The effect of UV-C light and heat treatments on fungi and germination of *Pinus contorta*, *Pinus sylvestris* and *Pseudotsuga menziesii* seeds

Project number: 1407

Duration of the project: April 2022 – December 2022 (extended until October 2023 as agreed on with the project partners and Partnerskap Alnarp)

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Project aim

The reduction of potential plant pathogens in seeds is crucial to ensure production of healthy seedlings and successful forest regeneration. Environmentally friendly treatments of seeds, e.g., treatments based on application of UV-C light or heat seem promising, but more research is needed to determine their efficacy in eliminating fungi and their effects on seed germination.

In this project, we assessed the effects of heat and UV-C light treatments on fungal communities and germination of conifer seeds. We focused on seeds of three tree species used in Swedish forestry: lodgepole pine (*Pinus contorta*), Scots pine (*Pinus sylvestris)* and Douglas fir (*Pseudotsuga menziesii*). Seed fungal communities were examined using metabarcoding targeting ribosomal RNA (rRNA) molecules, which allowed for the simultaneous identification of many, exclusively viable and active, fungal taxa within and across samples. Fungal communities and seed germination were assessed and compared before and after the seed treatments to determine that the treatments do not affect seed germination and provide maximal reduction of fungal inoculum.

Methods

Seeds used in this study were obtained from Svenska Skogsplantor and represent certain seed lots used for forest regeneration in Sweden. We focused on five seed lots belonging to three conifer species, *P. contorta* (seed lots PC14 and PC15), *P. menziesii* (seed lot PM) and *P. sylvestris* (seed lots PS18 and PS20). *P. menziesii* was used instead of Norway spruce (*Picea abies*) as it was not possible to acquire Norway spruce seed in time for the experiment. Detailed information on collection year and site is provided for each seed lot in Breza (2023).

Seed treatments

Five samples of 300 seeds each were taken from untreated (control) seeds and from seeds subjected to dry heat treatment at 55 °C for 8 h (later referred to as “heat treatment”) and two

ultraviolet (UV)-C treatments. The UV-C treatments consisted of running the seeds through a CleanLight machine once or three times; later referred to as “UVC1” and “UVC3”, respectively or “UVC” when referring to both). Heat treatments were done at the Forest Pathology Lab in Alnarp and UVC treatments at Svenska Skogsplantor in Lagan. The treatments are described in detail in Breza (2023). Treatments were done for all seed lots except for PC14 which was not UVC treated, and for PS20 which was not heat treated. Seed were obtained and treated during April-September 2022.

Assessment of fungi and germination tests

Fungal assessment and germination tests were done for the seed samples taken from untreated and treated seeds. Fungal assessments were done at the Forest Pathology Lab in Alnarp, and germination tests were done by the project partner, Svenska Skogsplantor, at the Seed Unit in Lagan.

One sample of 300 seeds from untreated and treated seeds from each seed lot was used for fungal assessment by traditional *in vitro* culturing (17 samples in total, each consisting of 300 seeds). The procedures and results of this work are described in Breza (2023), as well as the methodology and results of the germination tests. Lab work for this part was done in April-August 2022, and data analyses and thesis writing were done afterwards with the final thesis published in October 2023. Additional costs for the fungal assessments using traditional *in vitro* culturing were covered by a Forest Damage Centre Grant.

Within the Partnerskap Alnarp project reported here, fungal assessments were done from three samples per seed lot of untreated and treated seeds (51 sample in total, each consisting of 300 seeds) using rRNA metabarcoding as described below. This part was done during September 2022 until August 2023 and included optimization of lab protocols including total RNA extraction protocols and complementary DNA (cDNA) synthesis, processing of samples, sequencing and bioinformatic analysis.

Assessment of fungi using rRNA metabarcoding

Seed samples were ground with mortar and pestle on liquid nitrogen and stored at -80°C until RNA extraction. All equipment used during grinding was kept on liquid nitrogen and washed and cleaned with RNase AWAY (removes RNase & DNA contamination) in between samples.

