 65 to 100%. When stratified seeds were planted in conditions conducive to disease development, however, little disease attributable to *Fusarium* spp. resulted. A subsequent study was conducted to determine whether a biological control agent applied during imbibition could reduce the proliferation of *Fusarium* spp. during stratification. Unstratified Douglas fir seeds were imbibed for 24 h in a suspension of *Pseudomonas chlororaphis* isolate RD31-3A, a rifampicin-resistant fluorescent pseudomonad with previously demonstrated biocontrol activity against *F. oxysporum*. This treatment reduced the proliferation of *Fusarium* spp. during cold stratification without significantly affecting subsequent seed germination. The greatest reduction in poststratification populations of seedborne *Fusarium* spp. was achieved when preimbibition treatment with hydrogen peroxide was followed by seed imbibition in live bacteria. Seed imbibition in bacterial suspensions may be an effective means to deliver biological control agents to Douglas fir seeds.

Additional keywords: seed sanitation

Fusarium spp. are often found on and within conifer seeds, including loblolly pine (5,28), longleaf pine (34), slash pine (2), spruce (19), true fir (27), and Douglas fir. Estimates of the percentage of Douglas fir seeds with *Fusarium* spp. vary from seedlot to seedlot, ranging from less than 30% of seeds (4,12,13,22,27,30) to 60% or more (4,30). This variation probably reflects initial differences between seedlots, but it also may be influenced by such factors as cold stratification and the culture medium on which seeds are plated (4; M. H. Hoefnagels and R. G. Linderman, *unpublished*). For example, *Fusarium* populations on Douglas fir seeds increased on four of five seedlots after 3 weeks of cold stratification (4). The same authors reported greater detection of *Fusarium* spp. when seeds were plated on Komada's medium amended with benomyl and adjusted to pH 7.0 than on the same medium adjusted to pH 4.0.

Another unresolved issue regarding *Fusarium* spp. on Douglas fir seeds is the extent to which these fungi cause disease in seedlings. Most studies of the pathogenicity of seedborne *Fusarium* spp. have involved the inoculation of single, seed-derived isolates onto surface-disinfested or *Fusarium*-free seeds. Under these conditions, several pathogenic isolates have been identified. For example, *F. moniliforme, F. subglutinans*, and *F. proliferatum* from slash and loblolly pine seeds caused damping-off when applied to slash pine seeds (17), and *F. oxysporum* from Douglas fir seed was associated with preemergence damping-off in Douglas fir seedlings (13). In another study, isolates of *F. oxysporum* and *F. moniliforme* from Douglas fir seeds consistently caused disease on Douglas fir seedlings under laboratory conditions, whereas isolates of *F. avenaceum, F. sambucinum, F. lateritium*, and *F. acuminatum* caused little or no disease (4).

To our knowledge, only one study has tested the pathogenicity of seedborne *Fusarium* spp. in quantities and species mixtures that occur naturally on seeds (4). In that study, cold-stratified Douglas fir seeds (up to 99% contaminated with *Fusarium* spp.) were planted in standard container nursery cultural conditions. No disease attributable to seedborne *Fusarium* spp. occurred, even though several isolates from the seeds caused disease when inoculated individually on seeds or germinants in laboratory conditions. While this result suggests that seedborne *Fusarium* spp. may not cause disease in nurseries, it is also possible that environmental conditions in the nursery were not conducive to disease development. We know of no studies in which the pathogenicity of seedborne *Fusarium* spp. in naturally occurring quantities and species mixtures was tested under conditions known to be conducive to disease development.

Because some seedborne *Fusarium* spp. are pathogenic, it may be advantageous to reduce their proliferation during stratification. Prestratification sanitation of conifer seeds by treatments such as hydrogen peroxide, ethanol, bleach, or running-water imbibition has been used to reduce populations of seedborne pathogens (4,10,12,19,36). Seed sanitation combined with treatment with appropriate biological control agents, however, may have advantages over seed sanitation alone. Seed treatment with biological control agents during imbibition and stratification may not only help reduce the proliferation of *Fusarium* spp. during stratification, but also help protect seedlings from soilborne pathogens after planting. Although the idea of conifer seed treatment with bacteria is not new (11,16), to our knowledge this study represents the first attempt to introduce biological control agents to conifer seeds during imbibition and stratification.

The objectives of this study were to: (i) confirm and extend the results of previous studies on the effects of culture medium and cold stratification on isolation of *Fusarium* spp. from Douglas fir seeds; (ii) determine whether seedborne *Fusarium* spp. cause disease in the quantities and species that occur on stratified seeds; and (iii) determine whether *Pseudomonas chlororaphis* RD31-3A, applied during

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Accepted for publication 1 June 1999.

Publication no. D-1999-0629-01R

seed imbibition, can colonize Douglas fir seeds and reduce the proliferation of *Fusarium* spp. during stratification. Portions of this work have been presented elsewhere (15).

MATERIALS AND METHODS

Isolation of *Fusarium* spp. from cold-stratified Douglas fir seeds. Three Douglas fir seedlots were supplied by Weyerhaeuser Corp. (Rochester, WA). Seedlots DH2026 and DG2064 were collected in 1982 near Springfield, Oregon (2,501- to 3,000-ft elevation) and Twin Harbors, Washington (501- to 1,000-ft elevation), respectively; seedlot DJ8002 was collected in 1988 near Coos Bay, Oregon (1,001- to 1,500-ft elevation). Seeds were stored dry and unstratified at –15°C until use.

On each of 4 consecutive days (blocks), 100 cold-stratified (see stratification procedures below) seeds from each seedlot were plated on each of three semiselective culture media (10 seeds per plate): Komada's medium (24), pH adjusted to 3.8 ± 0.2; Komada's medium without pH adjustment (average pH = 6.80; range = 6.70 to 6.86); and modified Nash-Snyder medium (29; average pH = 6.36; range = 6.33 to 6.40). The 90 plates from each of the resulting four blocks were randomly arranged on separate trays and incubated in diffuse daylight on a single laboratory bench (23 to 25°C).

