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Fungal colonizers and seed loss in lodgepole pine orchards of British Columbia

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Abstract

Lodgepole pine is an important lumber species in Canada and seed orchards are expected to meet the increased demand for seed. However, seed production has been consistently low in the Okanagan region orchards of British Columbia. To determine whether the fungal microbiome contributes to seed loss, histological and molecular approaches were used. Seed production was studied at seven Okanagan orchards, all outside the natural range of lodgepole pine, and at one near Prince George, within its natural range. Seed losses were highest in the Okanagan, compared with Prince George. The role of fungal colonizers in consuming seed during the last stages of maturation is described. Fungal hyphae were frequently observed at all locations in developing seed, particularly once storage substances accumulated. Fungi identified from host tissues using molecular and morphological techniques included *Alternaria*, *Cladosporium*, *Fusarium, Penicillium*, and *Sydowia*. The opportunistic foliar pathogen *Sydowia polyspora*, which is known to have a variable biotrophic status, was detected at most orchards within different host tissues (seeds, needles and conelets), in association with pollen and in the air column. Reduced seed viability observed in Okanagan orchards is most likely due to a combination of factors, including the composition of the fungal microbiome.

Keywords

Microbiome, endophyte, *Pinus contorta* var. *latifolia* Engelm. ex S. Watson, *Sydowia polyspora* (Bref. & Tavel) E. Müll.

Introduction

Most mature conifer seeds are associated with abundant fungal spores that may cause disease under conditions conducive to spore germination and fungal growth (Lilja et al. 1995). These epiphytic fungi often cause seedling diseases, such as root rot and damping off (Fraedrich 2001), or they may be necrotrophic and produce toxic metabolites that harm seeds (Leishman et al. 2000). When seed-borne fungi penetrate seed coats, they can either destroy embryos and megagametophytes (Fraedrich 2001), or reduce germination vigour (Campbell and Landis 1990) and the long-term viability of seeds (Leishman et al. 2000). Some seed-borne epiphytic fungi have been described as both foliar pathogens (Talgø et al. 2011), as well as endophytes of conifers (Pirttilä et al. 2003; Terhonen et al. 2011). With the exception of cone and seed rusts (Sutherland 1991), most serious seed-borne pathogens are generalists; such pathogens often cause different diseases in different hosts (Ogoshi 1987) and are capable of dispersing among susceptible host species. Thus, their opportunism, their ubiquitous distribution and abundant production of asexual propagules make seed-borne fungi difficult to effectively control.

Fungal endophytes are widespread among most vascular plants and have been characterized for many conifer species (Carroll and Carroll 1978; Pirttilä et al. 2003; Arnold et al. 2007; Terhonen et al. 2011). These fungi follow a range of ecological strategies that include strict mutualists with a high host specificity and vertical transmission, to generalist species that display a broad host range and more often horizontal transmission among hosts. The latter category may adopt a gradient of biotrophic strategies that can include mutualism to parasitism and even pathogenic behavior within their host (Christian et al. 2017; Michal Johnson and Oelmüller 2009). Endophytes constitute a major component of the broader microbial biome, i.e. the microbiome that can be found within a host plant. The broader microbiome is also composed of other recognized plant symbionts, pathogens, parasites, saprophytes and epiphytes (Porras-Alfaro and Bayman 2011). Member species of the fungal microbiome will interact in association with their host and influence the biological processes occurring in host tissues. A consequence is that they have an indirect influence on plant growth, health and reproductive fitness (Michal Johnson and Oelmüller 2009). The external biotic and abiotic conditions experienced by the host will also shape the nature of these interactions (Christian et al. 2017). Consequently, there is also a concern about changing climatic conditions and how they may modify growing conditions for the host, the composition of the microbiome and the prevalence of both native and invasive pathogens in forest ecosystems (Aitken and Whitlock 2013; Woods et al. 2010).

Seed orchards produce phenotypically superior seeds for conifer nurseries and reforestation programs (El-Kassaby and Ritland 1996). In Canada, the production of lodgepole pine seed is imperative due to the commercial value of this lumber species (MacLachlan et al. 2017). In the past several decades, both natural and plantation populations of lodgepole pine have experienced drastic population declines due to extreme, unprecedented events that included the mountain pine beetle (*Dendroctonus ponderosae* Hopkins) epidemic (Dhar et al. 2016), and an increasing incidence of *Dothistroma* needle blight (Woods et al. 2010). Repopulating lodgepole pine forests is a priority, and 45% of current public land reforestation efforts in the provinces of British Columbia (BC) and Alberta are dedicated to this species (MacLachlan et al. 2017).

Unfortunately, seed orchards in the Okanagan region of BC have not been providing the expected lodgepole pine seed yield in the past few decades (Owens et al. 2005; Woods 2012). The shortfall in seed remains a persistent problem in these particular seed orchards, which, it is important to note, are located outside of the natural range of lodgepole pine. Putting lodgepole pine seed production orchards in the northern part of the Okanagan Valley would, it was thought, avoid problems of pollen contamination from surrounding forests. Because the climate of this region experiences a hotter spring and summer than the rest of British Columbia, it has been proposed that heat stress may be a contributing factor (Owens et al. 2005). However, no experiments have been carried out to test whether cone or seed development is, in fact, altered by heat stress and this idea remains conjecture. A first step to understanding seed losses would be to sample during the period in which seed losses occur.