All RNA extractions were done using E.Z.N.A.® Plant RNA kit (OMEGA BIO-TEK) following manufacturer`s protocol for difficult sample types. Multiple extractions per sample were done to obtain enough RNA (~1 ng) for the synthesis of cDNA by reverse transcription. Quantity and quality of RNA extracts was assessed by a DS-11 UV-Vis Spectrophotometer (DeNovix) and multiple extracts obtained from the same sample were pooled. Additional analyses of the

quality of pooled RNA extracts were done using Agilent RNA 6000 Pico Kit with an Agilent 2100 bioanalyzer (Agilent) and quantities were measured with the Qubit Fluorometer using Qubit™ RNA Broad Range (BR) Assay Kit (Invitrogen™) following manufacturer`s instructions. DNA contamination from RNA samples was removed prior to cDNA synthesis using TURBO DNase (Invitrogen™) following a modified manufacturer's protocol where 4 U of DNase was used per reaction and each reaction was done for 60 min instead of 30 min. After DNase treatment, a 35 cycle PCR targeting the internal transcribed spacer (ITS) was run with the primer pairs ITS1 and ITS4 using the DreamTaq (Thermo Scientific™) following the manufacturer's protocol. The absence of the PCR products, and therefore, DNA in the RNA extracts was confirmed by visual inspection of the PCR products on an agarose gel (1.2% in TAE). RNeasy MinElute Cleanup Kit (QIAGEN) was used for the cleanup and concentration of RNA which was then used for the synthesis of cDNA by reverse transcription using the SuperScript™ IV First-Strand Synthesis System (Invitrogen™). cDNA synthesis was done using the included random hexamer primers and including the optional RNA removal with RNase H. cDNA was then cleaned using AMPure XP beads (Beckman Coulter) using a 1.4 bead:sample ratio. After cleanup, cDNA was stored at -80 °C until submission for sequencing.

Sequencing and bioinformatics

Samples containing cDNA were sent for sequencing of the ITS2 region on Illumina Novaseq 6000 platform at BMKGENE (Germany) using primers ITS3 (F:GCATCGATGAAGAACGCAGC) and ITS4 (R:TCCTCCGCTTATTGATATGC). Demultiplexing and bioinformatic analyses were done by Novogene. Raw reads were first filtered by Trimmomatic v0.33 (Bolger et al., 2014). The primer sequences were then identified and removed by cutadapt 1.9.1 (Martin, 2011). This step also removed Illumina adaptor sequences and poor-quality base calls. Quality filtering, chimera checking, and paired end read joining was done using dada2 (Callahan et al., 2016). Obtained amplicon sequence variants (ASV) were then clustered into operational taxonomic units (OTUs) using vsearch (Rognes et al., 2016) and identified using Naive Bayes Classifier against the Unite data base (Nilsson et al., 2019).

Data analyses

All analyses were done in R version 4.3.1 (R Core Team, 2023). All visualizations were done using ggplot2 package (Wickham, 2016).

The differences in number of fungal OTUs (i.e., OTU richness; based on presence absence data) per sample were assessed visually 1) across treatments (i.e., control, heat treatment and UVC treatment) and 2) across treatments and seed lots. The changes of fungal community composition between control samples and samples that were subjected to different treatments

were assessed visually using non-metric multidimensional scaling (NMDS) with metaMDS function from vegan package (Oksanen et al., 2018). All samples were rarefied to 51,033 reads which represents the minimum number of reads found in a sample. Sørensen dissimilarity (based on presence absence data) was used as diversity measures and differences in between sample dissimilarities were assessed 1) across treatments and 2) across seed lots.

To better explore the effects of treatments on fungal community composition, rare OTUs were removed from the original data set by keeping only the OTUs with the read count greater than 1% of the total reads in that sample. Venn diagrams were used to show the number of OTUs unique to, or shared between, treatments for each seed lot (reads from three samples from the same treatment were pooled per seed lot). Furthermore, the relative number of reads belonging to OTUs assigned to different fungal genera was plotted across treatments and seed lots to visually demonstrate the effects of treatments on fungal community composition. For this, the reads belonging to each OTU were averaged for three samples per seed lot-treatment combination.

Fungal genera identified from a filtered data set were parsed to guilds using FunGuild (Nguyen et al., 2016). For the fungal genera which were not parsed to a guild in FunGuild, a google scholar search was done to determine if they are potential plant pathogens.