After 4, 8, and 12 days incubation, 30 plates per block were randomly selected, and the most prominent fungus growing from each seed was subcultured to corn meal agar (CMA; Difco Laboratories, Detroit, MI). Among the subcultured fungi, colonies of *Fusarium* spp. were counted, and the presence of other common fungal genera was noted. Plates were wrapped in Parafilm and stored in the dark at 4°C until *Fusarium* species were identified. For each seedlot, 44 to 48 colonies were randomly selected for identification from among the colonies isolated 12 days after seeds were plated. To identify *Fusarium* species, single germinated spores were transferred to Spezieller, Nährstoffarmer Agar (SNA; 31) and potato dextrose agar (PDA; Difco Laboratories) and compared with species descriptions (29) and isolates of known species. Identities of selected isolates were confirmed by Paul Nelson at the Fusarium Research Center at Pennsylvania State University.

The percentage of seeds with *Fusarium* spp. was analyzed separately for each seedlot and sampling date by logistic regression with contrasts between media. When logistic regression models fit the data poorly because the percentage of seeds with *Fusarium* spp. approached 100%, Fisher's exact test was used to make pairwise comparisons between media (SAS version 6.10, SAS Institute, Inc., Cary, NC). Because one-third of the 90 plates in each block were randomly selected at each of the three sampling dates, by chance some seedlot × culture medium combinations in block 2 were not represented at 8 and 12 days after plating. Data from block 2 were therefore omitted from statistical analysis.

Effect of imbibition and cold stratification on isolation of seedborne *Fusarium* spp. To improve the applicability of our results to seed processors, seed imbibition and cold stratification methods were adapted for laboratory-scale studies from procedures used at Weyerhaeuser's seed processing plant at Rochester (26; P. Cameron, *personal communication*). In all experiments, seed portions (10 to 40 g) were imbibed for 24 to 48 h in 250 to 500 ml of sterile distilled water. After imbibition, seeds were drained and stored wet in sterile plastic bags for 6 weeks at 4°C. The tops of the bags were partially closed with metal twist-ties to leave openings 1 to 2 cm in diameter. The bags were gently shaken every other day to promote air and moisture circulation and to prevent formation of fungal mats on the seeds.

Seeds from each of seedlots DG2064, DH2026, and DJ8002 were plated (10 seeds per plate) on Komada's (pH 6.8) before seed imbibition, immediately after seed imbibition, after 3 weeks of cold stratification, and after 6 weeks of cold stratification. Seeds withdrawn after imbibition, during stratification, or after stratification were air-dried in a laminar flow hood before plating. Plates were randomly arranged on trays and incubated in diffuse daylight on a single laboratory bench (23 to 25°C). The proportion of seeds with colonies resembling *Fusarium* spp. was determined 10 to 21 days after plating.

The experiment was conducted twice, with minor differences between the two trials. Four 100-seed blocks were plated at each stage for each seedlot, except in the first trial, in which four 50-seed blocks of postimbibition seeds were plated. In the first trial, blocks represented subsamples from the same plastic bags (one bag per seedlot), whereas in the second trial, blocks represented four independent bags of seeds (one 100-seed sample was withdrawn from each bag at each sampling date). Seed imbibition periods were 27 and 39 h in the first and second trials, respectively.

Pathogenicity of *Fusarium* spp. on cold-stratified seeds. Willamette Valley alluvial loam (pH 6.4) was air-dried, sieved (<2 mm), and mixed with sieved river sand (<2 mm) in a 3:1 (vol/vol) ratio. This mix was steam-pasteurized for 30 min at 60°C and stored in surface-sterilized buckets until use. Soil was sprinkled on CMA or dilution-plated on Komada's medium (pH 6.8) to confirm the absence of *Fusarium* spp.

Seeds from seedlots DG2064, DH2026, and DJ8002 were imbibed and cold-stratified for 6 weeks as described above and air-dried in a laminar flow hood. Stratified seeds in nylon mesh bags were treated for 40 min in 30% H_2O_2 to eliminate seedborne *Fusarium* spp., rinsed 10 times in sterile distilled water, and air-dried in a laminar flow hood. Control seeds were treated for 40 min in sterile distilled water, rinsed and air-dried as above, or left untreated. Seeds (39 to 100 per treatment, depending on the number of seeds available) were plated on Komada's medium (pH 6.8) and incubated as described above. The proportion of seeds with colonies resembling *Fusarium* spp. was determined after 10 to 14 days, and seedlot germination potential was determined as described below.

For each seedlot, 25 ml of pasteurized soil–sand mix was placed in each of 80 glass test tubes (25 mm diameter). One peroxide-treated or control seed was placed on the soil surface (40 tubes per treatment) and covered with a 0.5-cm layer of sterile sand (sieved to <1 mm and autoclaved for 1 h on each of 2 consecutive days). Each test tube was watered with 5 ml of sterile distilled water and covered with a clear plastic cap. Test tubes were randomly arranged in two 40-tube racks (blocks) and placed in a growth chamber, with 8-h days controlled at 30°C and 16-h nights controlled at 20°C. Light (233 $\mu E \cdot m^{-2} \cdot s^{-1}$) was supplied by cool-white fluorescent and incandescent lights.

Seedling emergence and postemergence mortality were monitored for all seeds every 3 to 7 days after planting. Dead and dying seedlings were removed, and hypocotyls or cotyledons were plated on CMA to isolate potential pathogens. *Fusarium* spp. isolated from dead and dying seedlings were identified to species. When seedling emergence was judged to be complete (no additional emergence for approximately 1 week), seeds that had failed to emerge were examined to determine whether the seeds had failed to germinate or had died after germination. The experiments were terminated 50 to 55 days after planting.

