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Title FUNGI ASSOCIATED WITH DOUGLAS-FIR SEED DURING CONE DEVELOPMENT, SEED PROCESSING, AND STORAGE

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The Pacific Coast form of Douglas-fir in natural forest succession is an intermediate species thriving in the Coast Range and Cascade Mountains of the Pacific Northwest. This species constitutes 26 percent of the standing timber in the United States, and 24 percent of the nation's annual timber harvest. Characteristics of the timber type and its management in addition to utility are: an irregular seeding habit; clear-cutting as the popular harvest practice; and the rapid intrusion of brush into cut-over areas to the detriment of tree regeneration. These factors demand immediate and successful restocking of deforested areas. Such restocking of cut-over lands with the desired Douglas-fir requires a constant supply of quality seed.

Successful seed storage resulting in minimum subsequent reduction of vitality of seed and seedlings may be more nearly achieved through fuller knowledge of the effects of storage on the

fungi normally occurring in the seed and on their activities. This study was undertaken to disclose and identify fungi occurring on seed of Douglas-fir, and to determine the effects of different common storage treatments on the seed microflora. To this end, cones collected from four Douglas-firs on the eastern periphery of the Coast Range in Oregon were briefly soaked in a five percent solution of Clorox prior to storage. A similar group was untreated. Seed were extracted from both groups at intervals and stored at different temperatures. Periodically, stored seed were plated on potato dextrose agar following surface sterilization in 30 percent hydrogen peroxide. Fungi growing from the seed were identified and tabulated.

Twenty-six species were identified, including a number of known seed pathogens. Occurrence of the five most prevalent isolates was analyzed statistically. All were found to become significantly more numerous with delay of extraction of seed from the cones following harvest. These organisms decreased numerically with time of storage at room temperature, but never reached the level found in promptly extracted seed. However, when seed was stored in the refrigerator at minus 16°C, the fungal flora was soon reduced below the level occurring in the promptly extracted seed of the controls. This was true of seed held for both short and long periods of storage at room temperature, preceding refrigeration. Prevalence of different organisms varied widely between trees, but differed little

between filled and empty seed. Both Clorox treatment of cones and detergent treatment of seed, preceding sterilization, contributed to a reduced fungal flora. Many of the fungi became associated with the seed very early in cone development, and fungal floras differed little between extracted and naturally shed seed.

The following conclusions were reached. Refrigerated storage reduces the fungal flora of mature seed. Douglas-fir seed should be artificially extracted from the cones immediately after harvest. Additional investigation of surface sterilization of cones prior to seed extraction is warranted. Sterilized seed for experimental purposes can be readily obtained by a combination of refrigerated storage, detergent soak and hydrogen peroxide sterilization. Knowledge of the effects of fungi in seed during and after storage is needed in order to determine the desirability of seed sterilization prior to storage.

Fungi Associated with Douglas-Fir Seed During Cone Development, Seed Processing, and Storage

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FUNGI ASSOCIATED WITH DOUGLAS-FIR SEED DURING CONE DEVELOPMENT, SEED-PROCESSING AND STORAGE

I. INTRODUCTION

Douglas-fir, Pseudotsuga menziesii, (Mirb.) Franco, is one of five major softwood lumber trees in the United States. Most of the forested area containing this species lies in the Coast Range and the west side of the Cascade Mountains in Washington and Oregon. Douglas-fir constitutes approximately 26 percent of the standing timber in the nation, and about 24 percent of the annual timber cut (58, p. 55). This natural resource can be sustained at the present level of productivity only by prompt regeneration of harvested areas with trees of the appropriate species (59).

The environment of a forest site is greatly changed when timber is removed. Natural reforestation often is slow and as a consequence artificial methods of regeneration are necessary to give advantage to the preferred tree species and to retain productivity (59).

Clearcutting has been and continues to be the most employed cutting practice in the Douglas-fir region. This technique eliminates any seed-source except from those remaining trees outside the cutting boundary and is a practice not always nor everywhere conducive to natural regeneration. Artificial restocking, consequently,

frequently is necessary. To accomplish this, planting of seedlings has been favored in the past, but in recent years aerial seeding has become more popular. In either case, an abundant supply of high quality seed is required.

About 60,000 acres of commercial timberland is clearcut annually in the Pacific Northwest, and about 80 percent of this area is either directly seeded or planted the year of harvest. The direct seeding of de-forested land requires 8,000 to 40,000 seeds per acre when conditions are favorable to reestablish a stand of trees at a desirable density. The results of the first efforts of reforestation are not always satisfactory and reseeding is frequently necessary. The total amount of seed needed to satisfy these requirements is about 20,000 pounds annually in the Pacific Northwest (59).

Douglas-fir produces an abundant seed crop at intervals of two to four years (20, p. 158). Production in intervening years is poor or none (45). Irregular seed production, the importance of prompt restocking after harvest - for both ecologic and economic reasons - and the large amount of seed required, emphasizes the importance of ready availability of seed from storage. Clearly, an understanding of storage relationships and of factors influencing seed quality both before and during storage is desirable.

Commercial seed companies and governmental agencies store coniferous seed to be used during years of poor production. Storage

methods to retain viability have received considerable attention, but little attention has been paid to the effect of storage practices on the microflora of coniferous seed. Such relationships have been extensively studied on seed of agricultural crops to good advantage.

Storage problems of agricultural seed, collection and cleaning procedures designed to avoid seed injury, and seed-borne diseases, have all been investigated in some detail. Certificates of freedom from seed-borne diseases have been possible in many cases. Such control has not been developed for coniferous seed, except in rare cases of seed for export.

Viability of Douglas-fir seed during the storage period between maturation on the tree and a subsequent time of germination, may be enhanced by an understanding of the microfloral associations on and in the seed. Investigations of the metabolic activity of coniferous seed during dormancy have defined the best storage conditions for maintenance of quality. The preferred times of cone collection for maximum seed viability are well defined (51, 35), as are preferred methods for drying and dewinging the seed (35).

With respect to agricultural seed, Malone (33, p. 177) states "there is now hardly any cultivated crop where at least one seed-borne fungal parasite is not known." Coniferous seed is similar to agricultural seed in the many saprophytic organisms associated with it. It differs, however, in that recognized seed pathogens are

not known to occur with regularity (11, p. 69). However, sometimes destructive organisms usually known as saprophytes have been found to attack emerging radicles of coniferous seed. These fungi may also play a role in the deterioration of coniferous seed in storage, similar to the role of storage organisms detrimental to cereals (8). Fungal inhabitants of seed, although not directly pathogenic may damage stored seed by releasing toxins (33), inhibiting germination (60, 11) or conversely aiding the nutrition of the developing embryo (34), or even competing actively with soil-borne pathogens to benefit the germinating seed (55).

Malone (33, p. 105) states that by "declaring the actual amount of contamination present in a seed sample, the greatest risk of disease is known should the seed be sown under conditions which allowed the parasite to launch a successful attack." The purpose of work reported here is three-fold: 1) to identify the fungi closely associated with Douglas-fir seed, 2) to report relative occurrence of these organisms within or on the seed during the course of its development, and 3) to compare the effects of certain storage conditions on seed microflora.

II. MATERIALS AND METHODS

The Seed Collection Area

Cones and seed for this study were collected four miles north of Corvallis, Oregon, along the eastern edge of Oregon State University's McDonald Forest. This area is on the eastern foothills of the Coast Range where natural Douglas-fir forest borders on the agricultural Willamette Valley in Benton County, Oregon. The area has a population density characteristically rural. All trees providing cones were near a paved road, and were growing in pastures, fence rows, or on residential property. However, blocks of timber were within 20 chains of each tree selected for cone collection.

Media Employed

Potato dextrose agar, formulated following the recommendations of Lacy and Bridgman (26), using dehydrated mashed potatoes, was used throughout the study. This medium is hereafter referred to as PDA. Instead of straining the medium to clarify it before pouring plates or bottles, as was recommended in the recipe, the medium was allowed to settle and the sediment-free portion was decanted as the plates were poured. Approximately 20 ml of medium was used per plate or bottle. Although PDA was used for general culture work, developing fungal colonies were transferred to other

media to aid identification of certain species as the keys required (54, 5). Media other than PDA also were used to induce fruiting of refractory isolates as an aid to identification.

Collection and Treatment of Immature Cones for Examination of Associated Microflora

Before any bud scales had broken or any visible cone abortions had taken place, ten trees producing a large number of "female buds" were selected in the seed production area. From each of these, five immature cones were picked at intervals beginning March 23, 1964, and continuing until cone maturity in the fall. At each sampling time cones from each tree were taken to the laboratory in a sterile glass jar, promptly surface sterilized, dissected and plated, to culture microorganisms present. Colonies developing appeared to arise from tissue in close association with the developing seed. Fungi not easily identified upon isolation were transferred and stored at room temperature until there was opportunity for identification.

Female strobili were first collected while still in the bud. Bud-scales were aseptically removed and the naked bud was immersed in 30 percent hydrogen peroxide[1] for five minutes. The buds

[1] Produced by Baker and Adamson, General Chemical Division, assayed to be of 29 to 32 percent concentration. Hydrogen peroxide of this concentration must be used with great caution. Addition of any fast oxidizing material to the solution may cause explosive boiling and splattering. The material is highly caustic to the skin and particularly dangerous to the eyes. Rubber gloves and goggles are recommended where quantities are used.

were rinsed for 20 minutes in sterile distilled water, split longitudinally into quarters, plated on PDA and incubated at room temperature for ten days. Organisms developing during this period were recorded and identified. Cones appearing to be aborting at time of collection as indicated by darkened central tissue were discarded. Later, when a stage of maturity was reached in which the seed attained approximately one half the size of mature seed, the intact cones were immersed for five minutes in a 25 percent solution of Clorox[2] and rinsed for 20 minutes in running tap water. Then, with a sterile knife, 20 seeds were excised from each cone. The excised seed were rinsed during five minutes of vigorous shaking, four times in succession in 100 ml of sterile distilled water. Five one ml samples from each rinse were spead uniformly over the surface of individual innoculation bottles and incubated at room temperature. After seven days the number of colonies, their preliminary identification, and certain macroscopic characterisitics of each were recorded.