An understanding of when and why seeds fail is needed if seed yield is to be improved in these orchards. This is complicated, because lodgepole pine cones take three years from initiation of cone buds to seed maturity. We know that external agents can be responsible for catastrophic seed losses. A seed-destroying insect pest, *Leptoglossus occidentalis*, is known to diminish seed yield (Richardson et al. 2016). Internal factors in the host, such as selfing have also been proposed (Owens et al. 2005), but have been ruled out by genetic testing (Michael Carlson pers. comm.). More attention needs to be brought to other factors that may cause seed to fail (Webber 2012).

The purpose of this study was to answer the following questions: how variable is seed shortfall between a selection of seed orchards in the Province of BC and does the fungal microbiome play a role in the deterioration of lodgepole pine seed.

Materials and Methods

Study sites and sampling regimes:

Cones were sampled from eight seed orchards (O) at seven sites (Table 1) in British Columbia (BC). Seven orchards were selected from the Okanagan region of southern BC and one seed orchard was selected from central BC (O-223). The latter orchard, which is located in the Prince George Tree Improvement Station, was included for two reasons: it is outside of the Okanagan (i.e. 500 km further north) and it is within the natural range of lodgepole pine, unlike orchards in the Okanagan.

Study of seed quality

In 2013, a general seed quality survey was carried out in orchards O-218, O-223, O-238, O-241, O-307, O-313 and O-339. For each orchard, two cones from 10 different genotypes were collected (20 in total per orchard) and stored at 5 ˚C. Filled and total seeds per cone were recorded to determine the number of filled seeds per cone (FSPC). A one-way ANOVA was run on the data ($\alpha = 0.05$), followed by pairwise comparisons among means (Tukey) to detect significant differences among sites for seed yield, with the assistance of Minitab Statistical Software (version 17.3.1).

Studies of seed development

In 2012, the first year of study of lodgepole pine seed development, samples were taken only from O-307. Samples were collected weekly between mid-July and mid-September. Four cones each were removed from 10 genotypes (40 in total) and stored at 5 ˚C. Cones were subsequently dissected and their seed removed for further processing (see below).

In 2013, a histological study was done in parallel with the seed quality survey. Cones were collected from orchards O-218, O-223, O-238, O-241, O-307, O-313 and O-339. Seed samples were processed for microscopy from three collection points: mid-July, mid-August, and mid-September. The numbers of seeds sectioned per sampling date varied by orchard as follows: for orchards O-223, O-238, and O-241, $n = 56$ seeds; for orchards O-218, O-307, O-313, O-339, $n = 24 - 32$ seeds.

In 2014, a histological study was done that was begun earlier in the growing season and included not only seeds, but ovules, too. Cones were collected from five seed orchards (O-223, O-230, O-238, O-307, O-241). Seed samples were processed for microscopy at four sampling times: mid-May, early June, mid-June, and early July. For each sampling time point, a minimum of 30 seeds from each orchard were examined. Processing for microscopy was done as follows: seeds were dissected from cones and their coats removed prior to placing megagametophytes in 2.5 % glutaraldehyde in 100 mM phosphate buffer at pH 7.5. These were stored at 5 ˚C until further processing. Samples were processed following a published protocol (von Aderkas et al. 2015). Briefly, they were washed to remove the glutaraldehyde, then dehydrated in series of increasingly concentrated ethanol solutions, after which they were infiltrated with glycol-methacrylate (Historesin, Reichert-Jung) and embedded for sectioning. Five-micron-thick sections were stained with either Amido Black10B, Lactophenol Blue, or Toluidine Blue O.

Isolation and culturing of fungi

Standard methods were followed to provide a qualitative survey of fungal species. Field-collected samples of lodgepole pine plant material (seeds, needles, and conelets) were processed to obtain pure cultures of plant tissue-associated fungi. In order to surface-sterilize plant material and remove all epiphytic fungi, plant tissue samples were suspended in a 1 % solution of NaOCl for varying lengths of time (5 min for seeds, 4 min for needles, and 10 min for conelets). Subsequently, samples were rinsed twice in sterile distilled water before transfer to plates of potato dextrose agar (PDA). Sterilized seeds and needles were plated whole, while conelets were cut in half longitudinally before plating. Subcultures of fungal colonies growing from plant material were aseptically transferred to fresh PDA plates to obtain pure cultures for further study. Selected isolates were later used to inoculate Yeast Extract Peptone Dextrose (YPD) liquid medium to produce fungal mycelia for use in DNA extraction protocols and sequence analysis. Both agar and liquid cultures of fungal isolates were incubated at 24 °C to provide material for analysis.