Results

Firstly, there were no negative effects of heat or UVC treatments on germination. In this report, we do not describe these results in detail as they are already described in Breza (2023). Instead, we focus on the results of rRNA metabarcoding for characterization of fungi in untreated and seeds treated by heat and UVC light.

rRNA metabarcoding revealed 4,146,714 raw reads (81,308 ± 839 reads per sample; mean ± SE). Of these, 84% remained after quality filtering (3,475,483 reads in total and 68,147 ± 756 reads per sample; mean ± SE). The filtered reads were assigned to 1,769 fungal OTUs. Almost 70% of obtained OTUs belonged to the phylum Ascomycota (1,214) and 25% to Basidiomycota (451), while the rest belonged to 13 other phyla. The top six classes to which 75% of all OTUs were assigned included: Sordariomycetes (375; 21%), Dothideomycetes (329; 19%), Agaricomycetes (173; 10%), Eurotiomycetes (159; 9%), Leotiomycetes (153; 9%) and Tremellomycetes (131; 7%). The rest of the OTUs were assigned to one of the 44 classes. Twenty-two OTUs were not assigned to any class. Almost 85% of all OTUs (1,505) were assigned to one of the 771 genera, and of those, 63% (1,107) to one of 1,106 species.

Overall, the number of fungal OTUs was similar in treated as in the control seeds (Fig. 1a; 265 ± 18; overall mean ± SE). On a seed lot level (Fig. 1b), a reduction of the number of OTUs was

only observed after the heat treatment in the seed lot PC14, although the samples showed high variation in OTU richness in control seeds of PC14 indicating that the trend is not robust. In all other seed lots, the number of OTUs after the treatments was either similar or even higher than in control seeds (Fig. 1b). Differences in the number of OTUs in untreated (control) samples were observed among seed lots with PS18 having double, and PC14 having almost triple the average number of OTUs per control sample compared to PC15 and PM (Fig. 1b).

Fungal communities did not show any clear pattern in OTU composition across treatments, as suggested by the NMDS plot (Fig. 1c). The only differences were observed on a tree species level where fungi associated with *P. menziesii* clearly differed from fungal communities associated with *P. contorta* and *P. sylvestris* seed lots (Fig. 1d).