After the fourth sterile water rinse, the seeds were surface sterilized one minute in 30 percent hydrogen peroxide, rinsed twice in sterile distilled water, and plated four per plate on PDA to culture

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[2] The solution was composed of one part Clorox, a commercial solution containing 5.35 percent sodium hypochlorite and three parts sterile distilled water.

any organisms present. Records were made after seven days at room temperature.

Mature Cone Collection

In September of 1964, cones were collected from each of four trees located in the area described earlier. Seed in these cones was all mature, but the cones of trees numbered 3 and 4 were green and tightly closed, while those of trees 1 and 2 were partially dried and beginning to shed seed. Collection of the latter was completed just prior to natural seed release. The cones were hand picked and dropped to the ground. Six hundred cones picked as nearly as possible at random from the upper crown of each tree were gathered in a burlap bag. Each bag was identified by a plastic tag and wired closed for delivery to the storage area for subsequent treatment. On three of the trees from which the cones were collected, and one from which no cone collection was made, cone-bearing branches were covered with burlap bags, and the seed was allowed to shed naturally into the bags. Seed from these bags was collected in October, dewinged, cleaned and stored in the same manner as seed collected in the cones.

Pretreatment and Storage of Seed-bearing Cones

Within 24 hours after the cones were picked, those from each

of the four trees were divided into two lots of 250 to 300 cones each, and put into burlap bags. These eight bags were labeled, fastened with twine, and those of one lot were immersed in a five percent solution of Clorox for one hour. The bags were hung up to drain for 20 minutes before they were stored on the concrete floor of the storage shed along with the untreated cones. The shingle-roofed, wood-sided storage shed sheltered the cones from rain but relative humidity and temperature were comparable to those outside. This type of storage is representative of the treatment that Douglas-fir cones commonly encounter in commercial practice prior to seed extraction.

Seed Storage Treatments

Cones and seed received the treatments in Table 1. A 25 cone sample from each of the eight lots of 250 cones was dried and the seed extracted at once as described hereunder ("control" in Table 1). Additional 25 cone samples were taken from each of the eight lots of stored cones after one week, three weeks, and eight weeks. Extracted seed from each sample of cones were dewinged and cleaned for seed storage. Four samples of 25 seeds each were taken from each seed lot within 24 hours of extraction. These seeds were surface sterilized and plated on PDA for micro-floral assay. The remaining seed from each lot was divided in half. One-half was

Table 1. Treatments applied to cones and seed from four Douglas-fir trees prior to microfloral assay.

Tree No. and seed characteristics	Cone treatment	Cone storage	Seed storage	Samples of 25 seed plated
	Chemical	Weeks	Weeks	Number
Hand picked and stored				
A*	Hypochlorite dip*	1*	0*	4
B		3	3	
C	Untreated*	8	10**	
D				
Hand picked, immediate extraction (Control)				
A*	Hypochlorite dip*	0	0	4
B				
C	Untreated			
D				
Hand picked, refrigerated				
A*	Hypochlorite dip*	0	10	4
B				
C	Untreated	3	22	4
D				
Naturally shed				
A	No cone treatment	No cone storage	No* seed storage	4
B				
C				
E				

* Each item in noted column received treatments of succeeding column.

** Occurred only in the three week cone storage sample.

stored in loose capped, 100 ml bottles at room temperature. The other half was stored at -16° C, a refrigeration temperature commonly employed in commercial seed storage ("refrigerated seed" in Table 1). Seed stored at room temperature was assayed for microflora after three weeks. Refrigerated seed was assayed after 10 and 22 weeks. The seed that was naturally discharged into burlap bags was assayed for microflora immediately after collection.

Seed Extraction from Stored Cones

Stored bags of cones were sampled by removing 25 cones, three to five at a time, from different levels within each of the eight bags. The remaining cones within the bags were disturbed as little as possible during the sampling. Each of the cone samples was put into a clean paper bag for drying and seed extraction. A ventilated oven set at 42°C was employed to dry the cones to ease seed removal. The cone samples were dried at 42°C for 72 hours, simulating the recommended practice of commercial cone drying (45, p. 223). Cone samples were removed from the oven once for rearrangement in order to facilitate more uniform drying and easier seed extraction. Each opened cone was inverted and tapped on a hard surface to remove the still partly encumbered seed. Seed that could not be freed in this way were loosened by holding the base of the cone and twisting the distal portion. Additional tapping usually

released remaining seed.

After extraction, the seed was dewinged by rolling them gently between the palms of the hands with enough pressure to just maintain the seed's position. This manual method was employed because of the impracticality of using a commercial dewinger on such small quantities of seed and because it was felt that there would be less damage to the seed by hand dewinging. The dewinged material was put through a seed blower (52) adjusted to remove most of the chaff and none of the seed. This level of cleaning differs from the customary procedure of allowing the empty seed to blow away with the chaff (45). The reasons for retaining the "empty seed" are considered later.

Culture of Microorganisms from Mature Seed

The extracted mature Douglas-fir seed were plated and incubated on PDA to reveal associated fungi. Ten seeds were distributed evenly over 15 ml of medium following the Ulster method (37) for assaying pathogens on flax seed. Four random samples of 25 seeds each from each seed lot were immersed in 30 percent hydrogen peroxide for 10 minutes, rinsed for 10 minutes in sterile distilled water, and plated on 15 ml of PDA. No obviously broken or injured seed was used, but small and light seed were included. Plates were incubated for 10 days at room temperature on a lighted

shelf. Intermediate observations were made at three and seven days. At each observation the origins of visible colonies arising from the seed were marked. Colonies of fast-growing Trichoderma Pers. ex Fr. and Rhizopus Ehrenb. ex Corda that were distinguishable at earlier observation dates, and which would overrun slower growing fungi on the plates, necessitated early isolation of the latter. Because of the numerous organisms inhabiting the seed after sterilization, it seemed probable that some slow-growing fungi were suppressed after there was no longer fresh media for them to colonize. It was felt that a more penetrating sterilization treatment might change the order of prevalence of the microflora previously observed. To attempt detection of any slowly developing fungi, beginning with the 8-3[3] seed lot, 50 seeds were soaked for 10 minutes in a solution of 10 gm of commercial detergent and 90 ml of distilled water. Seed were then soaked for 10 minutes in sterile distilled water before being treated with the hydrogen peroxide and the subsequent sterile water rinse.

Determination of Seed Quality

One measure of the quality of coniferous seed is the percent

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[3] This notation refers to a storage treatment of eight weeks of cone storage and a subsequent three weeks of seed storage. This form will be used throughout the remainder of the paper when a particular treatment is referred to.

filled seed in the sample. Seed-coat development in Douglas-fir is independent of fertilization, and non-fertilized seed forms a hard coat which maintains the outward appearance of viable seed. These infertile seed have a flattened or missing endosperm and can be differentiated from sound seed by dissection in a "cutting" test (38, p. 10). In normal seed processing most of the light and empty seeds are removed with the chaff during cleaning operations. In this experiment, however, the empty seed was intentionally retained. It was hoped that a comparison of microfloral populations on empty seed as compared with filled seed might provide an additional clue as to the part played by the endosperm as a substrate for seed pathogens as reflected by differential colonization by organisms. The seed were bisected and those with a complete endosperm counted to determine the percentage of filled seed. "Cutting tests" provide an estimate of the interior condition of the seed and are widely used as a preliminary estimate of germination quality in the field (38, p. 17) (27). Tests were run on five random samples of 100 seeds each from each tree and some of the seed plated for assay of microflora were subsequently cut for endosperm examination. (The cutting test disclosed no evident differences in quality between naturally shed seed and seed extracted artificially from cones of the same tree.)

Terminology

It becomes apparent from reviewing the literature that usage of the term "infected" and "infested" is confused. The confusion appears to arise from lack of understanding of 1) the developmental state of the organisms at the time of isolation or 2) activities of the organisms within the seed during their presence there.

The dictionary meaning (62) of "infect" is "to contaminate with a disease producing substance." Walker (61, p. 6) follows this meaning in that the term "...does not imply the production of disease symptoms." Ainsworth & Bisby (1), on the other hand, defines the term "infect" to mean "...to enter and establish a pathogenic relationship with an organism (host)." To "infest" (62) is to spread in or over in a troublesome manner or live in or on as a parasite." Because the manner in which these fungi, largely "saprophytes," affect seed is not yet adequately understood, there is no assurance that either of these terms is entirely accurate. The term "perthophyte" as used by Cartwright & Findlay (6, p. 67) for fungi decaying the heartwood of living trees--which is generally similar to the dead parts of living seed--may be more appropriate.

Until the specific activity of the organisms isolated from Douglas-fir seed is known, decision as to the correct designation in each case will remain unsettled. The term "infect" will be used throughout this work because some of the organisms were seen to invade the seed during the formative stages.

III. LITERATURE REVIEW

Organisms Commonly Found on Agricultural Seed

Both saprophytic and pathogenic microorganisms have been found in the seed-coats and in the endosperm of seed of agricultural crops. Observations of these fungi and bacteria by numerous workers has led Malone and Muskett to state (33, p. 179) "there is now hardly any cultivated crop where at least one seed-borne fungal parasite is not known." Pathogenic organisms associated with the seed of agricultural crops have been extensively investigated but the role of saprophytes has received little attention. Recently Andersen and Leach (2, p. 453) stated "viability of seeds in storage may be reduced by molds, which are unimportant in the field but may attack dormant seeds." This view is illustrated by the studies of destructive action of normally saprophytic fungi during the storage of cereals (8, 9, 39, 57). Several species of Aspergillus Mich. ex Fr. were found to be damaging. Aspergillus also was found to damage pea seed (13) and peanuts (64) in storage.

Species of Alternaria Nees ex Wallr. represent another common genus found on seed of many plants as a saprophyte, but these may be weakly pathogenic. Alternaria is particularly associated with cereals and grasses (23, 57, 25). Fulton, Bollenbacher and Templeton (17) found that species of Alternaria on cotton produce

a metabolite that causes chlorosis of the cotyledons of developing cotton seedlings. Harris and Ellett (21) isolated a species of Penicillium Link ex Fr. from diseased soybean plants that reduced germination and deformed soybean seedlings when applied to the seed. Leach (29) mentions that germinating legume seed can be attacked by various saprophytic organisms during germination tests. Accuracy of the tests may be affected.