We sampled fungi for three summers. In 2013, we limited ourselves to isolating fungal cultures from both external and internal surfaces of the seed, i.e. seed coat and megagameteophyte, respectively. A subsample of seeds collected in September from orchards O-241, O-238 and O-223 were processed to isolate fungal cultures, as described in the general methods (see above). In 2014, we sampled for fungal species from orchards O-223, O-230, O-307, O-241 and O-238. Maturing female cones were collected every 2 to 3 weeks over a period from mid-May to the end of August. Two cones from each of ten lodgepole pine genotypes (20 in total) were collected from the five seed orchards. Cones were stored at 5 ˚C until processed. To determine whether infection could be occurring during pollination, the sampling interval was increased to include conelets, i.e. pollinated, but unfertilized cones. Needles were also periodically sampled from May to July in all orchards. For a qualitative evaluation of communities of airborne fungal spores, sterile Petri dishes of PDA were exposed to the ambient air for one minute at selected seed orchards during the month of June. We also collected pollen from male cones at anthesis from a single tree at O-223 in late May. The pollen was distributed on a sterile Petri dish of PDA to determine presence of fungal inoculum. In 2015, we sampled for fungal species from one Okanagan orchard (O-338). Host tissues from ten lodgepole pine genotypes were sampled in mid-May and mid-June and included seeds, conelets, pollen (mid-May) and needles. Plant material was surface-sterilized before use in culturing procedures, as described above.

Identification of fungi

To initially confirm the presence of seed-associated fungi within living tissues, whole megagametophytes were ground and stored as individual samples in liquid nitrogen. Total DNA extractions were done according to manufacturer's protocols for the DNeasy Plant Kit (Qiagen, Inc., Toronto, ON). Purity and concentration of DNA were measured with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) prior to preparing DNA template dilutions for PCR reactions. A portion of the ribosomal RNA small subunit (*rRNA SSU*) region was amplified using the fungal rRNA-specific primers nu-SSU-0817 (5'-TTAGCATGGA ATAATRRAATAGGA-3') and nu-SSU-1536 (5'-ATTGCAATGCYCTATCCCCA-3') developed by Borneman and Hartin (2000). These primers selectively amplify fungal DNA. Preparation of PCR reactions and their parameters were done according to methods outlined by Borneman and Hartin (2000). Amplifications were completed using the Eppendorf Mastercycler® Gradient model 5331 (Eppendorf Canada, Mississauga, ON).

Pure cultures derived from other plant tissue-associated fungi were identified using both morphological and molecular approaches. In addition to observing morphological features of pure cultures grown on agar medium, representative fungal mycelia produced in liquid culture were harvested by centrifugation to remove liquid medium, ground in liquid nitrogen and their DNA extracted according to DNeasy Plant Kit (Qiagen) manufacturer's instructions. DNA purity and concentration were determined with a Nanodrop ND-1000 spectrophotometer prior to preparing DNA template dilutions for use in PCR reactions to amplify the internal transcribed spacer (*ITS*) of the rDNA cistron with the universal primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White et al. 1990). Each PCR reaction was performed in a 10 µL reaction volume using 10.0 ng of template DNA, one unit of DNA polymerase (Fermentas Dream Taq, Fermentas Canada Inc, Burlington, ON), a 1 x concentration of Dream Taq reaction buffer, 10 mM for all dNTPs (Promega, Madison, WI), and 10 µM of each PCR primer. Reactions were completed in an Eppendorf Mastercycler® Gradient model 5331. Reaction conditions included an initial denaturation step (2 min at 94°C), followed by 35 cycles of denaturation (10 *s* at 94 °C), annealing (10 *s* at 56 °C), and extension (30 *s* at 72 °C), and a final extension (2 min at 72 °C). Post reaction samples were held at 4 °C until processed.

For all PCR reactions, a 5.0 µL volume of each PCR product was mixed with 2 µL of 1:10 diluted loading dye (0.25% w/v bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol in dH_2O) and loaded into a 1.5% (w/v) agarose gel. Amplification products were separated by electrophoresis (150 volts for 1 hr and 30 min) that included a 100 bp DNA ladder (New England BioLabs, Inc., Ipswich, MA) for size estimation that was visualized with GelRed nucleic acid gel stain (Biotium, Hayward, CA) for 60 min (3x staining solution from 10,000x stock, w/v). This step was followed by illumination under UV light and digital image capture using the GelDoc XR+ Gel Documentation System. Amplified products (20-40 ng/µl) obtained for the ITS region were sequenced using the Sanger DNA sequencing process by Eurofins MWG Operon (Operon, Huntsville, AL, USA), following purification using the QIAquick PCR purification Kit (Qiagen, Germantown, WI, USA), according to the manufacturer's instructions. Amplified products for the fungal *rRNA SSU* region obtained from megagametophyte analysis were similarly purified and then ligated into a pGEM-T vector (Promega) to obtain single amplicons for sequencing, as described by Borneman and Hartin (2000). All nucleotide sequence results were manually analysed and manipulated using MEGA (version 6.0) Bioinformatic Software (Tamura et al., 2013). The opposing strands for each submitted sample were sequenced in each direction and aligned to verify nucleotide sequence quality, and to obtain a consensus sequence for use in searches. These sequences were then subjected to a BLASTn (nucleotide query) search, using default parameters of the National Centre for Biotechnology Information database (NCBI, web resource). Identity was based on the quality of the matched sequences in the NCBI database (threshold E-value < 1E-4 and sequence identity 98-100%). All identified nucleotide sequences will be submitted to the Genbank public sequence database.

Results

Seed losses

Okanagan seed orchards experienced substantial seed losses. Compared with O-223 in central British Columbia, all Okanagan seed orchards (O-218, O-223, O-238, O-241, O-307, O-313, O-339) had significantly lower seed yield (Table 2). By September, genotypes planted in the Okanagan had between 2.0 and 9.7 FSPC, whereas O-223 averaged 22.8 FSPC.