The experiment was conducted twice for each seedlot. Emergence and postemergence mortality for peroxide-treated and control seeds were compared using chi-square analysis, or Fisher's exact test when chi-square expected values were less than five. In the absence of significant differences between blocks, data were combined and analyzed together. All statistical analyses were completed on SAS (version. 6.10, SAS Institute).

Seed treatment with RD31-3A. The objective of this experiment was to determine the effect of seed imbibition in a suspension of *Pseudomonas chlororaphis* strain RD31-3A on poststratification isolation of seedborne *Fusarium* spp. *P. chlororaphis* strain RD31-3A (supplied by Paige Axelrood at B.C. Research Inc., Vancou-

ver, B.C., Canada) is a rifampicin-resistant strain of *P. chlororaphis* that was isolated on King's medium B (23) from the rhizosphere of a 1-month-old disease-escape Douglas fir seedling growing in British Columbia forest soil previously inoculated with *F. oxysporum*. In previous studies, this isolate increased the percentage of healthy seedlings when inoculated onto Douglas fir seed and grown in *Fusarium*-inoculated planting mix (P. Axelrood, *personal communication*).

For production of bacterial inoculum, test tubes each containing 10 ml of tryptic soy broth (Becton Dickinson Microbiology System, Cockeysville, MD) were inoculated with RD31-3A and incubated at a 45° angle on an orbital shaker at 1,200 rpm for 48 h at room temperature. Aliquots (200 µl) were spread on each of 135 10-cm petri plates of half-strength tryptic soy agar (20 g of TSA + 7.5 g of agar per liter). Plates were incubated in the dark at 28°C. After 48 to 72 h incubation, plates were randomly divided into three blocks. Each plate was flooded with 10 ml of 10 mM KH_2PO_4/K_2HPO_4 (phosphate) buffer, and cells were scraped free with a flamed glass rod. Cell suspensions were dispensed into sterile reagent bottles (45 plates contributed to each of three bottles). Ten ml was withdrawn from each bottle for inoculum counts before autoclaving half the contents of each bottle to produce the killed controls.

Treatments included imbibition in live and killed suspensions of RD31-3A. A preimbibition seed treatment with hydrogen peroxide also was included to test whether the presence of native seedborne organisms affected colonization of seed surfaces by RD31-3A. The experiment was arranged as a 2 × 2 factorial in a split-plot design, with initial seed treatment (peroxide versus water) as the main plot and bacterial treatment (live versus killed *P. chlororaphis* isolate RD31-3A) as the subplot. There were three replicates per treatment combination.

Douglas fir seeds (seedlot DJ8002; 15-g portions in small nylon mesh bags) were soaked for 40 min in 30% H_2O_2 or sterile distilled water (three portions per treatment). After nine rinses in sterile distilled water, seeds were air-dried in a laminar flow hood. One hundred seeds from each of the six portions were plated on Komada's medium (pH 6.8) for preimbibition counts of *Fusarium* spp., and an additional 50 seeds from each sample were withdrawn for determining preimbibition bacterial populations.

The remaining seed portions were divided in half and placed in plastic bags. Seeds were imbibed on a laboratory bench for 24 h in 100 ml of live or killed bacterial suspensions. After imbibition, the seeds were drained and cold-stratified in the plastic bags for 6 weeks at 4°C, as described above. Bags of seeds were shaken every other day to maintain circulation of air and moisture. Immediately after imbibition and again after stratification, seed samples were withdrawn and air-dried in a laminar flow hood. Fifty seeds per treatment were plated on Komada's medium (pH 6.8) for postimbibition and poststratification counts of *Fusarium* spp. In addition, 50 seeds per treatment were withdrawn for determination of postimbibition and poststratification bacterial populations. Also, after stratification was complete, 100 seeds per bag were withdrawn for standardized seed germination tests.

For determination of seedborne bacterial populations, 10 air-dried seeds were placed in each of five test tubes containing 9.5 ml of sterile phosphate buffer. Each tube was