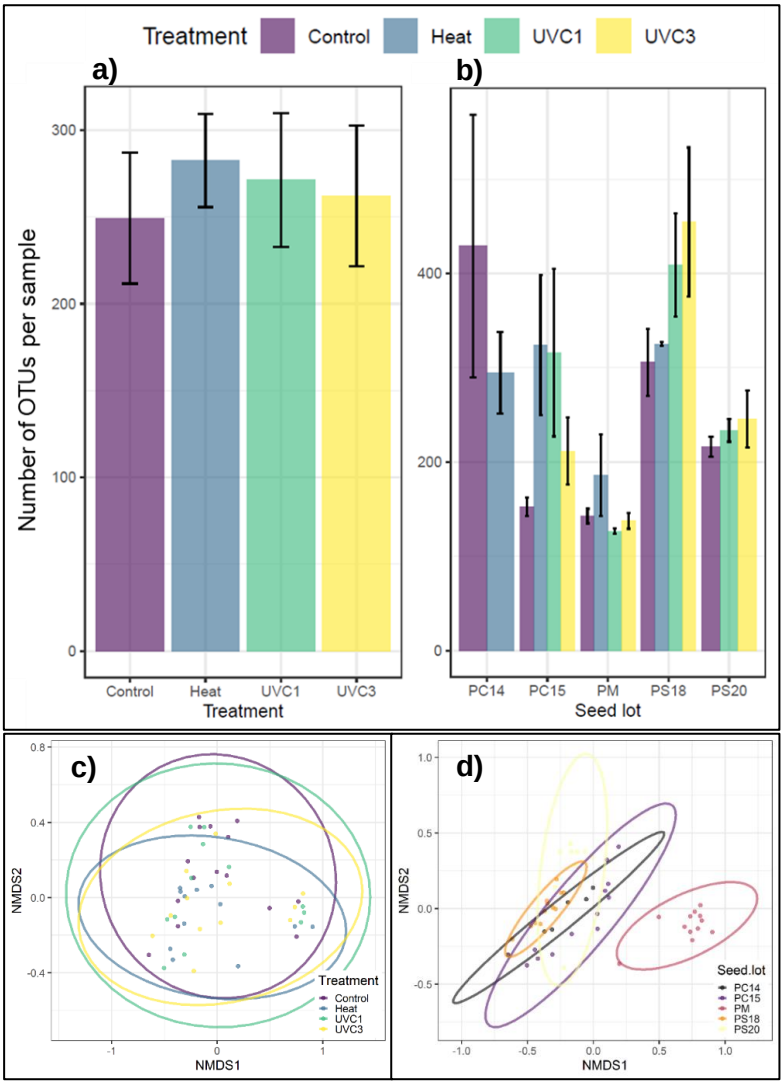

Figure 1 OTU richness (i.e., number of OTUs) per sample across treatments (a) and treatments and seed lots (b) and non-metric multidimensional scaling (NMDS) plots based on Sørensen`s dissimilarities (c, d). Treatments are control, heat treatment at 55 °C for 8 h, UVC treatment repeated one time (UVC1) and three times (UVC3). Seed lots belong to *Pinus contorta* (PC14 and PC15), *Pseudotsuga menziesii* (PM) and *Pinus sylvestris* (PS18 and PS20). In NMDS plots (c, d) points represent samples and ellipses represent 95% confidence intervals based on standard error of the group centroids (mean). Data are colored by treatments (c) and seed lots (d).

Discarding OTUs that comprised less than 1% of the total reads in each sample resulted in an elimination of 97% of OTUs with the filtered data set consisting of only 45 OTUs. These remaining OTUs represent the dominant community in our samples, and based on their taxonomy (i.e., 37 OTUs were assigned to 32 genera and eight OTUs were not assigned to a genus), twelve of them were assigned to a potentially plant pathogenic fungal genus — *Alternaria* (OTU 255), *Botryosphaeria* (OTU 12), *Cercospora* (OTU 82), *Clonostachys* (OTU 885), *Colletotrichum* (OTU 859), *Diaporthe* (OTU 845, OTU 847), *Diplodia* (OTU 15), *Fusarium* (OTU 945, OTU 947), *Gibberella* (OTU 961), *Neocatenulostroma* (OTU 114), *Periconia* (OTU 214), *Trichothecium* (OTU 931). Examining the Venn diagrams showing shared and unique OTUs between treatments across seed lots (Fig. 2) and relative number of reads (i.e., relative abundance) of OTUs belonging to different genera across treatments and seed lots (Fig. 3) reveals that only a few OTUs appear in the control seeds but not in treated seeds, and, thus, represent those OTUs that were likely eliminated by the treatments.