Organisms on Coniferous Seed

Shea (49) isolated more than 40 different fungi from Douglas-fir seed in 1960. Eighteen species of saprophytic fungi were able to destroy the seed. Among those most commonly found were members of the Penicillium oxalicum series, the Aspergillus niger and A. flavus groups, Thamnidium Link ex Wallr., Pullularia Berk. = Aureobasidium, Trichoderma, Trichothecium Link ex Fr. (Shea, 48), Rhizopus and Mucor Mich. ex Fr.

Timonin (55) isolated Alternaria, Aspergillus, Cephalosporium Corda, Chaetomium Kunze ex Fr., Cladosporium, Link ex Fr., Cylindrocarpon Wollenw., Fusarium Link ex Fr., Gliocladium Corda, and Penicillium from seed of Pinus banksiana Lamb., P. contorta Dougl. and Picea glauca (Moench) Vass. He also reported observing symptoms of the known pathogens Pythium Pringsh., Phytophthora deBary and Rhizoctonia D. C. ex Fr. although these organisms were

not isolated and identified from the seed.

In 1941 Fisher (13) observed nurseries where sown coniferous seed failed to germinate. Soil conditions were apparently satisfactory and damping-off fungi appeared not to be appreciably active. He noted that these failures were more common in cold wet soils where emergence was slow. Seed that gave a good germination test in the laboratory failed in the field to appear above ground and subsequently were found to be rotted. From these observations he suspected that organisms other than damping-off fungi may decay seed. The organisms he successfully isolated from the decayed seed of Pinus and Picea were predominantly saprophytes. The following reduced germination without visible evidence of decay: Aspergillus, Alternaria, bacteria, Botrytis Pers. ex Fr., Cephalosporium, Cladosporium, Fusarium, Helminthosporium Link ex Fr., Mucor, Penicillium, Rhizopus, Rhizoctonia, and Pestalozzia de Not. Fisher also found that Aspergillus niger, some species of Penicillium, and Pestolozzia occasionally destroyed the radicle in tests on germinating seed. Gibson (19) reported pine seed losses from seed-borne saprophytes penetrating injured seed-coats. Losses decreased with increased germination rate. Leach (30, p. 178) says that "other factors being constant, the relative growth rate of the host and pathogen determine to a considerable degree the severity of the pre-emergence damping-off infection at different temperatures."

Porter (42) concurred with Gibson that coniferous seed with seed-coat injuries allowed ready entrance to fungi. He found, as did Fisher and also Harris and Ellett, the latter working with soybeans, that species of Penicillium reduced seed germination. He presumes this damage to result from organisms depleting the food supply of the embryo, or producing toxins detrimental to the seed. Hartley, Murill and Rhoads (22) mentioned as early as 1918 that dormant conifer seed was sometimes killed by saprophytic fungi. Their observations were based on seed of Pinus banksiana seriously molded by species of Penicillium. Dorogin (11) in 1923 listed the principal organisms he identified on fir seed and Prisjáznjuk (40) in 1960 analyzed seed of pine and spruce to determine when, to what extent, and by which organisms these seed were infected. He found mostly saprophytes of which Penicillium glaucum was the most common. Contrary to the apparent significance of the findings reported above, Baker (3, p. 220) felt that the losses of coniferous seed from fungi was unimportant. Shea (49, p. 1), however, remarks that "Molds are a major hazard to Douglas-fir seed between harvest and sowing."

Effects of Microflora on Coniferous Cones

In 1960, Shea (49) observed that increased mold counts of the Douglas-fir cones indicated a reduction of seed viability within the cone. Seed viability was greater in seed from cones dipped in a

fungicide than in the seed of untreated cones. Prisjaznjuk (40) in work that possibly sheds light on Shea's findings, found no seed infection in unopened cones of Scots pine on the tree, but infection began as the cones started to open, and finally 100 percent of the seed was infected. Salisbury (46) concurs generally with the portion of Prisjaznjuk's findings involving the absence of microorganisms in tightly closed cones when he found 93 of 108 seeds free of organisms. Shea (47) reports that molds may invade cones and seed through insect punctures and galleries in the cones. He also found saprophytic organisms on the cone scales while the cones were still attached to the tree, and there was a relatively close association of the fungi with the seed at this time. Shea (47, 49) reported the scales of drying cones to develop a heavy growth of mycelium including Penicillium, Pullularia, a fluffy white and a dark appressed hyphomycete. He found that these molds developing on cones in storage increased the difficulty of seed extraction. The fungi he isolated from cones during this period were Penicillium, Trichoderma, Aspergillus, and Schizophyllum Fr. Schizophyllum formed a white mycelial mat over the cones and Shea felt this fungus to be important in seed deterioration.

Surface Sterilization of Cones and Seed

Numerous efforts have been made to remove fungi from coniferous seed. In 1925 Metcalf (36) sterilized seed of Sequoia sempervirens (D. Don) Endl. by gas fumigation with potassium permanganate and formaldehyde. He also soaked seed in formalin, bichloride of mercury, Semesan, sulfurous and sulfuric acids and hydrogen peroxide. Each of these treatments, although seemingly adequate to reduce the microflora of the seed, inhibited germination to various degrees. Prisjaznjuk (40) attempted sterilization of coniferous seed employing solutions of formalin and potassium permanganate, and dusts of Thiram, dinitro-trichlorobenzene, Grandosan, and Merkuran. He found formalin most fungicidal while Grandosan and Merkuran increased the number of fungi on the seed. No explanation was offered for the latter behaviour and he made no report of the effect of the treatments on seed germinability. Trappe (56) has recently reported a 30 minute soak in 35 percent hydrogen peroxide without a subsequent wash in distilled water, to remove contaminants from seed of Pinus, Picea, Larix Mill., Pseudotsuga and Tsuga (Endl.) Carr. Protection of the decontaminated seed during transfer along with decomposition of the hydrogen peroxide shortly after treatment are great advantages of this material. Trappe felt that seed could tolerate the peroxide for as long as an hour to remove microorganisms. The treatment stimulated germination.

Rediske and Shea (43, p. 1) attempted to reduce deterioration of the seed by treatment of Douglas-fir cones with Actidione BR, Phygon XL, Orthocide 75 and Dithane Z-7. Shea (49) found greater viability at the time of normal extraction in cones dipped in a fungicide as compared with untreated cones. However, Lavender (27) reported no reduction in germinability of untreated cones in burlap sacks after four months storage.

IV. RESULTS

Presence of Microflora in Immature Cones

Immature female cones from six of the 10 trees were heavily contaminated with fungi and bacteria when plated on PDA following surface sterilization. Many of the fungi belonged to the same genera common in the mature seed. Platings of the diluted rinse water from the immature seed (Table 2B and 2C) yielded a preponderance of bacteria over fungi. The relative prevalence of Aureobasidium pullulans (deBary) Arnud. was considerably higher on exised immature seed than on the extracted mature seed, but species of Penicillium and Asperigillus were approximately the same in each case. Female conelets of Douglas-fir have four main parts (67, p. 472) (24): (1) The bud-scales that form a capsule protecting the developing bud from its initiation the year prior to seed-shed until just before pollenization, (2) The cone-scales, sometimes more precisely called the ovulate scales, each supporting two developing ovules, (3) A cone bract on the abaxial side of each cone-scale, and (4) The pair of seeds on the adaxial side of each cone-scale. The conelets appeared free of both fungi and bacteria while the bud-scales sealed the developing cones. Organisms were present in the bud-scales but not the underlying tissues. As the buds began to open and the capsule of bud-scales fell away, the interior surfaces of the

Table 2. Prevalence of bacteria and fungi, as determined by plating methods, on immature female buds and excised seed of Douglas-fir and in water used to rinse infected buds and seed.

A. Halves of immature female buds (6 replications of 20 halves each - 2 trees)

	Number infected						Total
Encapsulated bud	18	20	20	20	20	20	118
Bud less capsule	--	0	0	0	4	8	12
Autoclaved unopened bud	0	0	0	2	-	0	2
Opened bud	20	20	20	20	20	--	100
Autoclaved opened bud	0	0	0	0	0	0	0

B. Colonies cultured from water of four consecutive rinses of excised immature seed (each of the seven replicates is from one tree)

Rinse number	Number							Total
1	5	4	5	4	3	5	4	30
2	2	1	5	2	0	1	2	13
3	2	0	4	1	2	0	2	21
4	3	1	5	0	0	0	0	9

C. Colonies cultured from water of four consecutive rinses of autoclaved excised seed (control for B)

Rinse number	Number							Total
1	1	4	1	0	1	0	0	7
2	2	0	5	0	0	0	0	7
3	0	3	0	0	0	0	0	3
4	0	1	0	0	0	0	0	1

cone-scales became contaminated. This was prior to pollination. As the pollinated seed developed, organisms grew from cultured, surface-sterilized seed and from the adjoining tissue which inevitably accompanied the excised seed. The cone-scale bracts were not plated and any association of organisms with them is not known.

Male strobili also became contaminated after the buds broke. Shed pollen also was contaminated by A. pullulans, Penicillium spp. and Aspergillus spp.

Fungi Found on Douglas-fir Seed

Fungi in the following list were isolated from mature seed cultured on PDA during the various stages of storage and treatments presented in Table 1. They are arranged according to decreasing frequency of occurrence:

An unidentified, sterile Hyphomycete

Penicillium spp.

Aureobasidium pullulans (deBary) Arnud. = Pullularia pullulans (deBary) Berkh.

Rhizopus stolonifer (Ehrenb. ex. Fr.) Lind. = R. nigricans Ehrenb.

Trichothecium roseum Link ex. Fries

Rhizopus oryzae Went & Gerlings

Trichoderma virid Pers. ex. Fr.

Aspergillus oryzae (Ahlburg) Cohn

Aspergillus sp. (Flavus-gryzae group)

Aspergillus flavus Link

Mucor racemosus Frescenius

Papulaspora Preuss

Rhizopus arrhizus Fischer

Alternaria consortiale (Thom) Groves & Hughes

Verticillium Nees ex Wallr.

Alternaria tenuis Auct.

Phoma Sacc.