In 2013, high losses for most Okanagan seed orchards were obvious even by the first sampling date in mid-July (Figure 1). Okanagan seed orchards had FSPC values well below those from the orchard O-223 in central BC. A few Okanagan seed orchards, such as O-238 and O-313, had, in early July, FSPC values similar to those of O-223, but their seed yield steadily diminished in the following six weeks. By mid-September, all Okanagan seed orchards had a mean FSPC of less than 10. In contrast, O-223 from central British Columbia not only maintained a high percentage of FSPC throughout the study period but also produced the highest mean FSPC (Table 2).

Embryology and seed health

Sectioning provided information on seed development and fungal presence. The earliest sample date was in late May: at this time lodgepole pine ovules were unfertilized. Megagametophytes were healthy. Aborting megagametophytes were not observed, and fungal filaments were never seen, which means that infection of seed was not likely associated with pollination in the previous year. Fertilization had taken place in late May or early June (sections not shown). Embryogenesis occurred normally: zygote stage was followed by proembryo development in early to mid-June. Embryological development and storage accumulation in seed of trees in the most northerly orchard, O-223, had a relatively delayed development compared with trees in the more southern orchards. Seed development in all genotypes (northern or southern) was normal. Eventually, large precotyledonary embryos developed into cotyledonary mature embryos. As embryos developed, the megagametophyte began to accumulate storage reserves, such as lipid bodies, protein bodies and starch. By the time the embryos were precotyledonary, megagametophytes had accumulated substantial reserves. Healthy seeds sampled in early July were normal in appearance (Fig. 2A).

Seed degeneration and fungal infestation

Unhealthy seeds were readily identified and common after mid-July. There were two types of histological evidence: degeneration of cells and occurrence of fungal filaments. Degeneration was hierarchical, occurring first in megagametophytes and then in embryos. Megagametophytes showed increased separation of cells (Fig. 2B), followed by localized tissue death (Fig. 2C-D). In comparison, immature stages, such as unfertilized megagametophtyes or recently fertilized megagametophytes, did not show cell separation or degeneration. One major difference between immature and mature megagametophytes was the presence of starch, lipid bodies and protein bodies. The earlier stages, such as megagametophtyes in unfertilized ovules, and early developing seed had no evident storage reserves compared to mature seed. Fungi were noticeable only after the proembryo stage, when seeds had accumulated rich storage reserves (Table 3). Our histological study determined that fungal hyphae were observed in sections of approximately half of all seed from all seed orchards over the summer (Figure 3). By the end of August and early September, all tissues inside the integument of contaminated seed, with the exception of the megaspore wall, had been well-colonized by these fungi.

Identification of fungi

The selective amplification, cloning and sequencing of fungal DNA from within dissected megagametophyte tissue confirmed the presence of seed-associated fungi in mature lodgepole pine seed; the species *Sydowia polyspora* was detected in megagametophyte tissue obtained from seed collected in O-241. In subsequent analysis of other host tissue samples, species of fungi identified from degenerating seed included *Alternaria* sp., *Cladosporium* sp., *Fusarium* sp*.,* *Penicillium* sp., and *Sydowia polyspora*. Fungi were also present in surrounding tissues of conelets; maturing cones, needles and pollen, all of which appeared to be asymptomatic. Table 4 provides a summary of the fungal species and their locations within the tree. Only mature seed was destroyed.

Sydowia polyspora was the most common fungus detected (Table 4). It was often found within different surface-sterilized host tissues, demonstrating that it occurs frequently inside the living tissues of lodgepole pine. The verification of its widespread presence in seed at the end of the summer supports the histological study results that lodgepole pine seed tissues are infested with fungi, which are consuming the megagametophytes and embryos.

Discussion

A survey of seven seed orchards in the Province of BC detected variable levels of seed viability accompanied by an increase in the incidence of fungal colonization in mature seed of lodgepole pine. While all seed orchards demonstrated substantial fungal colonization of seeds in maturing cones, only those six orchards located in southern BC showed a significant decline in FSPC. The single orchard located in central BC near Prince George (O-223) showed a much higher level of FSPC, which agrees with previous findings for the period of 2006-2012 (Webber 2012). The O-223 site has a long and consistent history of superior seed yields, while the southern orchards in the Okanagan have been identified as problematic, when it comes to their significantly lower seed set (Woods 2012).

The incidence of fungal contamination of seed from the best of the seed orchards, O-223, was approximately 30 % and accounts for a large portion of failed seed. To get a measure of what this means in terms of seed losses, this number can be compared with the number of potentially fertilized megagametophytes. A study of potential seed (i.e. developing ovules in pollinated but unfertilized ovules) was done on eight genotypes from O-223, a northern orchard, comparing potential seed of ramets of identically-aged trees of the same eight genotypes growing in O-307, a southern orchard. The potential seed was found to be 40, regardless of location (von Aderkas and Kruithof 2010). In our current study, we were unable to compare identical genotypes in both locations, because many had died in the interim. However, if we allow comparison of genotypes at each site, some interesting conclusions can be drawn. Using the data in our current study, and the data from von Aderkas and Kruithof (2010), we know that average FSPC for O-223 is 22.75, which represents 22.75/40 realized seed/potential seed, or 57 % of potential seed, and its inverse, 43 % failed seed. By comparison, O-307 has an average of only