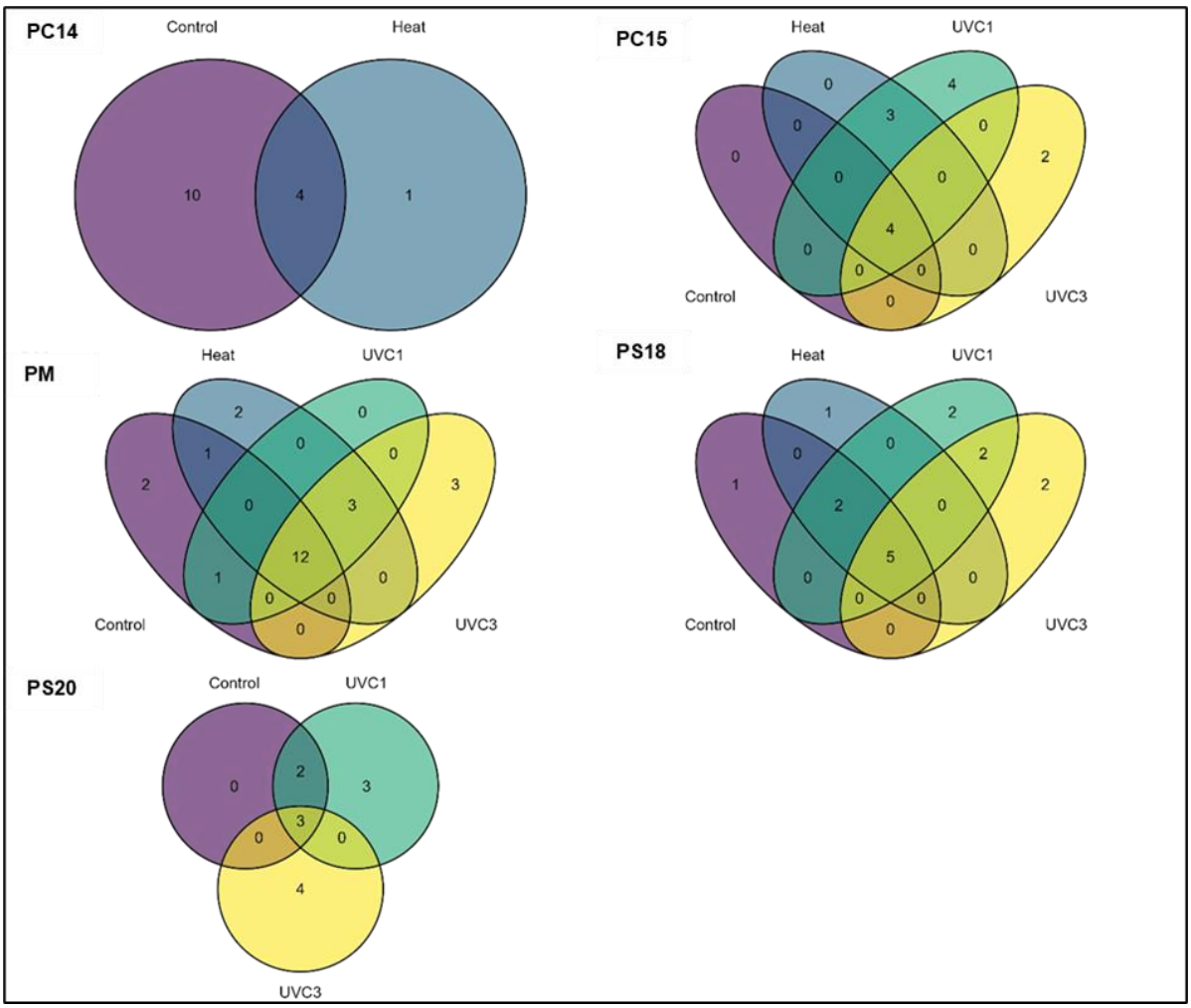

Figure 2 Venn diagrams showing the number of OTUs unique for each treatment or shared between the treatments. This is shown for each seed lot separately. Seed lots belong to *Pinus contorta* (PC14 and PC15), *Pseudotsuga menziesii* (PM) and *Pinus sylvestris* (PS18 and PS20). The data set consists of OTUs representing 99% of reads per sample and three samples belonging to the same seed lot and treatment are pooled.

This number was the highest for *P. contorta* PC14 seed lot in which only four out of total 14 OTUs that appeared in control seeds were not eliminated by the heat treatment (*Ramichloridium* sp., *Pleonectria* sp., *Hormonema* sp. and one unidentified OTU). On the other hand, 10 OTUs were detected in the control but not in heat-treated seeds and those were likely eliminated by the heat treatment. Those OTUs belonged to genera *Vishniacozyma*, *Kazachstania*, and potentially pathogenic, *Neocatenulostroma, Gibberella*, *Colletotrichum* and *Alternaria*. In addition, three of those 10 OTUs were not identified to genus. One OTU belonging to the potentially pathogenic genus *Cercospora* was detected in heat-treated, but not in control seed and thus represents possible contamination from the post-treatment seed handling.

In *P. contorta* PC15 seed lot all OTUs present in control seeds were found in the treated seeds suggesting that neither UVC nor heat treatment was able to eliminate those fungi (i.e., *Ramichloridium*, *Pleonectria*, and *Hormonema*). In addition, several OTUs were found in treated, but not in control seed and as such might represent contaminants introduced during post-treatment seed handling. Those potential contaminants included two OTUs belonging to genera *Alternaria* (potentially pathogenic) and *Vishniacozyma*, and one unidentified OTU which were found in heat treated, but not in control seeds. The same OTUs were found in UVC1 treated seed which also contained three additional OTUs belonging to genera *Cladosporium*, *Colletotrichum* (potentially pathogenic) and *Kazachstania*, and one OTU not