Hormodendrum Bon.

Aspergillus chevalieri (Magnin) Thom & Church

Epicoccum purpurascens Ehrenburg ex. Schlect

Fusarium poae (Peck) Wollenweber

Sepedonicum Link ex. Fr.

Chaetomium gangligerum Ames

Phomopsis Sacc.

Aspergillus fumigatus Fries

Aspergillus phoenicis (Corda) Thom

Syncephalastrum Schroet.

The fungus isolated most often proved refractive to all efforts to stimulate sporulation. It therefore remained unidentified and is hereafter referred to as the sterile fungus. Several fungi not listed were isolated only once or very rarely and were lost before identification was possible. Because of their rarity they are assumed to be of minor importance to the study. This could be a fallacious assumption in that (1) no medium is ideally suited to isolation of all possible organisms and (2) significant unevaluated and unknown competitive influences among the organisms arising from the plated seed could have distorted the chances of isolation.

Location of Organisms Within the Mature Seed

In one experiment in which the seed was surface sterilized and the endosperm separated from the seed coat, the endosperm was resterilized and both parts were plated separately on PDA. A "T" test of isolation data from 100 seeds showed a significant difference in fungus recovery between the endosperms and the seed coats, the latter being the more infected. In no instance was the condition reversed and on numerous pairs the seed coat only was infected.

Distribution of Organisms on Filled Versus Empty Seed

As reported earlier, the seed was cleaned so as to retain the light seed. Either a quantitative or qualitative differential in organisms might occur between filled and empty seed. Such a differential could have significance if the empty seed harbored exceptionally large numbers of organisms, or a few distinctively important ones. Such seed might provide a substrate in storage for contamination of healthy seed.

Five samples of 100 seeds each from each tree were cut to determine the percent of "sound seed" among seed employed in the storage treatments. Lots of the same size of naturally discharged seed also were examined. By establishing the seed as either filled or empty, a binomial population was indicated. The percent confidence

interval was determined following the example given in Freese (16, p. 61). The results appear in Table 3.

Table 3. Seed quality by trees of extracted and naturally discharged Douglas-fir seed.

Extracted		Naturally discharged	
Tree	Percent	Tree	Percent
1	40.6 ± 2.2	1	53.0 ± 6.0
2	70.4 ± 4.1	2	71.4 ± 4.0
3	36.4 ± 3.7	3	41.2 ± 4.4
4	21.4 ± 3.9	5	35.2 ± 4.3

Seed lots similar to those in the cutting test from each of the four trees were plated on PDA for assay of fungal infection. All lots were highly infected and there appeared to be no significant difference in infection frequency between filled and empty seed. The level of infection was found to be of the same order in naturally shed seed as in extracted seed after one week of cone storage. The results appear in Figure 1. A build-up of organisms apparently accompanies storage of the seed in the cones and naturally shed seed attains the same high level of infection as occurs in seed remaining within the cones for one week after harvest.

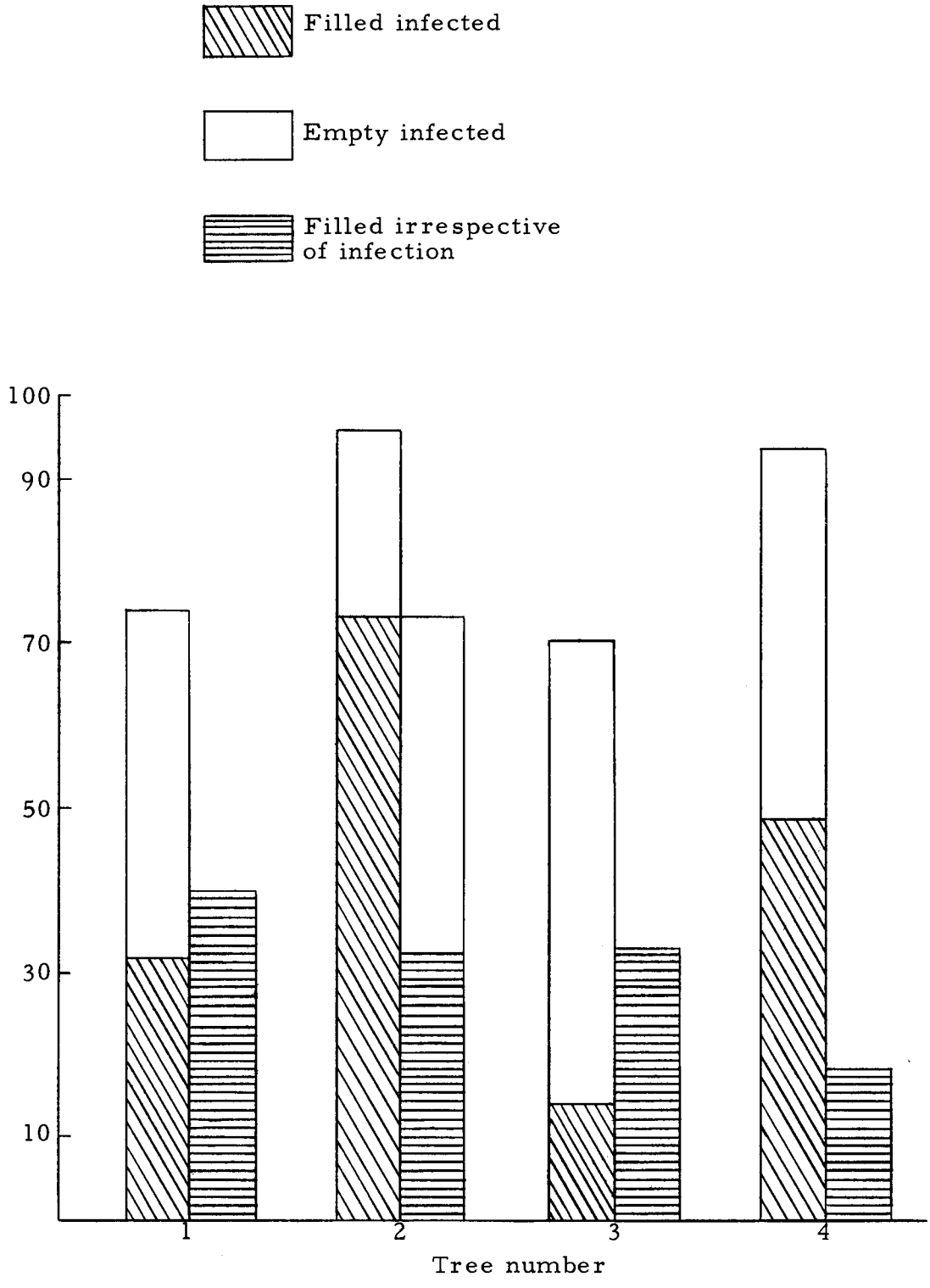

Figure 1. Comparative frequency of infection of filled vs. empty seed and the percentage of filled seed in seed lots from each of four trees.

Analysis of Variance of Factors Influencing the Microflora of Douglas-fir Seed

Seed were treated according to the schedule in Table 1, Materials and Methods. Consequently the following analysis of variance comprised four replications of 25 seeds comparing the following "treatments": Trees, Cone disinfection (Hypochlorite), Interval of Storage both in the cones and after extraction, or combinations of these treatments, thus involving 320 observations of 25 seeds each. Data appear in Appendix A.

The original intent of this work was to determine the effect of the 10 storage treatments on the microflora of Douglas-fir seed in general although the conclusions rest on a sample of only four trees. It was intended that we be able to extrapolate to all Douglas-fir seed, hence the use of the mixed model for the analysis of variance was indicated with the factor "Tree" being fixed and the other factors random. The results of the mixed model analysis indicate that the microfloras of the seed of the four trees employed were widely divergent but statistically probable. As the results of this mixed model analysis have clearly established differences which must be accepted among the four trees and between them and the Douglas-fir population, the mixed model is omitted in future discussions.

The seed treatments (storage) are significant at the one percent

level and the treatment of the cones with the hypochlorite dip interacted with the storage treatments at the five percent level, although the cone treatment alone was not significant. This interaction tends to make the significance of the main factor for seed treatment unclear except for the overpowering F value of that factor.

Table 4. Mixed model analysis of variance of the effect of cone and seed storage on seed microflora. (Randomized block 3x2x2 factorial experiment.)

Factor	Sums of squares	df	Mean square	F
Tree	851.784	3	283.928	14.223**
Cone treatment (chemical)	50.043	1	50.043	2.506
Tree-cone treatment	90.120	3	30.040	1.504
Seed-treatment (storage)	10,889.878	9	1,209.986	60.614**
Cone-treatment - seed-treatment	522.113	9	58.012	2.906*
Tree-seed treatment	2,087.216	27	77.304	3.872**
Tree-seed to tree-cone treatment	893.068	27	33.076	1.656*
Total	15,384.222	319		
Error	3,625.781	240	19.962	

* Significant at the 5% level
** Significant at the 1% level

The Effect of Storage on the Prevalence of Specific Fungi

The sums of squares in the preceding analysis of variance can provide additional useful information about the individual storage treatment effects when the Shortest Significant Range test (SSR) is employed (31, p. 238, 53, p. 251). Figures representing each storage treatment on the following tables are the average number of infected seed per 100 of both chemically treated and untreated cones for the four trees combined. Each storage treatment figure consequently represents the average number of infected seed per 100 for 800 seed cultured on PDA. In each of the following tables for fungi, data for the different treatments are placed in the order of decreasing percentage of infected seed. All treatments that are not significantly different at the five percent level are joined by the standard notation, a vertical line on the right of the figures.

When reference is made to these tables or others in this paper concerning significance between figures, the term "Significant" refers to a significance at the five percent level and "Highly Significant" to the one percent level.

In each of the following tables the quotes "Storage Treatment" column is divided into two parts, one for the time seed was stored in the cone and the other for subsequent storage of the extracted cleaned seed. The two treatments marked "Refrigerated" refer to

seed storage after extraction.

Figure 4 is a graphic presentation of the following five tables in a composite form to illustrate the relationships between the most prevalent fungi found on Douglas-fir seed under different storage treatments.