2/40 seed/potential seed, or 5 % of potential seed, and its inverse, 95 % seed loss. How much of this failed seed is consumed by fungi? If we use the values in Figure 3, in O-223 about three-quarters of the failed seed is consumed by fungi (30 % seed have fungi present/43 % failed seed), compared with O-307, in which fungi consume about 51% of failed seed (49 % fungi present/95 % failed seed). In short, fungi account for most of the seed loss in O-223, but only about half the seed loss in the Okanagan. Consideration of seed losses to *Leptoglossus occidentalis* was not part of our study, but based on previous studies of Okanagan orchards, insect-related losses are likely to constitute a substantial percentage of the remaining portion (Richardson et al. 2016). Abiotic factors, such as high temperatures, may have an influence (Woods 2012). We did not include climatic factors in our study, but this may be an informative avenue to pursue. Studies using climate modeling and historical lodgepole pine seed production have shown that climate factors influence seed production (Lew et al. 2017).

Geographical location can make a difference to some aspects of development. Von Aderkas and Kruithof (2010) found that the number of sterile ovuliferous scale-bract complexes is significantly higher in cones from seed orchards in southern BC (O-307), compared with cones from the identical genotypes sampled in central BC (O-223). In short, the cones in southern BC were similar in size but composed of many more parts. This is not linked to seed development, as the number of ovules was indistinguishable. It does illustrate that biological differences in one of the target tissues of the fungi – conelets – are correlated with location. Again, we do not know the influence of climate on cone morphology, but it appears that abiotic factors can influence more than one biological process.

Seed loss shows stage specificity. Events that contribute to seed loss in conifers may occur at different stages of embryological development (Sarvas 1962), a process that takes over three years to complete in lodgepole pine. Long-shoot buds form male and female buds in the first year (Owens et al. 2005; von Aderkas et al. 2007). The latter expand in the spring of the next year and these conelets become receptive to pollen. By the end of the second growing season, megagametophtyes have begun to differentiate inside ovules. In the third growing season, megagametophtyes form eggs, which are then fertilized by male gametes. The resulting seeds are histologically mature by mid-July and seed set levels are usually acceptable at this time (Owens et al. 2005). It is at this final stage (early August) when seed set can decline rapidly and significant seed losses occur, the onset of which depends on the tree genotype (Giampa 2012). The current study demonstrated a similar pattern of seed loss in August and September for the Okanagan orchards.

The widespread occurrence of *S. polyspora* and other fungi in the maturing seeds has not been documented previously. Our results suggest their presence in seed is common in lodgepole pine. The other genera that we found, i.e. *Alternaria*, *Cladosporium* and *Fusarium* spp. (anamorph of *Gibberella avenacea*) contain a number of important plant pathogens, but are also known to include common endophyte species with a broad host range for many plant taxa, including *Pinus* spp. (Chandra Paul and Yu 2008). They are considered to be horizontally transmitted, although there is evidence for vertical transmission via spores associated with plant pollen (Christian et al. 2017). Given the variable biotrophic strategy among species of these genera, it is difficult to know whether the isolates detected in our study were strict mutualists, parasites, or opportunistic pathogens. Although fungal species occurrence was not quantified in this study, the ascomycete species *Sydowia polyspora* (anamorph *Hormonema dematioides*) was often found in host tissues: it was detected in most orchards, in all tissue types sampled, as well as in association with pollen. This fungus has been detected previously in the foliage of a wide range of conifers, including pine, fir, spruce, hemlock, larch and cedar (Smerlis 1970; Funk et al. 1985; Talgø et al. 2010*a*, 2011). It is known to be an opportunistic foliar pathogen causing current season needle necrosis (CSNN) in *Abies* spp. (Talgø et al. 2010*b*) and in *Pinus halepensis* Mill. (Tinivella et al. 2014). *Sydowia polyspora* is a common needle endophyte in both *Pinus sylvestris* (Pirttilä et al. 2003; Terhonen et al. 2011) and *Picea abies* (L.) H. Karst (Müller et al. 2001). This species has been recorded more frequently when fungi from the genera *Alternaria*, *Cladosporium* and/or *Epicoccum* spp. were found in *Pinus* spp. (Kowalski 1993; Ganley and Newcombe 2006; Terhonen et al. 2011). There is limited evidence to indicate that the fungus will infect seeds, but it is not known to have a negative impact on seed health (Cho et al. 2007; Talgø et al. 2010*a*, 2011). In the current study, the detection of *S. polyspora* in surface-sterilized seeds, as well as the decline in seed viability associated with fungal colonization, suggests that this species may reduce lodgepole pine seed health under certain conditions. Although this species was found in different host tissues, its biotrophic status appeared to be stage- and context-dependent. Fungi were found in conelets (receptive cones), which had ovules with few accumulated resources. As the megagametophytic storage products accumulated in the maturing seeds, fungal colonizers consumed this nutrient source. Further study is required to confirm that nutritional resources in maturing seeds facilitate the growth of fungi already present within cones.