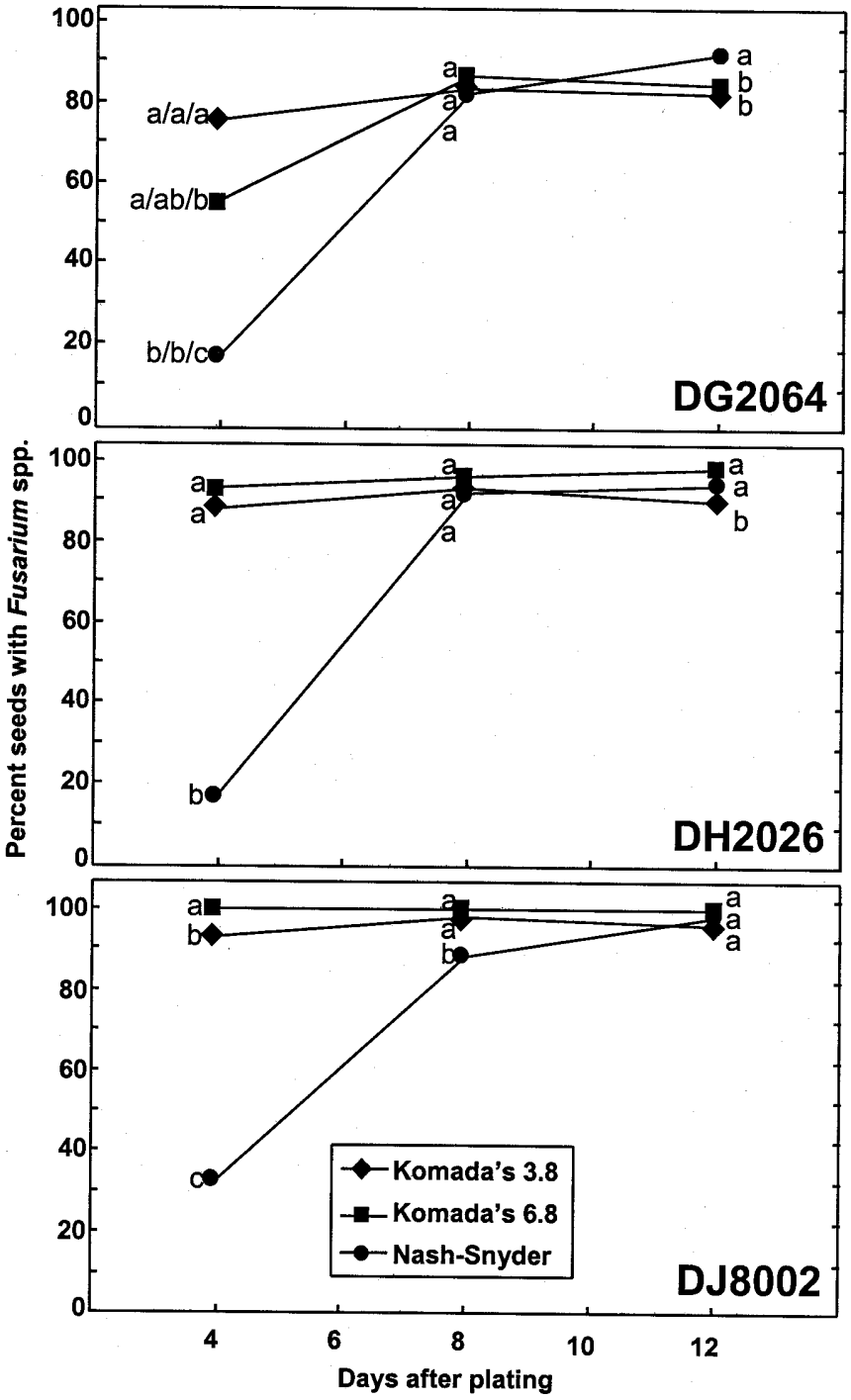

Fig. 1. Percentage of Douglas fir seeds with *Fusarium* spp. 4, 8, and 12 days after plating on three culture media. Data points represent averages of three 100-seed blocks taken from seedlots DG2064, DH2026, and DJ8002 (data from one of four blocks were eliminated from analysis). Within each seedlot and sampling date, data points accompanied by different letters are significantly different ($P < 0.05$). For seedlot DG2064 at the first sampling date, a significant block × medium interaction remained after eliminating block 2 for data analysis (see text for details). The three letters accompanying the data points are given as block 1/block 3/block 4.

vortexed for 1 min and sonicated for 5 min before making 10-fold serial dilutions. In the first trial, only populations of rifampicin-resistant bacteria were determined, and dilutions were plated on half-strength TSA amended with 100 µg of cycloheximide, 30 µg (active ingredient) of benlate, and 100 µg of rifampicin (TSA_{CBR}) per ml. In the second trial, dilutions were plated on TSA_{CBR} as well as on the same medium without rifampicin (TSA_{CB}) to determine total bacterial populations. All dilutions were plated in duplicate on each medium, and only plates with 30 to 300 colonies were counted. The dilution series resulted in detection limits of 3×10^2 to 3×10^5 CFU per seed, depending on the dilutions plated. For statistical analysis, when rifampicin-resistant bacteria were not detected on seeds, a value of half the detection limit was used.

Rifampicin-resistant bacteria were isolated from some seeds receiving killed RD31-3A. To compare the phenotypes of these bacteria to that of RD31-3A, eight colonies (representing the range of morphological variability among the colonies) were streaked on King's medium B and compared with eight colonies isolated from seeds receiving live RD31-3A. The presence or absence of fluorescence under ultraviolet light was noted. In addition, each colony was tested for the oxidase reaction (25).

The experiment was conducted twice. Analysis of variance tested for treatment effects on the proportions of seeds germinated in standard germination tests (arcsine-square root transformed) and seedborne bacterial populations (natural logs). Proportions of seeds with *Fusarium* spp. (post-imbibition and poststratification) were analyzed by weighted least squares analysis on response marginals. All statistical analyses were carried out in SAS (version 6.10, SAS Institute). Except for bacterial population data, results from the two trials were similar and thus were combined.

For poststratification germination tests, seeds were planted on the surface of Sunshine Mix Plug 5 potting medium (Fisons Horticulture, Inc., Vancouver, B.C.) in clear, plastic 13 × 13.5 × 3.5 cm germination boxes (100 seeds per box). Conditions were as specified by Association of Official Seed Analysts protocols for Douglas fir (3), except that incandescent and fluorescent lights maintained light levels at 233 $\mu E \cdot m^{-2} \cdot s^{-1}$. A seed was considered fully germinated when cotyledons were visible, and tests were terminated when no new instances of seed germination were observed (3 to 4 weeks after planting). Percent germination is reported as the average of two to four blocks of 100 seeds each. Whenever possible, fungi from aboveground lesions on dead and dying germinants were isolated and identified to species.