The Effect of Time of Extraction and Method of Storage on Fungal Flora

The total microfloral level of the seed remaining in the cones, increases sharply to its highest level one week after harvest, as indicated by the (1-0) treatment compared to the control (0-0). The number then begins to drop to lower levels (Figure 2 and Table 5). When the seeds are retained in the cone beyond the initial week, the number of the total fungal colonies makes a highly significant drop to the third week (3-0). Decline continues at significant levels through the eighth week (8-0) under continued cone storage. The trend toward a lower level of infection with prolonged storage appears to continue with storage of the extracted seed as well as with storage of the cones. Seed from cones stored one week show a significant decrease in infection with three weeks of seed storage as compared to none. Similarly with cones stored eight weeks there is a significant decrease in infection of the extracted seed at three weeks of seed storage as compared to none. This trend seems not to hold, however,

Table 5. Prevalence of all fungi in extracted Douglas-fir seed following various periods of storage in the cone and subsequent seed storage in glass jars.

Storage treatment in weeks		Seeds yielding fungi	
Cone storage (weeks)	Seed storage* (weeks)	Number per 100 seeds**	
1	0	83.1**	
1	3	68.6	
3	3	59.2	
3	10	57.4	
3	0	52.8	
8	0	46.8	
8	3	22.9	
0	0 (control)	22.5	
3	22 (refrigerated - 16°C)	14.7	
0	10	11.6	

* Room temperature except as indicated

** Number per 100 or 800 seeds cultured from the four trees combined.

*** Shortest significant range test by Li (31, p. 238-240): P = .05

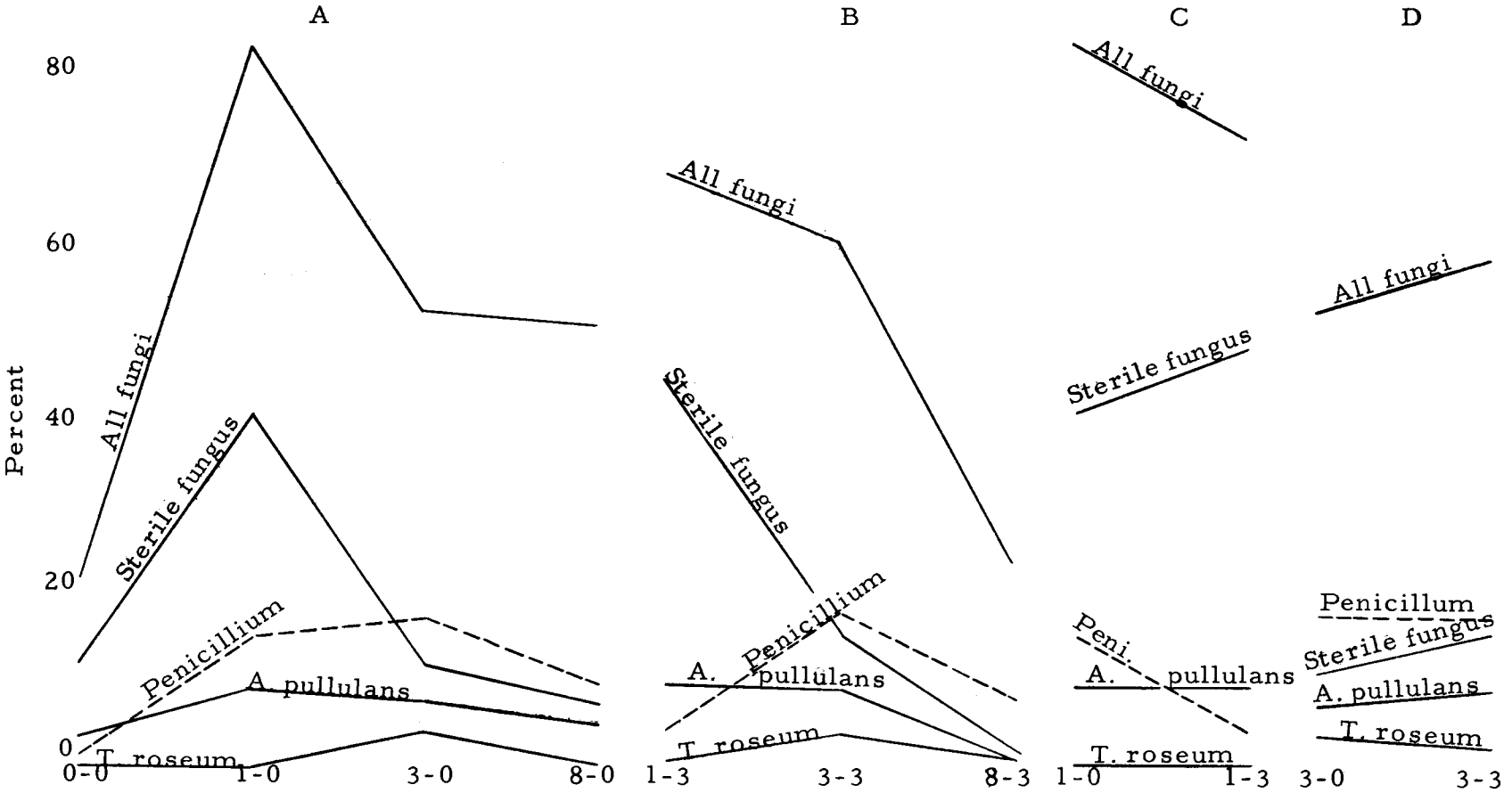

Figure 2. Relative prevalence of individual organisms after various periods of seed storage or seed and cone storage.

with continuing seed storage of cones stored for three weeks. Here there are no significant differences in flora between extracted seed stored for three and ten weeks and the infection in these is barely significantly greater than in the unstored seed from the same cone storage. Reference to Tables 6 to 9 shows considerable variation among the performance of different organisms with different periods of seed storage following three weeks of storage in the cones. Fresh seed from fresh cones has a very low level of infection, lower than all but the treatments involving refrigerated storage. This low level of infection, however, is further significantly decreased with refrigerated storage. Even seed with the very high level of infection characteristic of the three week period of cone storage show greatly reduced seed infection following refrigerated storage at -16°C.

The sterile fungus was most prevalent on seed from cones stored for a week followed by three weeks seed storage at room temperature. This high infection incidence was closely followed by the (1-0) and (3-10) storage treatments, although both were significantly less infected than the (1-3) treatment. The level of infection decreased sharply between the one week cone storage (1-0) and the three week (3-0). This decrease continued through the eighth week of cone storage, dropping to a level not significantly greater than that in storage under refrigeration.

This sterile fungus was most prevalent of all fungi isolated;

Table 6. Prevalence of the sterile fungus on tree-ripened Douglas-fir seed following various periods of open storage in the cone and subsequent seed storage in glass jars.

Storage treatment in weeks		Seeds yielding the sterile fungus
Cone storage (weeks)	Seed storage*	Number per 100**
1	3	47.3
1	0	41.2
3	10	40.1
3	3	15.5
0	0 (control)	12.0
3	0	11.0
3	22 (refrigerated-16°C)	8.6
8	0	7.0
0	10 (refrigerated-16°C)	6.6
8	3	2.5

* Room temperature except as indicated.

** Number per 100 of 800 seeds cultured from the four trees combined.

Table 7. Prevalence of Penicillium spp. in tree ripened Douglas-fir seed following various periods of open storage in the cone and subsequent seed storage in glass jars.

Storage treatment		Seeds yielding Penicillium
Cone storage (weeks)	Seed storage* (weeks)	Number per 100**
3	3	16.6
3	0	16.2
1	0	15.4
8	0	9.9
8	3	7.4
3	10	6.2
1	3	3.4
3	22 (refrigerated-16°C)	2.2
0	10	1.4
0	0 (control)	1.2

* Room temperature except as indicated.

** Number per 100 of 800 seeds cultured from the four trees combined.

Table 8. Prevalence of Trichothecium roseum Link from tree-ripened Douglas-fir seed following various periods of open storage in the cone and subsequent seed storage in glass jars.

Storage treatment		Seeds yielding T. roseum
Cone storage (weeks)	Seed storage* (weeks)	Number per 100**
3	0	3.5
3	3	2.1
8	0	1.4
8	3	1.1
1	3	0.4
0	10 (refrigerated-16°C)	0.4
3	10	0.1
3	22 (refrigerated-16°C)	0
1	0	0
0	0 (control)	0

* Room temperature except as indicated.

** Number of colonies per 100 of 800 seeds cultured from the four trees combined.

Table 9. Prevalence of A. pullulans (deBary) Arnud. in tree-ripened Douglas-fir seed following various periods of open storage in the cone and subsequent seed storage in glass jars.

Storage treatment		Seeds yielding A. pullulans
Cone storage (weeks)	Seed storage* (weeks)	Number per 100**
1	3	9.4
1	0	9.2
3	3	9.2
3	0	6.9
8	0	5.2
3	10	4.8
0	0 (control)	3.9
8	3	1.8
3	22 (refrigerated-16°C)	1.2
1	10 (refrigerated-16°C)	0.1

* Room temperature except as indicated.

** Number per 100 of 800 seeds cultured from the four trees combined.

consequently it has a strong numerical influence on the arrangement of the treatment responses as they appear in Table 6.

The most notable departures for the frequency of this fungus from the frequencies of all fungi is: (1) the high incidence of the sterile fungus in the control (0-0) where it represents more than half the total, (2) its extremely transitory nature with prolonged storage particularly in the cone after the first week, and (3) its apparently minor tendency to be influenced by refrigeration.

Frequency of the species of Penicillium followed the same rising and then falling curve with time as occurred with the sterile fungus and with species of Trichothecium and Aurobasidium which will be described later. The rise in number of infected seeds with cone storage was not so rapid as in the case of the sterile fungus. Prevalence of Penicillium infection reached its peak after three weeks in the cone, but declined quite markedly at the eighth week. This decline, however, was only approximately a 50 percent reduction, and proportionately was not nearly so great as occurred with the sterile fungus. Room temperature storage of the extracted seed also reduced the amount of Penicillium. Refrigerated storage was even more effective in bringing about a reduction in these fungi.

Trichothecium roseum behaved very similarly to Penicillium. Both fungi increased to a maximum at three weeks in the stored

cones and then decreased by approximately 50 percent with eight weeks storage. There also appeared some tendency for Trichothecium roseum to decrease with continuing room temperature storage of the extracted seed. The fungus was not as responsive to reduction in refrigerated storage as any of the other fungi, but somewhat resembled the sterile fungus.