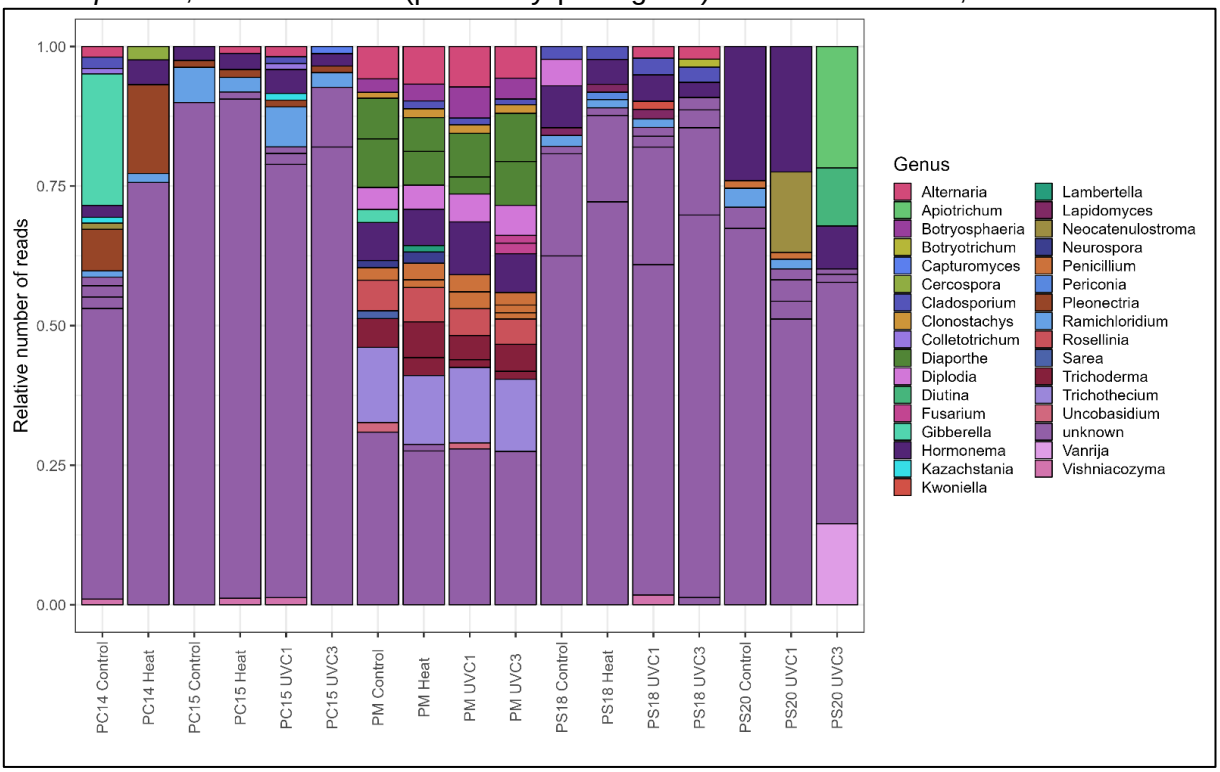

Figure 3 Relative number of reads (i.e., relative abundance) of OTUs belonging to different genera across treatments and seed lots. The data set consists of OTUs representing 99% of reads per sample. The reads belonging to each OTU were averaged for three samples per seed lot-treatment combination.

identified to the genus. UVC3 treated seed contained one OTU belonging to the genus *Capturomyces* and one unidentified OTU which were not found in control seed.

In *P. menziesii* PM seed lot, twelve out of 16 OTUs found in control seeds were still present after all treatments and those belonged to genera *Hormonema*, *Penicillium*, *Rosellinia*, and potentially plant pathogenic *Alternaria, Botryosphaeria*, *Clonostachys*, *Diaporthe*, *Diplodia*, *Trichoderma*, *Trichothecium*, and one unknown OTU. On the contrary, some OTUs were likely eliminated by all or some treatments; two OTUs appeared only in control seeds and not in the treated seeds (potentially pathogenic *Gibberella* and non-pathogenic *Neurospora*), one OTU appeared in control and heat-treated seeds, but not in the UVC treated seeds (*Cladosporium*) and another OTU appeared in control and UVC1 treated seeds, but not in the heat and UVC3 treated seed (*Uncobasidium*). Eight OTUs were found only in treated, and not in control seeds (and thus might represent post-treatment contaminants) — genera *Cladosporium*, *Penicillium* and *Trichoderma* were represented by one OTU each in all treated seeds. In addition, one OTU identified as *Lambertella* sp. and one unidentified OTU were found in heat treated seed, and two OTUs belonging to the genus *Fusarium* (potentially pathogenic) and one OTUs belonging to the genus *Penicillium* were found in UVC3 treated seed.