The correlation of seed viability with the geographical location of the orchards poses interpretive problems. It is difficult to separate the differential effects of climate on growth of either seed or of the various tissue-borne fungi. Serious abiotic stress is known to increase susceptibility of a plant host to fungi (Desprez-Loustau et al. 2006). If we compare the climate in the Okanagan Valley region with central BC, the Okanagan has a combination of higher average temperatures and lower precipitation during summer months. However, this correlation does not prove causation and further work is required. Another possibility that may have geographical underpinnings is the distributions of fungi. Where they overlap, interactions among fungal species may be possible. For example, one of the fungal species that we identified from lodgepole pine seeds and tissues – *S. polyspora* – both enabled *Dothistroma* needle blight in *Pinus ponderosa* and increased the severity of disease symptoms (Ridout and Newcombe 2015). In Norway spruce, Rajala et al. (2013) observed a mixture of fungi in healthy needles that included known endophytes, e.g., *Lophodermium piceae*, and potential pathogens, e.g., *Phoma herbarum*. We know very little of the fungal microbiome of lodgepole pine trees. However, any future study that investigates the role of *S. polyspora* in seed decline needs to consider the dynamics of the endophyte community within the host. Although our research did not aim to study endophytes within lodgepole pine, the presence of a number of different fungal species inside seeds and tissues hints at a larger fungal microbiome. This study used a culture medium (PDA) suitable for the growth of many fungi; however, some fungal taxa are more restricted in their growth requirements and this likely limited the diversity of species detected. In order to obtain a more complete assessment of the fungal microbiome, a metagenomics approach would be the next logical phase in this study. It would allow a more comprehensive and effective quantification of the fungal community in host tissues, as well as the evaluation of community structure over time as cones develop.

In other plant species the composition of endophytic species is known to vary over time as host tissue ages (Pirttilä et al. 2003; Scattolin and Montecchio 2009), among different host tissues (Carroll and Carroll 1978), under different host growing conditions in different geographical regions (Ganley et al. 2004; Terhonen et al. 2011), and among different genotypes of a single host species (Rajala et al. 2013). Factors that influence the composition of fungal endophytes and the broader microbiome are complex and not entirely understood. There is likely to be an important role for host genotypic variability and species' interactions among members of the fungal community. Both of these factors are shaped by environmental conditions. Environmental factors can shape the composition of the microbiome by providing conditions that favour the growth of some fungal taxa that will exploit a susceptible host. Conditions of host biotic or abiotic stress may modify the biotrophic habit of the fungal species. For example, *Sphaeropsis sapinea*, normally a fungal endophyte of *Pinus*, can cause significant losses in many other conifers (Burgess et al. 2001; Flowers et al. 2003). The composition of the fungal community can influence the success of pathogens present in host tissues. In a study of *Pinus ponderosa*, common fungi isolated from the host microbiome were inoculated on to newly emerging needles of seven-year-old trees (Ridout and Newcombe 2015). Five of six fungi significantly modified disease severity. One fungus (*Penicillium goetzii*) reduced the severity of foliar disease, whereas four fungi (*Sydowia polyspora*, *Bionectria ochroleuca*, *Penicillium raistrickii*, and *Elytroderma* sp.) increased severity. In a similar study that examined the role of common foliar fungi isolated from wild populations of *Populus trichocarpa*, Busby et al. (2016*a*) identified common, non-pathogenic fungal species that influenced the severity of *Melampsora* leaf rust disease in both controlled experiments and field surveys. These fungi included both pathogen antagonists and facilitators, among them common broad host-range endophytes, such as *Cladosporium*, *Epicoccum* and *Alternaria* spp.. Non-pathogenic endophytic fungal species in host tissues are therefore likely to influence disease development. The composition of the community of non-pathogenic endophytic fungi will in turn be influenced by both host and environmental conditions (Busby et al. 2016*b*; Christian et al. 2017).

In the current study, we have shown that seed health of lodgepole pine genotypes declines during histological and physiological maturation stages in seed orchards, especially in those sited in the Okanagan. Our report of consumption of failing seed by resident endophytes is a finding that should encourage further study of fungal community structure within lodgepole pine host tissues. The biotrophic status of some fungal species will likely vary and be influenced by microbial interactions, host status and environmental conditions. Understanding how these factors interact will allow us to better understand tree health in relation to seed reproduction.

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Table 1: Seed orchard locations.

Orchard number	Orchard location
O-218	Vernon Seed Orchard Company, Vernon, BC
O-223	Prince George Tree Improvement Station, Prince George, BC
O-238	Kettle River Seed Orchard, Kettle River, BC
O-241	Sorrento Seed Orchard, Sorrento, BC
O-307, O-230	Kalamalka Seed Orchard, Vernon, BC
O-313, 0-338	Pacific Regeneration Technology, Armstrong, BC
O-339	Eagle Rock Seed Orchard, Armstrong, BC

Table 2: Mean FSPC at each sampling interval (*n* = 20) and the pooled mean of the five sample intervals (*n* = 100) for each orchard site, for maturing cones collected in seven BC seed orchards from July to September of 2013.

	Weekly sampling interval (date)*					
Seed orchard	**1 (Jul 15)**	**2 (Jul 22)**	**4 (Aug 5)**	**6 (Aug 19)**	**9 (Sep 9)**	**Pooled data**
O-218	7.1 b	7.8 ab	6.9 c	4.1 c	5.5 bc	6.3 cd
O-223	17.5 ab	19.8 a	19.1 a	23.3 a	22.8 a	20.5 a
O-238	20.3 a	19.9 a	16.9 ab	11.9 b	6.0 bc	15.0 b
O-241	12.6 ab	14.5 ab	8.1 bc	3.0 c	3.8 bc	8.4 c
O-307	5.8 b	5.05 b	3.1 c	2.7 c	2.3 c	3.8 d
O-313	17.1 ab	18.7 a	11.3 abc	11.2 b	9.7 b	13.6 b
O-339	10.3 ab	8.2 ab	7.5 c	2.3 c	2.0 c	6.0 cd

* Significant differences among sites are indicated by differing letters within a single column (Tukey, $\alpha = 0.05$).