Except for marked suppression of Aureobasidium pullulans by refrigerated storage, this fungus developed very much like the sterile fungus; there was a relatively high incidence in the controls and development was exceedingly rapid in the cones reaching a peak after one week of storage. This peak was followed by a decline to approximately 50 percent with eight weeks of cone storage. This fungus also seemed to decline with continuing room temperature seed storage following seed extraction. This was most apparent following eight weeks of cone storage. Refrigeration was particularly effective in suppressing this fungus.

From the preceding section (Table 5) it is apparent that the fungal flora of Douglas-fir seed is at a peak (83 percent) in seed extracted from cones one week after harvest. This represents an increase of approximately 60 percent above the level on seed from newly harvested cones. The question arises as to whether this increase is a natural phenomenon or is a consequence of artificial harvest. To clarify this point the fungal floras are compared among

seed lots assayed at cone harvest one week following harvest and assayed after natural seed discharge (one month after cone harvest) into clean burlap bags. The results appear in Figure 3 where the percent of infected seed on each of the four source trees is graphed separately. The first family of bars represents the control (0-0) time, and indicates a significantly low number of infections in the fully mature cones at harvest, as compared with subsequent treatments involving cone storage and natural seed shed. The second family of bars illustrates the condition of infection in seed extracted from the cones one week after harvest (1-0), and the third family shows the prevalence of infection in naturally shed seed (bagged cones) collected and assayed one month later than family (0-0). Infection was significantly greater than the control (0-0) but not significantly different from the seed stored for one week in the cones. Naturally shed seed from the fourth tree was not available.

Comparison of the Fungal Flora of Seed by Species Between Trees and Between Artificially Extracted and Naturally Shed Seed

Figure 4 illustrates the prevalence of the different microfloral populations in the seed of each of four Douglas-firs. As was indicated by the Mixed Model Analysis of Variance (Table 4), there was a highly significant difference between the microorganisms infecting the seed of the four trees and a Shortest Significant Range test

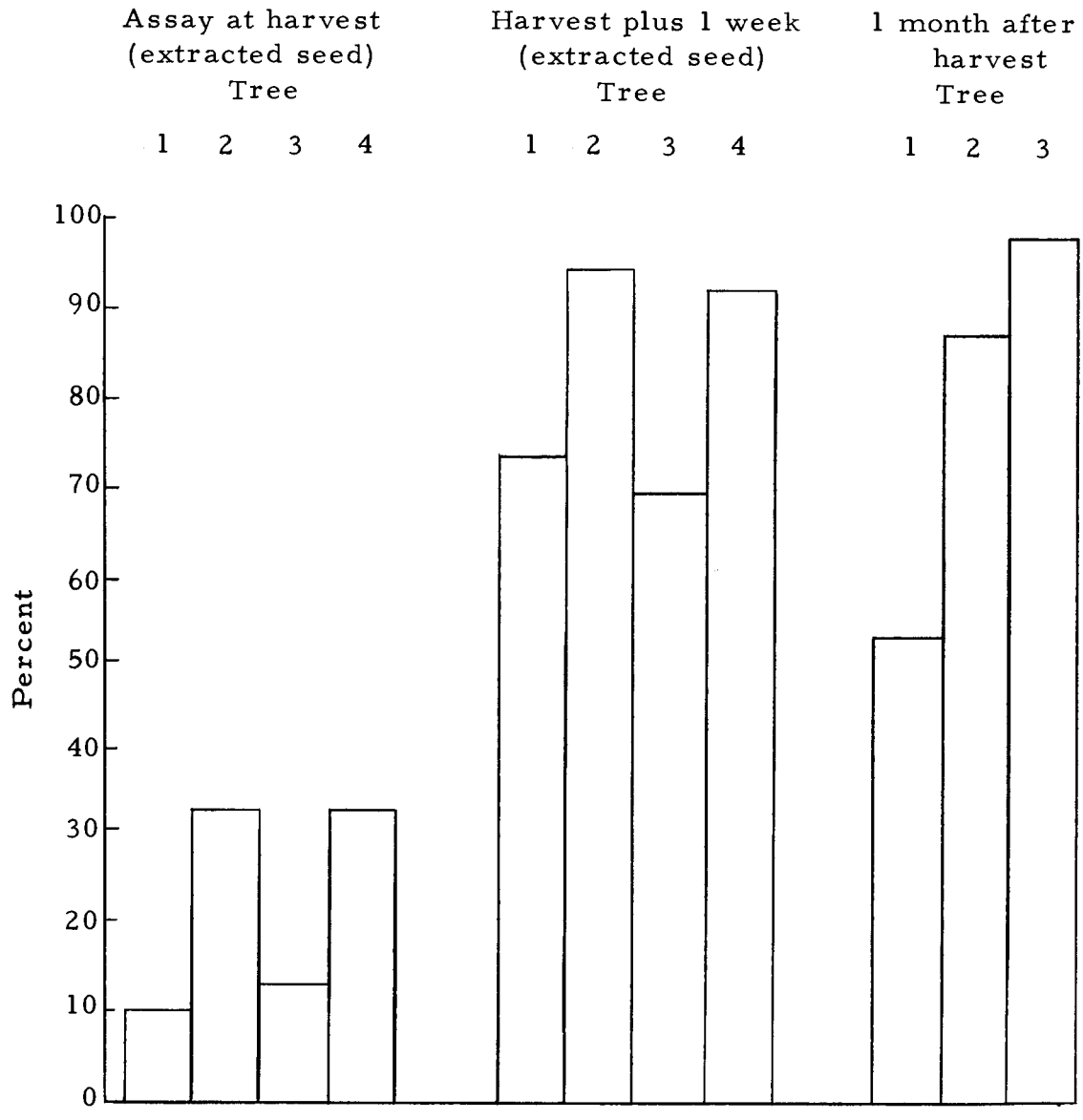

Figure 3. Comparison of the fungal flora of naturally shed seed and seed extracted 1 week after cone harvest, with seed assayed at cone harvest time. Four trees recorded independently.

employing the mixed model error term showed the seed of no two trees in this experiment to be "statistically" from the same population. Additional study involving the role of each species of organism found to inhabit the seed and also their relationships one to another and between individual trees is needed to clarify these points.

Although most of the fungal inhabitants are ubiquitous and are commonly found on Douglas-fir needles (44), their population fluctuations tend to follow those of Garrett's "sugar fungi" (17, p. 96-101) when one considers their rapid initial build-up in numbers of colonies and subsequent slower decline (Table 5, Figure 2).

Each bar in Figure 4 represents the total number of infections in 2000 seed from each of the four trees. In addition, each bar is subdivided into the portions of the total infection that each of the most prevalent fungi plays in the seed-infection of that tree. The segment of the bar that is left unshaded includes the many remaining species listed previously as well as the bacterial infections that occurred. When comparing the microflora quantitatively, the lack of similar association in the relative percent of infection between trees or between different organisms within the same tree obscures any significance of these seed-borne organisms other than that of being conjunctive symbionts.

Cones on trees "1" and "2" were early seed producers and the cones were opening and beginning to shed seed at the time of

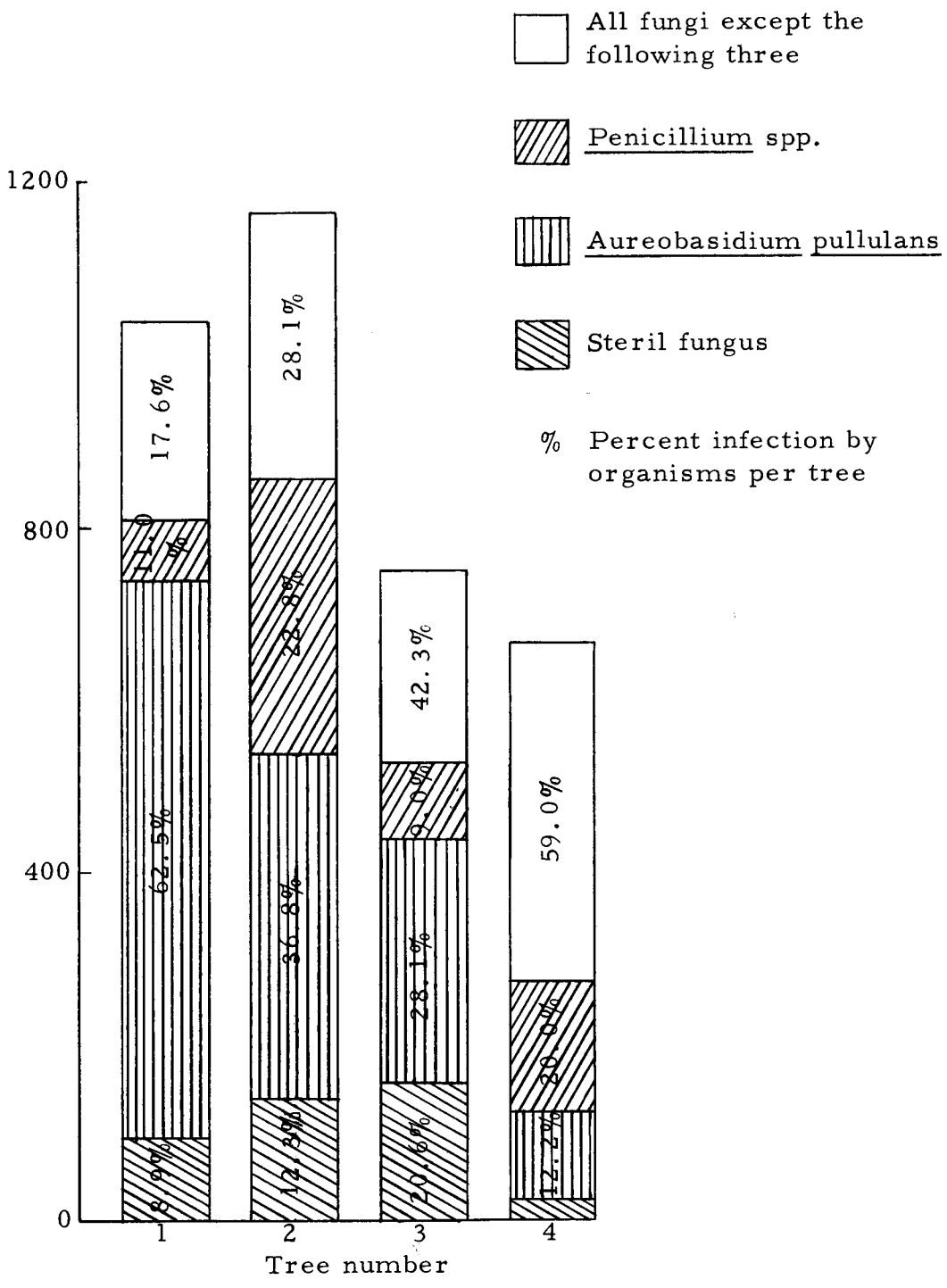

Figure 4. Relative prevalence of most frequently occurring fungi in 2000 extracted seed of each of four Douglas-fir trees after the combined storage treatments.

cone collection. Cones of the other two trees were "late," being green and tightly closed at harvest. The relative prevalence of the different fungi, however, failed to disclose any relationships between the seed from the green and dry cones, with one notable exception: Penicillium species appear in a lower concentration in the seed of the cones that were dry when harvested than in seed from the green cones.