RESULTS

Isolation of *Fusarium* spp. from cold-stratified Douglas fir seeds. Isolation of *Fusarium* spp. from all three seedlots approached 100% on all three culture media by 12 days after plating (Fig. 1). Nine *Fusarium* species were isolated from the three seedlots (Table 1). *F. lateritium* was the most frequently isolated species on seedlot DG2064, although *F. poae* and *F. tricinctum* also were represented. Six species were isolated from seedlot DH2026; the most frequently occurring were *F. lateritium* and *F. tricinctum*. Six species also were isolated from seedlot DJ8002; *F. sambucinum* and *F. avenaceum* were most common (Table 1). The most obvious differences among the three culture media occurred in seedlot DH2026, where *F. lateritium* was isolated more frequently and *F. tricinctum* less frequently on Komada's medium at pH 6.8 than on the other two media (Table 1). Six isolates of *F. lateritium* (L-379, L-380, L-381, L-382, L-383, and L-384) and one isolate of *F. culmorum* (R-9491) originating from the three seedlots have been deposited at the Fusarium Research Center at Pennsylvania State University.

The most common genera of contaminant fungi on Nash-Snyder medium were *Gliocladium* and *Trichoderma* for seedlots DG2064 and DH2026 and *Oidiodendron* for seedlot DJ8002. On Komada's medium at pH 3.8 and pH 6.8, the most common genus of contaminant fungi from all three seedlots was *Trichoderma*.

Effect of imbibition and cold stratification on isolation of seedborne *Fusarium* spp. The percentage of seeds with *Fusarium* spp. increased steadily during cold stratification from less than 25% before imbibition to 65 to 100% after 6 weeks stratification (Fig. 2). Isolation of *Fusarium* spp. from seedlots DH2026 and DJ8002 increased in response to both imbibition and cold stratification. For seedlot DG2064, the proportion of seeds with *Fusarium* spp. was not affected by seed imbibition but increased in response to cold stratification (Fig. 2).

Pathogenicity of *Fusarium* spp. on cold-stratified seeds. Seed treatment with hydrogen peroxide removed most seedborne *Fusarium* spp. without reducing seed germination in standard germination tests (Table 2). In the pathogenicity assay, the hydrogen peroxide treatment did not affect seedling emergence or the proportion of seedlings that died after emergence (Table 2).

In the pathogenicity assay, seedling mortality was infrequent and rarely associated with *Fusarium* spp. Of the 480 seeds planted in the six experiments combined, 17 died after emergence. Of these 17 seedlings, *Fusarium* spp. were isolated from four, all of which were planted in the first experiment. *F. avenaceum* was isolated from one of those four (DH2026), whereas *F. oxysporum* was isolated from the other three (DJ8002). *Fusarium* spp. also were isolated from two control seedlings that did not die after emergence. In these seedlings, mycelium of *F. lateritium* (DG2064, first experiment) and *F. oxysporum* (DH2026, second experiment) emerged from seed coats and covered the cotyledons to which the seed coats were attached. In both cases, however, the mycelium remained confined to a single cotyledon for the final 3 to 4

Table 1. *Fusarium* species isolated from Douglas fir seeds on three culture media[z]

	Seedlot								
	DG2064			DH2026			DJ8002		
	K 3.8	K 6.8	NS	K 3.8	K 6.8	NS	K 3.8	K 6.8	NS
Species	Number of isolates								
F. avenaceum	0	0	0	2	0	0	6	8	0
F. culmorum	0	0	0	0	0	0	0	1	1
F. lateritium	16	15	13	1	8	1	2	1	2
F. moniliforme	0	0	0	1	1	0	0	0	0
F. poae	0	0	1	0	0	0	0	0	0
F. proliferatum	0	0	0	0	1	0	0	0	0
F. sambucinum	0	0	0	0	0	0	6	5	12
F. solani	0	0	0	1	3	0	0	1	0
F. tricinctum	0	0	1	9	2	14	2	0	1
Total identified	16	15	15	14	15	15	16	16	16

[z] Seeds from seedlots DG2064, DH2026, and DJ8002 were cold-stratified and plated on Komada's medium at pH 3.8 (K 3.8) and pH 6.8 (K 6.8) and Nash-Snyder medium (NS), and 14 to 16 *Fusarium* colonies per seedlot were randomly selected from each culture medium for identification to species.

weeks of the experiments, and the seedlings remained otherwise healthy.

Disease was infrequent and not quantified in seed germination tests, but occasionally fungi were isolated from diseased germinants. *F. lateritium* (DG2064 and DJ8002), *F. avenaceum* (DG2064 and DJ8002), *F. proliferatum* (DH2026), and *F. oxysporum* (DH2026) were isolated from diseased germinants in germination tests. Various other genera of fungi also were associated with ungerminated seeds and seedlings that died after emergence but did not occur consistently enough to relate symptoms to particular fungi. Fungal genera observed included *Cylindrocarpon, Gliocladium, Penicillium, Aspergillus, Trichothecium, Talaromyces,* and *Trichoderma*.

Seed treatment with RD31-3A. Live suspensions of *P. chlororaphis* strain RD31-3A averaged 1.50×10^9 CFU ml^{-1} and 2.50×10^7 CFU ml^{-1} in the first and second trials, respectively. Bacteria were not detected in killed suspensions streaked on half-strength TSA.

Data from the two trials were combined for statistical analysis and presentation of results, except that data for seedborne bacterial populations are presented for the second trial only. One reason for omitting bacterial population data from the first trial is that we counted only rifampicin-resistant (not total) bacteria in the first trial. Also, in the first trial, we did not anticipate the presence of rifampicin-resistant bacteria on control ($-H_2O_2$) seeds treated with killed bacteria, and we therefore plated dilutions containing too many colonies to accurately count. This problem was addressed in the second trial by plating a greater range of dilutions of seed washings. Where plate counts of rifampicin-resistant bacteria were obtained in the first trial, however, they were similar (within an order of magnitude) to those obtained in the second trial.