Table 3: Timelines of seed development, accumulation of storage products and detection of fungal hyphae in five lodgepole pine seed orchards.

		Sample date			
Seed orchard	**Type of observation***	**May 22**	**June 4**	**June 19**	**July 3**
O-223	Embryological stage	unfertilized	unfertilized	proembryo	precotyledonary
	Storage products	none	none	moderate	abundant
	Fungal hyphae	none	none	evident	evident
O-230, O-307	Embryological stage	unfertilized	proembryo	precotyledonary	cotyledonary
	Storage products	none	moderate	abundant	abundant
	Fungal hyphae	none	none	evident	evident
O-238	Embryological stage	unfertilized	unfertilized	proembryo	precotyledonary
	Storage products	none	none	moderate	abundant
	Fungal hyphae	none	none	evident	evident
O-241	Embryological stage	unfertilized	proembryo	precotyledonary	cotyledonary
	Storage products	none	moderate	moderate	abundant
	Fungal hyphae	none	none	evident	evident

* A minimum of 30 seeds were examined for each orchard at each sampling time point.

Table 4: Summary of identified fungal isolates obtained from different plant tissues and orchards.

	Seed orchard[*]				
Tissue	**O-223**	**O-238**	**O-241**	**O-307**	**O-338**
Seed	*Sydowia polyspora* *Alternaria* sp.(2) *Penicillium* sp. (1)	*Sydowia polyspora* *Alternaria* sp. (8) *Cladosporium* sp. *Penicillium* sp. (2)	*Sydowia polyspora* (8) *Alternaria* sp. (3) *Fusarium* sp. (2)	*Sydowia polyspora* (5) *Cladosporium* sp. *Fusarium* sp. *Penicillium* sp.	*Sydowia polyspora* (32) *Alternaria* sp. (3)
Wild seed[†]	*Sydowia polyspora* (8)				
Megagametophyte[‡]			*Sydowia polyspora* (2)		
Conelet	*Sydowia polyspora* (2)	*Sydowia polyspora*(2)	*Sydowia polyspora* (2)	*Sydowia polyspora* (2) *Alternaria* sp. (2)	*Sydowia polyspora* (10) *Alternaria* sp. (2) *Cladosporium* sp. (2) *Fusarium* sp. (2)
Cone	*Sydowia polyspora*	*Sydowia polyspora*	*Sydowia polyspora*	*Sydowia polyspora*	*Sydowia polyspora*
Pollen[§]	*Sydowia polyspora* (2)				*Sydowia polyspora* (6)
Needle	*Sydowia polyspora* *Alternaria* sp.	*Sydowia polyspora* *Alternaria* sp.	*Sydowia polyspora* (2) *Alternaria* sp. *Cladosporium* sp.	*Sydowia polyspora* (2) *Alternaria* sp.	*Sydowia polyspora* (7) *Alternaria* sp. (6) *Cladosporium* sp. (2) *Fusarium* sp. (1)
Air column[‖]	*Sydowia polyspora* (1) *Alternaria* sp. *Cladosporium* sp.	*Sydowia polyspora* (3) *Alternaria* sp. *Cladosporium* sp. (1) *Fusarium* sp.	*Sydowia polyspora* *Alternaria* sp. *Fusarium* sp.	*Sydowia polyspora* *Alternaria* sp. *Fusarium* sp.	

[*] Species identified by the examination of morphological features. The number of isolates for which identity was also confirmed by sequence analysis is indicated in parentheses.

[†] Sampled only from wild trees beside O-223.

[‡] Whole megagameteophytes were sampled only from O-241.

[§] Pollen was sampled only from O-223 and O-338.

[‖] Air column was not sampled in O-338

Figure captions

Figure 1. Scatterplot of average filled seed per cone (FSPC) for seven lodgepole pine seed orchards in British Columbia in 2013. Linear trend lines were fitted to the mean values calculated for each orchard, including O-218 (▲), O-223 (♦), O-238 (+), O-241 (⋇), O-307 (■), O-313 (x), and O-339 (●). At each sampling time point, mean FSPC values were calculated from 20 harvested cones (2 cones from each of 10 genotypes that are unique to each orchard). Sampling took place each week for 9 weeks beginning July 15 (sample 1) and ending September 9 (sample 9).

Figure 2A-D. Longisections of lodgepole pine seed. **A.** Healthy megagametophyte (m) and embryo (e). **B-C.** Degeneration was marked by the appearance of intercellular spaces (i) within megagametophyte tissue. **D.** Fungal hyphae (f) were visible in the larger intercellular spaces and within individual cells. Bar = 200 µm (A) and 50 µm (B-D).

Figure 3. Average percentage of seed in which fungal hyphae were observed, obtained from seven seed orchards in British Columbia that were sampled at three time points over the summer (mid-July, mid-August, mid-September, *n* = 24-56). Significant differences among sites are indicated by differing letters above each column (Tukey, *α* = 0.05).