The plating of naturally shed seed showed a sharp increase in Penicillium species and a poorly defined increase in the other prominent organisms as is shown in Table 10. The sterile fungus and A. pullulans varied differently between trees. The increased level of contamination in the naturally shed seed resulted in a better defined indication of possible competitive actions between the different fungi occupying the seed as a substrate. The totals of the fungi other than penicillium was in an inverse proportion to penicillium occurring on the seed. Whether this indicates an in vitro effect accounted for by the different medium on which the organisms were cultured, or a true indication of in vivo results, cannot be ascertained by these data.

Seed Sterilization with Hydrogen Peroxide

In a brief review of the seed sterilization treatments explained in the materials and methods section, 100-seed samples from each

Table 10. Comparative distribution of seed infecting fungi in extracted and naturally shed seed from three trees.

Organisms isolated	Tree #1		Tree #2		Tree #3	
	Naturally shed	Extracted	Naturally shed	Extracted	Naturally shed	Extracted
	Percent					
Penicillium spp.	30.4	9.0	42.6	22.8	75.0	20.0
A. pullulans	2.0	20.6	9.4	12.3	6.5	0.8
Sterile fungus	9.0	28.1	34.0	36.8	16.3	12.2
Sum of all others	58.6	42.3	14.0	28.1	2.2	59.0

storage time were treated for ten minutes in a surface sterilizing solution of 30 percent hydrogen peroxide and rinsed in sterile distilled water before plating. The seed samples of the last seven storage treatments were each divided in half prior to the peroxide treatment and one-half, or 50 seeds, were immersed in a solution of 10 percent volume/volume commercial detergent and distilled water for 20 minutes, then rinsed before being treated with the standard hydrogen peroxide sterilant. Neither the treatment of the cones, seed extraction method, nor storage temperature and time, were changed from any previous analysis. The data appear in Appendix B.

As seen in Table 11, under the conditions of the several treatments, all of the main factors were highly significant. Differences between the four trees were considered earlier in the thesis. The storage treatment consisting of (1) long refrigeration, (2) short refrigeration, and (3) room temperature storage of cleaned seed (in this case, the 1-0 treatment), was again divided by single degrees of freedom as shown at the bottom of the analysis of variance table. Although the two F values resulting from these single degree of freedom tests were also both significant, the difference between the two refrigerated storage periods and the room temperature storage being significant at the one percent level, was more important than that between the short and long term refrigeration.

Table 11. Analysis of variance of the effect of seed storage treatments and surface sterilization on the microflora of Douglas-fir seed as influenced by "tree", "storage", "cone treatment" and use of "detergent."

Factor	Sums of squares	df	Mean square	F value
Tree	158.464	3	52.811	13.655**
Storage	128.250	2	64.125	16.578**
Tree-Storage	309.068	6	51.511	13.317**
Cone Treatment (Chemical)	157.594	1	157.594	40.743**
Tree-Treatment	38.432	3	12.811	3.312*
Storage-Treatment	144.750	2	72.375	18.711**
Detergent	396.094	1	396.094	102.402**
Tree-Detergent	185.099	3	61.700	15.551**
Storage-Detergent	48.688	2	13.875	3.643*
Treatment-Detergent	49.594	1	49.594	12.821**
Second order interaction				
1. Tree-Storage-Treatment	49.599	6	8.266	2.138
2. Tree-Storage-Detergent	132.432	6	22.072	5.720*
3. Storage-Treatment-Detergent	9.750	2	4.875	1.260
Third order interaction				
Tree-Treatment-Storage-Detergent	49.029	6	8.172	2.112
Totals		95	1935.907	
Error	193.406	50	3.868	
Within Storage Periods				
Short vs. Long refrigeration			20.250	5.235*
Refrigeration vs. non-refrigeration			128.250	33.156**

The effect of soaking the seed-containing cones in hypochlorite also showed highly significant results statistically, contrary to other analyses performed concerning the activities of single organisms and of the combined microfloral population. Immersion of the extracted seed in detergent before surface sterilization is shown to aid the effectiveness of the sterilant in reducing the microflora (significant at the one percent level).

The first order interactions were all significant at the one percent level except for the tree-cone interaction, which was significant at the five percent level. The presence of these significant interactions obscures the true values of the main factors, but the sums of squares or F-values of the individual main effects were clearly so significant it was felt that they could still be useful for interpretative purposes.

The Number of Seed Found Infected with Fungi

In this investigation of 8,000 seeds plated on PDA, 7,691 seed were ultimately available for microfloral analysis. Of these plated seed, 3,312, or 43.1 percent, were contaminated with fungi or bacteria after a ten minute disinfectant soak in hydrogen peroxide. The total number of seed counted here was the combined number from the different seed storage lots. Many of these storage treatments influenced the microflora. These effects accessory to the

chemical treatment complicate the picture and may introduce considerable error into any figure representing a quantitative value for the fungal population of Douglas-fir seed. None of the treatments increased the microfloral level, however, so this figure can be considered a conservative estimate. Of the 750 other seed which by contrast were treated with a detergent soak before the hydrogen peroxide disinfection, 70, or 9.3 percent, produced colonies. This reduced level of infection indicates an effect due either to the detergent alone, or more likely the detergent in conjunction with the hydrogen peroxide soak.

V. DISCUSSION

Inoculum Source and Time of Infection

Several investigators, as mentioned in the literature review, concluded that immature Douglas-fir seed, while within the still tightly closed cones are free of either fungal or bacterial contamination. Shea, however, suggests that microorganisms can infect the young seed by distribution by insects in insect galleries. Other authorities have indicated that this can be implemented through the transport of spores on insect bodies (several genera of the Scolytoidea (7), aphids (44), et cetera.

This paper shows that many of the microfloral contaminants are present from the time the female strobil opens for pollination (Table 2). The paper further suggests that the Douglas-fir pollen can transport these cosmopolitan organisms to the receptive flower. Without precise evidence, it is also reasonable to assume that aphids carrying fungal spores between diseased needles visit the open flower disseminating inoculum.

The increased infection of the naturally shed seed over those seed extracted from the mature, green, but closed cone, the abrupt increase in the number of infections when the seed is retained within the cone for a week after harvest, and their eventual reduction after the initial build-up and colonization of the substrate, suggests that

the cone itself is the initial substrate for the fungal microflora infecting Douglas-fir seed. Similarly, the gradual reduction in the microfloral concentration in the seed after extraction also points to the importance of the cone tissue for initial colonization. Apparently the extracted seed provides either an inadequate food base, or an inadequate environment for their development. Normally we do not think of fungi of this character as particularly delicate; their rapid decline with storage therefore seems notable. How long they may actually persist in the seed and what their ultimate influences may be remain as yet unknown.

This paper also indicates that Douglas-fir seed, not unlike seed of agricultural crops, harbors many fungal microorganisms. Some of the organisms are known to be potential pathogens but the majority are apparently unimportant under normal conditions. These latter organisms can possibly assume pathogenic significance (1) when the seed is stored for extended periods as portrayed by Christensen in his investigations of stored cereals, and (2) as a source of inoculum and an infection point for the attack of seedlings in their initial stages of growth. Zak (66, p. 380) infers this possibility when he refers to the susceptibility of young root tissue during its initial few months after germination until its final naturally acquired resistance is established.

Effect of Refrigeration on Seed Microflora

The apparent reduction in the numbers of infections in seed after storage under refrigerated conditions is difficult to explain by the data presented here. This phenomenon was not anticipated at the outset of the experimentation and no direct controls were established. In referring to Figure 2A and 2B and Table 5, however, the data comparing the control storage time (0-0) and the (8-3) seed storage (a total of eleven weeks room temperature storage), the seed stored for this period developed a sharply fluctuating microfloral level but at no time was the number of fungal colonies below the level of the control. The seed in refrigerated storage for ten weeks showed a distinctly lower level of contamination (infection) than the seed stored at room temperature or the control indicating a "real" effect of refrigeration rather than time on the abundance of seed microflora. The reason why this suppression took place is not available from this data nor, to my knowledge, can it be obtained from any prior work.

On reviewing the literature in an effort to explain these results, Wolf and Wolf (65, p. 103) are found to say that "Experiments to determine the ability of fungi to survive when subjected to temperatures in excess (below) of those known to inhibit growth and reproduction are meager...(but)...fungi are much more tolerant of low than of high temperatures." They continue by saying that "Temperatures

employed in ordinary refrigeration and cold storage are very effective in inhibiting the growth of such fungi as those causing decay of meats, fruits, vegetables, and other food stuffs." They also state that (65, p. 98) "At 0°C the growth of fungi is completely checked." Greene and Barnett (32, p. 34) agree with this statement when they say "A few fungi are capable of growing below 0°C but for most species the minimum temperature is 0-5°C. Cochrane (10, p. 14), in referring to A. pullulans (a common fungus found in Douglas-fir seed) specifically, indicates that this organism shows vegetative growth (dry weight) between 5-37°C. None of the previously quoted authorities indicated any lethal temperatures, and although Wolf and Wolf present an extensive list of fungi with their minimum, maximum, and optimum growth temperatures, none of the organisms reported were found on Douglas-fir seed.