Preimbibition seed treatment with hydrogen peroxide reduced populations of native bacteria to below detectable levels, but after 24 h imbibition in live or killed RD31-3A, bacterial populations on all seeds had recovered to preperoxide treatment levels (Fig. 3). For seeds treated with live RD31-3A, however, postimbibition bacterial populations consisted primarily of rifampicin-resistant bacteria (approximately 10^6 CFU per seed), whereas rifampicin-resistant bacteria were not detected on peroxide-treated seeds imbibed in killed RD31-3A (Fig. 3). Rifampicin-resistant bacteria were, however, detected on control ($-H_2O_2$) seeds imbibed in killed bacteria (Fig. 3; approximately 10^4 CFU per seed). By the end of cold stratification, total bacterial populations had increased by about 10-fold relative to postimbibition levels, although populations of rifampicin-resistant bacteria in most treatment combinations did not show a corresponding increase (Fig. 3). The exception is that a population (approximately 10^4 CFU per seed) of rifampicin-resistant bacteria developed during cold stratification on peroxide-treated seeds imbibed in killed bacteria (Fig. 3). Total bacterial counts did not differ significantly among treatments after imbibition ($P = 0.2011$) or after stratification ($P = 0.3390$).

To determine whether the rifampicin-resistant bacteria isolated from seeds treated with killed RD31-3A were a result of native bacteria or contamination with live RD31-3A, 16 bacterial colonies isolated from seeds imbibed in live and killed RD31-3A were further examined. All eight colonies from seeds imbibed in live bacteria resembled RD31-3A on King's medium B (colony color, shape, and growth rates) and matched the RD31-3A phenotype of fluorescence under ultraviolet light and a positive reaction in the oxidase test. In contrast, none of the eight colonies from seeds imbibed in killed bacteria resembled colonies of RD31-3A on King's B, and only one matched the fluorescence/oxidase-positive phenotype. All rifampicin-resistant bacterial colonies isolated from seeds imbibed in killed bacteria were smaller and appeared much later on rifampicin-amended culture medium than RD31-3A.

Isolation of seedborne *Fusarium* spp. was reduced by hydrogen peroxide treatment immediately after seed imbibition and after cold stratification ($P < 0.0001$; Table 3). Seed imbibition in live bacteria did not significantly affect isolation of seedborne *Fusarium* spp. immediately after imbibition ($P = 0.8884$), but reduced post-stratification *Fusarium* populations regardless of peroxide treatment ($P <$

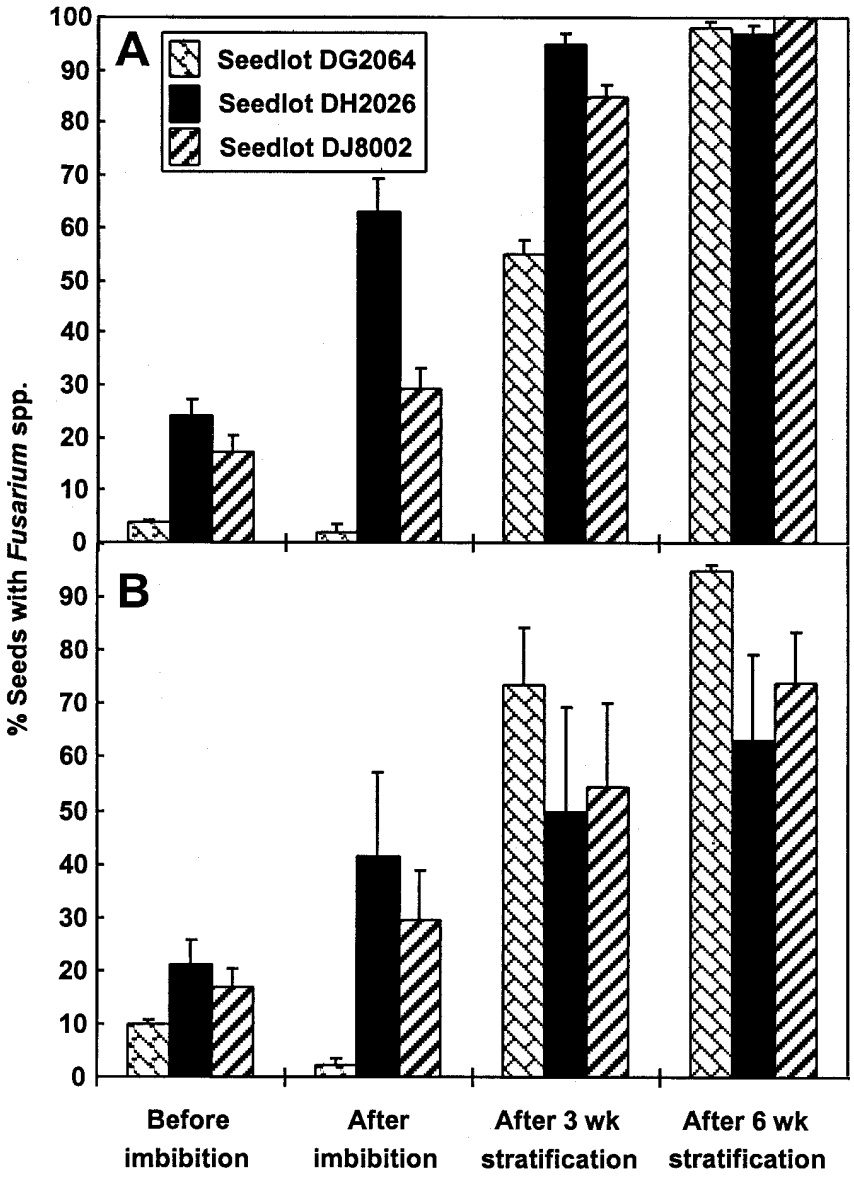

Fig. 2. Percent Douglas fir seeds with *Fusarium* spp. during different stages of seed imbibition and cold stratification. Bars represent averages of four 100-seed blocks taken from seedlots DG2064, DH2026, and DJ8002. Error bars represent one standard deviation. **(A)** First experiment. **(B)** Second experiment.