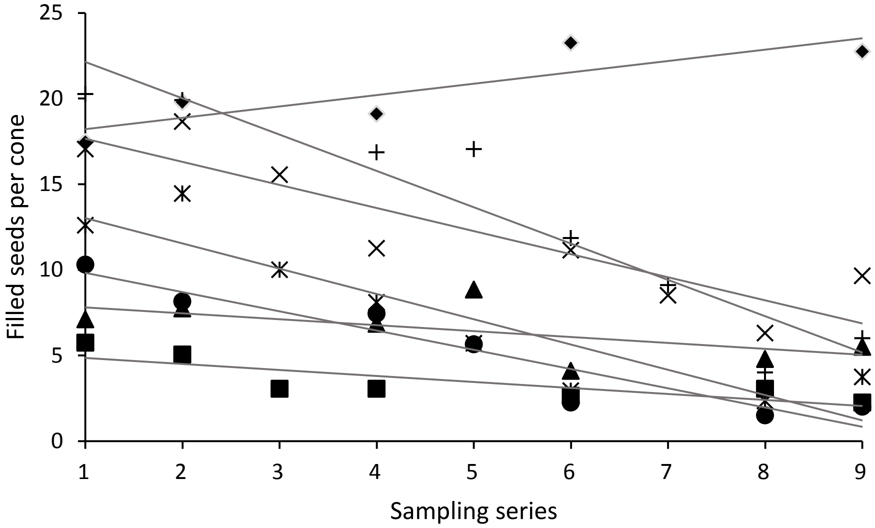
25
20
15
10
5
0
Filled seeds per cone
1
2
3
4
5
6
7
8
9
Sampling series

Figure 1.

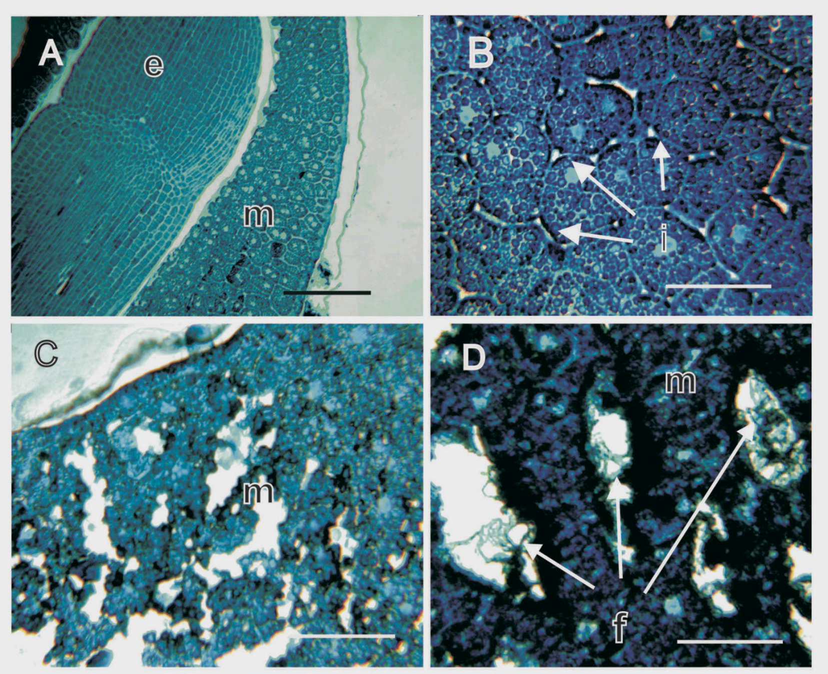

Figure 2A-D.

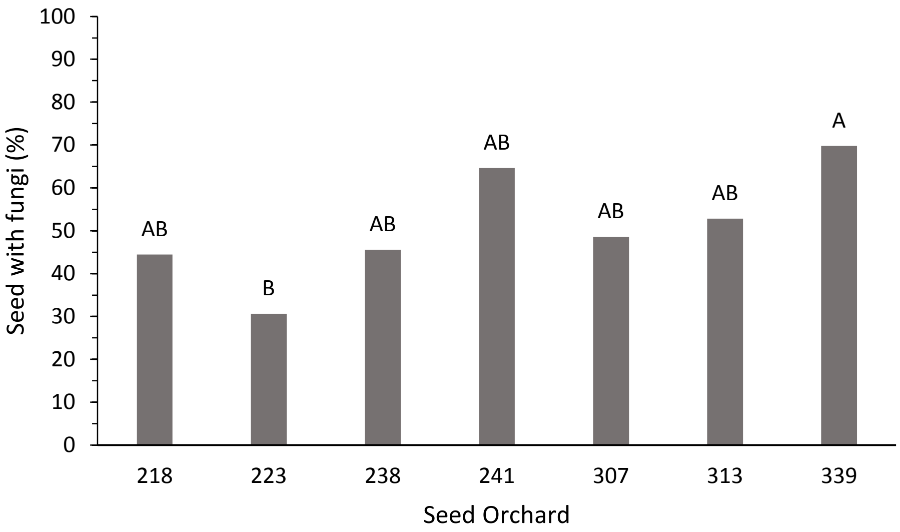
Seed with fungi (%)
100
90
80
70
60
50
40
30
20
10
0
AB
B
AB
AB
AB
AB
A
218
223
238
241
307
313
339
Seed Orchard

Figure 3.