The Microfloral Level Under Seed Storage

It is perhaps wise to mention here that this discussion pertains to the most prevalent fungi found in Douglas-fir seed. These isolates did not include any of the numerous known seed pathogens identified and listed previously. This might infer their lack of importance but for their identification as destroyers of germinating conifer seed.

The marked increase in the microfloral level of the naturally shed seed over that found in the artificially extracted seed immediately

after harvest indicates that (1) in the in situ status of the seed in nature, infection by microorganisms is prevalent and is apparently tolerable for one season, (2) additional steps need be taken to reduce the infection level of the "natural" condition if seed is to be stored for extended periods without extensive loss in survival of germinating seed due to the fungal attack referred to by Lawrence and Rediske (28) and Gibson (19). To minimize the fungal microflora level within the seed, extraction should take place immediately after harvest. The fungal organisms are apparently already inhabiting the cone tissue and infect the seed remaining within the cone after harvest. The numbers of organisms apparently does not increase appreciably in the seed during further storage after the seed has been removed from the cone. This is indicated by Figure 2C and 2D. The microfloral population does increase, however, when the seed is retained within the cone (Figure 2A).

Surface Sterilization

The possible usefulness of an application of a surface sterilant to the conifer cones upon harvest is indicated by the statistical results of the sterilization treatment (Table 11) even though the hypochlorite dip was not a sufficiently effective fungicide in the experiment to warrant commercial application at this concentration. In spite of the insufficiency of the cone disinfection treatment, the

susceptibility of seed-borne fungi to a fungicide applied to the cone on harvest is clearly demonstrated. The use of a fungicidal coating to the seed itself although not attempted in this work, does not appear to be of any particular use in controlling these organisms unless it is capable of acting through the seed-coat. In fact, it may be deleterious to the ultimate objective of reforestation by interrupting the natural microfloral competition between those organisms within the seed and pathogenic organisms attacking the sown seed from the soil. This latter possibility is reminiscent of the suspected effect of mycorrizha infecting new conifer roots and excluding the root decaying soil-borne organisms mentioned by Zak (66, p. 384).

Merit of Analyzing Seed Storage Treatments Statistically

The combined relatively large sample size, the clear separation of non-related variables from the main factors, and the significance of the "F" values, appears superficially to negate the necessity of statistical analysis of the data. Considering the lack of previous evidence to enable one to foresee the results obtained, however, and the large number of factors combined in the one experiment, the main effects of each treatment were somewhat obscured without mathematical separation of the effects of each factor. The interactions between the main effects were still more difficult to see from

observation of the raw data. Knowledge of these interactions is significant in that it indicates that the results of the main factors are not clearly indicative of their simple effects, and caution should be used in the interpretation of these results. This information could not have been ascertained from the raw data by other than an experienced eye. As mentioned in the results section, the clearly distinctive values of the main effects as compared to the first order interactions, should add credence to the effects of the main factors in spite of the interaction effects.

It should also be noted that all of these analyses were computed from only four statistically different trees as is shown in the Mixed Model Analysis of Variance. These trees were determined to be from different populations through the use of a theoretical combination of main and interaction effects. This point tends to indicate that although the sample size was adequate to describe the "Within Tree" variations, perhaps the variation between all Douglas-firs is wider than realized. This, in itself, indicates that the interpretation of the results of these experiments should be limited to the effects of the treatments on individual organisms and not indiscriminately to all Douglas-fir seed until more extensive experimentation is done.

Characteristics of the "Sterile Fungus"

The organisms herein referred to as the "Sterile Fungus"

because of its lack of fruiting structures on artificial culture, was included in the analyses because of its prevalence and corresponding significance in the analysis of "all fungi" in the different seed storage treatments. This point is quite clear when the curve of the "sterile fungus" is compared with that of "all fungi" (Figure 2).

The effects and relative importance of this organism on Douglas-fir seed, similar to the other fungal organisms reported here, is up to conjecture at this time. After many observations of this fungus, however, it is the author's belief that the compatibility between this organism and the other four most prevalent fungi found appears to be good. This fungus grew without interruption around and through any other single organism with the exception of a few Penicillium isolates that emitted a distinct inhibitive factor to the media. The vegetative growth of the fungus was moderate (about three cm. per week on PDA) and the hyphae were preponderantly aerial, floccose and tufted with age, and light tan in color. After a period of about two weeks the medium was stained from tan to deep brown and with flecks of deeper brown bearing a trace of green or black (possibly stained potato pieces). The most easily noted identifying characteristic observed about the fungus was the narrow, dark lines of demarcation produced at the junction of two colonies of this organism from distinct seeds. The periphery of the adjoining isolates appeared to stain the medium in the immediate vicinity dark brown. There was no appreciable

change in the aerial mycelium at this point other than a slight slowing of growth revealed by a depressed area. The fungus occurred frequently enough that the production of these lines upon meeting was used as one identification mark in these experiments.

The organism was reported and described in this paper because of its frequency of isolation and because this fungus has possibly been observed by other investigators but listed under "other organisms unidentified."

Silvicultural Applications

1. Douglas-fir seed should be extracted from the cone immediately after harvest to keep seed infection from the microfloral population already inhabiting the cone to a minimum. This would seem particularly important if the seed is to be stored for an extended period.
2. The extracted seed should be refrigerated immediately after removal from the cone to inhibit further fungal activity within the seed.
3. As a result of the apparent effectiveness of the sterilant applied to the cones at harvest in reducing the seed-borne microflora, the application of a cone fungicide appears favorable and should be explored further.
4. When sterile conifer seed is desired with minimal

stimulation or retardation to the seed, an immersion of the seed in a commercial detergent prior to sterilization with 30 percent hydrogen peroxide is recommended.

5. Investigation of the effects of fungal organisms within Douglas-fir seed is suggested to determine if any or all the microorganisms found could or should be eliminated prior to seed storage.
6. Efforts should be made to evaluate the beneficial effects, if any, of competition between the seed-borne microflora and soil-borne pathogens. The elimination of the seed-borne fungi or application of a seed-coating fungicide may be deleterious to seed sown in forest soil.

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APPENDIX

APPENDIX A

Seed Storage Treatment

Fungi found on four 25-seed samples of extracted, cleaned, and surface sterilized Douglas-fir seed.

All Organisms Combined

Tree No.	Treated*	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated
	(0-0)**		(1-0)		(1-3)		(3-0)		(3-31)	
1	3***	11	31	36	31	36	75	44	36	33
2	10	26	50	46	48	34	22	26	14	32
3	0	10	34	39	30	16	28	13	26	22
4	16	20	50	47	31	38	40	4	35	40
	(3-10)		(8-0)		(8-3)		S-refrigeration		L-refrigeration	
1	29	27	24	33	5	5	4	8	1	24
2	45	39	20	29	11	11	5	8	4	25
3	10	21	26	28	14	14	4	6	5	10
4	4	28	34	25	37	37	3	20	9	20

* Hypochlorite cone treatment.

** Seed storage treatment. first figure is weeks of cone storage. second is weeks of seed storage at room temperature. S-refrigeration and L-refrigeration are short and long refrigeration respectively.

*** Total infected seeds in four 25-seed samples.

APPENDIX A
(cont.)

Penicillium spp.

Tree No.	Treated**	Untreated***	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated
	(0-0)*		(1-10)		(1-3)		(3-0)		(3-31)	
1	0	0	8	14	3	4	8	61	13	34
2	3	1	8	5	0	1	5	10	6	3
3	0	1	6	24	0	5	26	0	18	20
4	3	1	12	46	7	9	20	0	7	32
	(3-10)		(8-0)		(8-3)		S-refrigeration		L-refrigeration	
1	9	4	5	13	1	3	1	0	3	0
2	0	3	3	10	1	3	4	1	3	5
3	0	6	8	6	0	3	0	2	1	1
4	3	25	10	24	25	23	0	3	1	4

APPENDIX A
(cont.)

Trichothecium roseum

Tree No.	Treated**	Untreated***	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated
	(0-0)*		(1-10)		(1-3)		(3-0)		(3-31)	
1	0	0	0	0	0	1	4	0	1	0
2	0	0	0	0	0	0	0	3	0	3
3	0	0	0	0	0	1	0	6	0	10
4	0	0	0	0	0	1	12	3	1	2
	(3-10)		(8-0)		(8-3)		S-refrigeration		L-refrigeration	
1	0	0	0	3	0	0	2	0	0	0
2	0	0	2	3	0	0	0	1	0	0
3	0	1	0	3	1	2	0	0	0	0
4	0	0	2	1	5	1	0	0	0	0

APPENDIX A
(cont.)

Alternaria spp.

Tree No.	Treated**	Untreated***	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated
	(0-0)*		(1-10)		(1-3)		(3-0)		(3-31)	
1	0	9	10	11	11	10	23	17	32	17
2	3	7	13	14	12	9	8	2	7	1
3	0	0	2	1	5	3	0	5	3	0
4	2	10	15	8	21	1	0	0	4	10
	(3-10)		(8-0)		(8-3)		S-refrigeration		L-refrigeration	
1	4	0	3	22	1	0	0	0	0	2
2	8	9	1	8	4	3	0	0	1	1
3	0	0	-	0	0	0	0	0	0	1
4	11	6	6	2	5	1	0	1	2	3

APPENDIX A
(cont.)

Sterile fungus

Tree No.	Treated**	Untreated***	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated
	(0-0)*		(1-10)		(1-3)		(3-0)		(3-31)	
1	0	2	31	46	31	50	18	6	12	13
2	7	41	92	31	85	62	26	21	20	46
3	1	8	27	11	25	14	3	0	1	6
4	12	18	35	67	44	61	2	0	8	32
	(3-10)		(8-0)		(8-3)		S-refrigeration		L-refrigeration	
1	25	40	1	1	5	0	0	10	1	14
2	88	51	11	23	2	4	7	19	0	23
3	4	8	0	3	2	0	1	1	1	7
4	40	46	12	10	2	5	2	13	5	15