0.0001). The greatest reduction in poststratification populations of seedborne *Fusarium* spp., however, was achieved when preimbibition peroxide treatment was followed by seed imbibition in live RD31-3A (Table 3).

Seed germination was not significantly affected by either preimbibition peroxide treatment (P = 0.0646) or bacterial treatment (P = 0.0821). The overall seed germination rate was 82% for the combined trials.

DISCUSSION

The results of this study confirm that diverse *Fusarium* spp. occur on Douglas fir seeds and that seedborne populations of *Fusarium* spp. can increase greatly during seed imbibition and stratification. Prestratification hydrogen peroxide treatment combined with the application of a bacterial biological control agent during imbibition and stratification can substantially reduce the proliferation of seedborne *Fusarium* spp. without reducing seed germination.

Although delivery of fluorescent pseudomonads to sweet corn seeds during "biopriming" (seed hydration combined with application of bacteria) has been demonstrated in previous studies (9,35), to our knowledge this study represents the first time bacteria have been introduced to Douglas fir seeds during seed imbibition and cold stratification. Bacterial seed treatment during imbibition, particularly after surface-sterilization, holds promise as a method for delivery of biological control agents to Douglas fir seeds. Although RD31-3A failed to protect Douglas fir seedlings against soilborne *F. oxysporum* in preliminary greenhouse studies (data not shown), other bacterial strains may be found that can reduce infection by both seed- and soilborne *Fusarium* spp. Also, further research is needed to determine whether RD31-3A or other biological control agents can be maintained on seeds during poststratification seed storage.

Our results reinforce previous reports that cold stratification can promote the proliferation of *Fusarium* spp. on seeds (4). Since

Table 2. Effect of poststratification seed treatment with hydrogen peroxide on isolation of seedborne *Fusarium* spp., seed germination in standard germination tests, and seedling emergence and postemergence mortality in the pathogenicity assay

	Seedlot					
	DG2064		DH2026		DJ8002	
	Exp. 1 (%)	Exp. 2 (%)	Exp. 1 (%)	Exp. 2 (%)	Exp. 1 (%)	Exp. 2 (%)
Seeds with *Fusarium* spp. (poststratification)						
H_2O_2	4 (90)[y]	0 (119)	0 (90)	5 (150)	2 (90)	0 (90)
Control	92 (90)	68 (120)	94 (90)	33 (150)	23 (39)	100 (90)
Germination in standard germination test						
H_2O_2	50	47	93	93	87	85
Control	47	48	83	91	82	89
Emergence (out of 40 seedlings planted)						
H_2O_2	58 a[z]	40 a	100 a	80 a	93 a	95 a
Control	53 a	60 a	93 a	90 a	95 a	95 a
Postemergence mortality (% of emerged seedlings)						
H_2O_2	0 a	6 a	0 a	0 a	19 a	5 a
Control	0 a	0 a	3 a	6 a	8 a	3 a

[y] Numbers in parentheses represent numbers of seeds plated.

[z] For emergence and postemergence mortality, values within each seedlot and experiment followed by the same letter are not significantly different (chi-square test, $P < 0.05$).

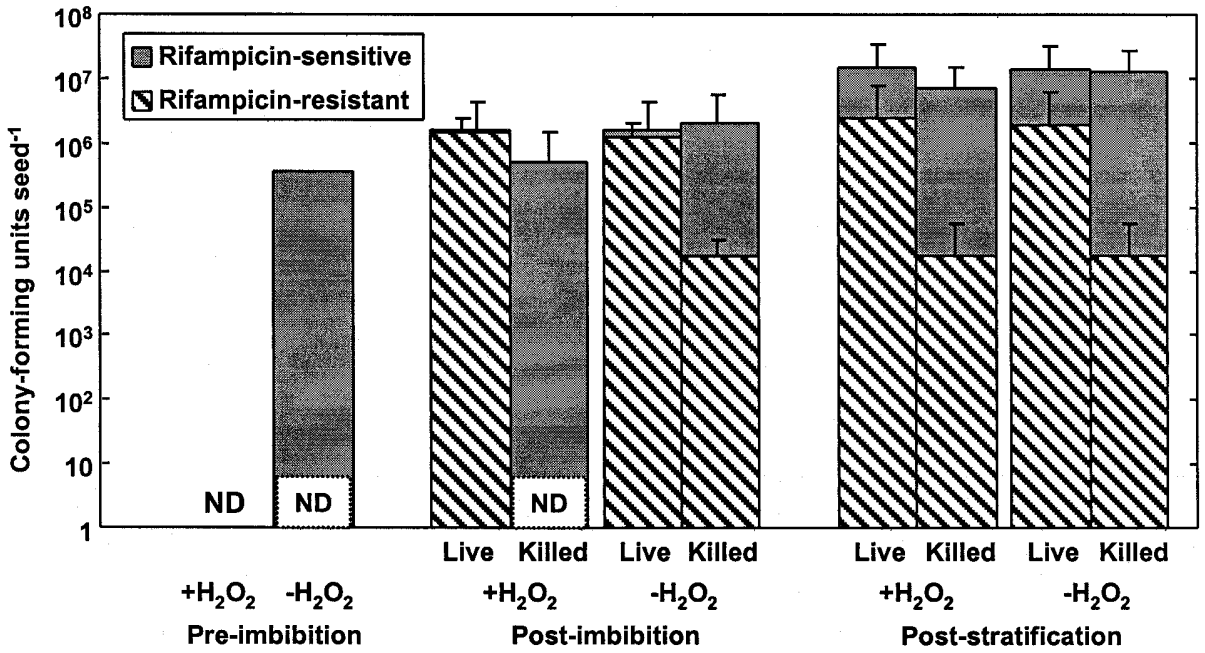

Fig. 3. Bacteria (total and rifampicin-resistant) isolated from Douglas fir seeds before and after imbibition in live or killed *Pseudomonas chlororaphis* strain RD31-3A, and after 6 weeks cold stratification. Seeds were treated with hydrogen peroxide or water before imbibition. Data are shown for second trial only. ND = rifampicin-resistant bacteria not detected (detection limit = 3,000 CFU per seed). Error bars represent upper limits of 95% confidence intervals for total and rifampicin-resistant bacteria.