In *P. sylvestris* PS18 seed lot, five out of eight OTUs detected in control seeds was also detected in all treated seeds (*Cladosporium*, *Hormonema*, and three unidentified OTUs) while one OTU appeared only in control seeds, and not in the treated seeds (potentially pathogenic *Diplodia*) and two OTUs appeared in control, heat and UVC1, but not UVC3 treated seeds (*Ramichloridium* and *Lapidomyces*). Of the OTUs that were not detected in control samples, one OTU belonging to potentially pathogenic *Periconia* was found only in heat treated seed, and one OTU belonging to *Alternaria* and one unknown OTU were found in UVC1 and UVC3 treated seed. In addition, *Kwoniella* and *Vishniacozyma* (one OTU each) were found in UVC1 treated seed, and one *Botryotrichum* OTU and one unknown OTU were found in UVC3 treated seed.

In *P. sylvestris* PS20 seed lot, three out of five OTUs detected in control seeds were not eliminated by the UVC treatments (*Hormonema* and one unknown OTU) and two OTUs were detected in control and UVC1 treated seeds, but not in the UVC3 treated seeds (potentially pathogenic *Periconia* and non-pathogenic *Penicillium*). Of OTUs not found in control seed, two unknown OTUs and one OTU belonging to potentially pathogenic *Neocatenulostroma* were found in UVC1 treated seed, and genera *Apiotrichum*, *Lambertella* and *Vanrija* were represented by one OTU each (and one unknown OTU) in UVC3 treated seed.

Summary of the main results and conclusions

No reduction in the number of OTUs was observed between control and treated seeds suggesting no seed treatment was effective at reducing fungi in the seed. On the contrary, similar, or even higher number of OTUs was observed in some cases after the treatment. Some of the fungi which were found in treated but not in the control seeds might have been introduced as contaminants during the post-treatment handling of the seed. However, we cannot exclude that certain fungi which were dormant within control seeds (and thus not detected) were activated by, and only detected after, the treatment.

High variation in the number of OTUs was observed between seed lots in control seeds, including same seed lots collected a year apart (e.g., lodgepole pine seed lots PC14 and PC15) suggesting that fungal communities might be very sensitive to several factors associated with seed collection (e.g., site, environment), the handling of seed during transport and drying, and seed storage. Furthermore, high variation in the number of OTUs was observed within seed lots (i.e., 3 samples collected from the same seed lot and treated in the same way revealed highly variable number of OTUs) indicating that optimization of sampling approach (e.g., determination of the number of technical and biological replicates) is needed for rRNA metabarcoding.

No differences in community composition were observed between control and treated seeds indicating that the majority of fungal OTUs present in the control seeds survive the treatments. Some fungal genera seem quite resistant to the treatments. For example, *Hormonema* appeared in untreated and treated seeds across seed lots, *Ramichloridium* appeared in control seeds and most treated samples of pines (except UVC3 treated *P. sylvestris* seed lots), *Pleonectria* was recovered from seeds despite the treatments in lodgepole pine seed lots (PC14 and PC15) and *Cladosporium* in *P. sylvestris* seed lot PS18. However, none of these genera are known to be pathogenic to plants so their persistence poses no phytosanitary concern.

Concern, however, may be warranted for *P. menziesii* seeds, which, although, having a lower number of OTUs per sample compared to other species (Fig. 1b), had more even relative abundance of OTUs which resulted in a diverse OTU community when the rare OTUs were removed (Fig. 4). In *P. menziesii* several genera persisted across samples despite the treatments including potentially pathogenic *Alternaria sp., Botryosphaeria* sp., *Clonostachys* sp., *Diaporthe* spp., *Diplodia* sp., *Trichoderma* sp., and *Trichothecium* sp. This result is rather interesting because as Breza (2023) reported, *P. menziesii* had the lowest fungal diversity based on *in vitro* isolations in nutrient media, compared to the other conifers tested, probably due to presence of the fast-growing *Trichoderma* sp.. Similar results were obtained by

Bergmann & Busby (2021) who reported low diversity of fungi associated with seeds of *Pseudotsuga menziesii* var. *menziesii* across provenances of the Pacific Northwest, USA using *in vitro* culturing with *Trichoderma* spp. and *Hormonema macrosporum*, representing more than 50% of the obtained isolates. In the same study, much higher diversity was obtained using rDNA metabarcoding than *in vitro* culturing (295 *vs* 12 taxa, respectively) highlighting the importance of culture-free methods for assessing the diversity associated with plant samples.