different *Fusarium* species are likely to react differently to stratification conditions, better techniques for quantifying seed colonization are needed to determine why this proliferation occurs. The practical importance of this proliferation is unknown, however, because the evidence from this and previous studies suggests that high levels of seedborne *Fusarium* spp. do not necessarily predict high levels of disease in seedlings (4,22), even if individual isolates can cause disease. Since environmental conditions in our growth chamber assay were known to be conducive to disease (M. H. Hoefnagels and R. G. Linderman, *unpublished*), it is possible that the *Fusarium* strains present on our seeds were not virulent. Different isolates of a given *Fusarium* species vary in virulence (7,8,20), and avirulent isolates of *Fusarium* spp. have been shown to protect other crops from pathogenic strains (1,14,33). The possibility that abundant nonpathogenic strains of seedborne *Fusarium* spp. may protect seedlings against pathogenic strains present on seeds or in soil warrants further investigation.

Among the nine species of *Fusarium* isolated from cold-stratified Douglas fir seeds taken from three seedlots, seven (*F. avenaceum*, *F. lateritium*, *F. poae*, *F. sambucinum*, *F. tricinctum*, *F. proliferatum*, and *F. moniliforme*) have been previously reported on Douglas fir (4,21). Among the other species found here, *F. solani* has been found on seeds of eastern white pine (32), longleaf pine (34), and slash pine (17); *F. culmorum* has not been previously reported on conifer seeds.

Also notable is the apparent absence of *Fusarium* species that have commonly been found on Douglas fir seeds by previous workers, particularly *F. oxysporum* (4,13,18,21) and *F. acuminatum* (4,18,21). In spite of the absence of *F. oxysporum* from our culture plates, however, this species was isolated directly from seedlings in the pathogenicity and germination tests and from water drained from seeds after imbibition (data not shown). *F. oxysporum* also was previously isolated from stratified seeds of seedlot DJ8002 plated on Komada's (pH 6.8) (data not shown). It is possible that *F. oxysporum* growing from seeds on our culture plates was masked by other, more abundant or faster growing species. More intensive sampling of all fungi (rather than the single most prominent fungus) growing from each seed might reveal whether any species were systematically excluded by the methods used in this experiment.

The presence of internal seedborne *Fusarium* spp. in the seeds used in this study is strongly suggested by the fact that *Fusarium* spp. could be isolated from some seeds that were treated for 40 min in 30% peroxide, supporting previous findings (6,13). Also, in preliminary studies, we isolated *Fusarium* spp. from 4% of internal seed contents aseptically dissected from stratified seeds (data not shown). Internal seedborne *Fusarium* spp. may be especially difficult to eradicate in seed sanitation programs.

In our three seedlots, the three culture media used did not strongly affect the isolation of seedborne *Fusarium* spp. However, contaminant fungi interfered to some extent with our ability to distinguish *Fusarium* spp. on all three media tested. Colonies of contaminant fungi were usually less prominent on Komada's medium adjusted to pH 6.8 than on the other two media, reinforcing previous reports that plating seeds on Komada's medium at pH 7.0 results in greater *Fusarium* visibility and fewer contaminant fungi than when seeds are plated on Komada's at pH 4.0 (4).

Taken together, these results confirm that *Fusarium* spp. proliferate during cold stratification but also show that this effect can be reduced if seeds are imbibed and stratified in the presence of bacterial biological control agents. Although RD31-3A did not protect seedlings from soilborne *F. oxysporum* in preliminary studies (data not shown), other biological control agents may inhibit both seed- and soilborne *Fusarium* spp. With additional research, conifer seed imbibition in suspensions of beneficial organisms may provide multiple benefits: eradication of seedborne pathogens, subsequent seed-surface colonization by the beneficial organisms, and protection of the developing seedling from soilborne pathogens.

ACKNOWLEDGMENTS

We thank Weyerhaeuser Corporation for supplying the Douglas fir seeds used in this study. We also thank Paige Axelrood and B. C. Research Inc. for providing RD31-3A. We thank Jean Juba and the late Paul Nelson of the Fusarium Research Center at Pennsylvania State University for identifying selected *Fusarium* isolates, and Bruce Mackey (USDA Biometrics) and Cliff Pereira (Oregon State University) for help with statistical analysis. Finally, we appreciate helpful reviews of early versions of this manuscript by Paige Axelrood, Douglas Gaffin, Willis Littke, Joe Marlow, Cynthia Ocamb, Jeff Stone, and Yasu Tanaka.

Table 3. Effects of preimbibition treatment of Douglas fir seeds with 30% hydrogen peroxide and seed imbibition in live or killed suspensions of *Pseudomonas chlororaphis* strain RD31-3A on percentage of seeds (±95% confidence interval) with *Fusarium* spp.

		Seeds with *Fusarium* spp. (%)	
Time of plating	**RD31-3A**	**$-H_2O_2$**	**$+H_2O_2$**
Preimbibition	…	16.0 ± 3.2	0.3 ± 0.9
Postimbibition	Live	8.3 ± 3.2	1.0 ± 1.1
	Killed	8.3 ± 3.2	0.7 ± 0.9
Poststratification	Live	25.7 ± 4.9	0.7 ± 0.9
	Killed	69.3 ± 5.3	17.7 ± 4.3

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