Another interesting finding is a presence of *Diplodia* sp. (supposedly *Diplodia sapinea*) in seeds of *Pseudotsuga menziesii*, as *Diploda sapinea*, a latent pathogen which can become pathogenic under stressful conditions, is known to be associated mostly with pines (Roy et al., 2022). However, the pathogen was recently isolated from blue-stained sapwood of dead large Douglas fir trees and was shown as pathogenic to young seedlings of this species, suggesting that the fungus might had contributed to the death of the large trees (Ritzer et al., 2023). Furthermore, to our knowledge, *D. sapinea* was never reported from *P. menziesii* seeds and this finding warrants the assessment of the role of *P. menziesii* seeds as a source of plant pathogenic *D. sapinea* for the seedlings and the environment.

Of pathogenic genera that were identified in this study, *Neocatenulostroma, Gibberella*, *Colletotrichum* and *Alternaria* were eliminated by the heat treatment in *P. contorta* PC14 seed lot; *Gibberella* was eliminated by all three treatments in *P. menziesii* PM seed lot; in *P. sylvestris* PS18 seed lot *Diplodia* was eliminated by all 3 treatments and similarly in *P. sylvestris* PS20 seed lot, *Periconia* was eliminated by the UVC3 treatment. The fact that *Diplodia* was eliminated in *P. sylvestris* PS18 seed lot, but not in *P. menziesii* PM seed lot suggests that the efficacy of the treatments might depend on tree-species specific factors such as the initial fungal community or the size or shape of the seed.

Finally, although the effects of the treatments on fungi in seed were not obvious, more research is needed with particular attention paid to replication to discern the within seed lot variation and to determine the number of seeds necessary to ensure that all the diversity with the seed lot is captured. Furthermore, to ensure the robustness of the results, more seed lots of a tree species of interest should be tested, because the initial fungal communities might differ across seed lots of the same tree species (and across tree species), which might affect the outcome of the treatments. To better understand the effects of the treatments on certain fungi, experiments using fungal isolates should be conducted. Finally, a consideration of seed size (larger seed might need higher thresholds of treatment) is necessary for determining optimal treatment conditions, as well as the testing of different time-temperature combinations for heat treatments, and different applications of the UVC treatments (one that would include

appropriate mixing of samples). All of this is important for finding the most appropriate method for the reduction of the fungal inoculum within seed while keeping the germination rates high.

Main outputs of the project

Delivered

- SLU newsletter describing the project was published in December 2022 (Appendix 1)
- Project partner meeting was hosted by Oskar Skögstrom in Lagan in winter 2022/2023
- Although not directly financed from this project, Wiktoria Breza completed her Master thesis describing the effects of the heat and UVC treatments of seeds on fungal communities as assessed by traditional *in vitro* culturing – an add-on to the funded project which made for very interesting comparison of traditional *in vitro* culturing versus high throughput sequencing analysis. (Appendix 2)
- The project was presented to the international community of researchers and stakeholders at the 19th meeting of the International Forestry Quarantine Research Group (IFQRG) which acts as an advisory group to the ICCP, FAO.
    - **Franić I**, Cleary M, Eschen R, Prospero S, Sherwood P, Skögstrom O **(2022)** Improved assessment and mitigation of phytosanitary risks associated with seed trade. **19th meeting of the International Forestry Quarantine Research Group (IFQRG).** September 2, virtual symposium. *Oral presentation.*

Planned

- A peer-reviewed publication describing the effects of the treatments on fungi and germination in the studied seed lots. We will combine the data obtained from *in vitro* culturing (Breza 2023) and RNA profiling and discuss not only the treatment effect but also methodology for conducting similar type studies.
- **Franić I**, Sherwood P, Skogström O, Cleary M (**2023**) RNA-based metabarcoding for the assessment of viable fungi in tree seeds. **20th meeting of the International Forestry Quarantine Research Group (IFQRG)**. November 7, virtual symposium. *Oral presentation*.
- **Franić I**, Sherwood P, Skögstrom O, Cleary M (**2024**) Environmentally friendly seed treatments to reduce pathogenic fungi in conifer seeds. **IUFRO World Congress 2024**. June 23–29, Stockholm, Sweden. *Oral presentation*.

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