Survey of the Phytophthora species diversity reveals *P. europaea*-like isolates as Phytophthora root rot pathogens in Québec Christmas tree plantations

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Abstract

Christmas trees are an economically and culturally important ornamental plant in North America. Many microorganisms are pathogens of firs cultivated as Christmas trees. Among those, *Phytophthora* causes millions of dollars in damage to plantations every year. In Canada, it is still not known which species or group of species is responsible for Phytophthora root rot (PRR) of the cultivated *Abies* species. Between 2019 and 2021, soil and root samples were collected from 40 Christmas tree plantations in Québec province. We used soil baiting and direct root isolation to assess the diversity of culturable *Phytophthora* spp. The obtained isolates were identified with a multi-locus sequencing approach and we used the sequencing data to place them along the *Phytophthora* phylogeny. A total of 44 isolates from 6 different *Phytophthora* species were identified, one of which was fitting the undescribed *P. sp kelmania*. There was also a group of 10 isolates which could not be assigned to a known Phytophthora species based on DNA sequences. Among the known species, a *Phytophthora europaea*-like was the prevalent isolated species associated with PRR symptomatic trees. The fulfillment of Koch's postulates confirmed the pathogenicity of *P. europaea*-like on both Fraser and balsam fir seedlings. Our studies provide a first snapshot of the *Phytophthora* diversity in Québec's Christmas tree productions and describe multiple first associations between *Phytophthora* species and *Abies balsamea* and *A. fraseri*.

Keywords: Christmas trees, *Abies fraseri*, *Abies balsamea*, *Phytophthora* root rot, ITS1-5.8S-ITS2, *Phytophthora europaea*, *Phytophthora abietivora*, Phytophthora diversity, Phytophthora isolation, Koch's posulates.

Introduction

With a little less than 1000 km^2 in cultivated surface, Christmas trees are an economically important ornamental plant for the USA and Canada (Statistics Canada 2021; USDA National Agricultural Statistics Service 2021). In eastern North America, Fraser fir is the preferred species cultivated as Christmas trees. Reasons for this preference include shape and growth, combined with its postharvest qualities such as needle retention and durability (Bates et al. 2004; Mitcham-Butler et al. 1988). Unfortunately, Fraser fir is also one of the most susceptible species to *Phytophthora* root-rot (PRR) (Benson and Grand 2000; Chastagner and Benson 2000). Hence, PRR is responsible for millions of yearly losses (Grand and Lapp 1974; Kuhlman and Hendrix 1963; Pettersson et al. 2017). The first symptom of PRR is a reddish-brown discoloration of the cambium region of the roots, which is difficult to detect unless roots are unearthed. As the infection progresses through the root system, symptoms such as chlorosis of foliage, wilting of new growth, branch flagging and stunting begin to show in the above-ground parts of the tree (Chastagner and Benson 2000). Unfortunately, it is usually too late to take actions at the onset of visible symptoms and infected trees will die within months.

In the last 40 years, most of the data concerning *Phytophthora* species associated with Christmas tree productions came from the US (Benson and Grand 2000; Chastagner et al. 1990; Grand and Lapp 1974; McKeever and Chastagner 2016). More than 20 *Phytophthora* species have been associated with more than 20 different species of true firs (Farr 2022), with at least 12 Phytophthora species known to induce PRR (McKeever and Chastagner 2016). Since the mid-2000s, data available for European countries

overlap with what is found in the US (Pettersson et al. 2019; Shafizadeh and Kavanagh 2005; Talgø et al. 2006; Talgø et al. 2007; Talgø et al. 2017), suggesting that either the association between these Phytophthora species and firs have been longstanding or that, as for other *Phytophthora* species, worldwide plant trade has contributed to their global dissemination (Bienapfl and Balci 2014; Brasier 2013; Jung et al. 2016). While the information currently available connects *Phytophthora* species to PRR symptomatic trees, few studies have shown their pathogenic potential with Koch's postulates (Frampton and Benson 2012; Hamm and Hansen 1982; Hoover and Bates 2013; McKeever and Chastagner 2016). Moreover, when Koch postulate are tested, the experiments are usually performed by injuring the tree and directly putting the pathogen at the wounded site (Li et al. 2019; McKeever and Chastagner 2016; Pettersson et al. 2019; Quesada-Ocampo et al. 2009), which does not capture all infection patterns (Hardham 2001; Judelson and Blanco 2005).

The province of Québec represents more than 35% of the total Canadian cultivated area used for Christmas tree plantations. Eighty-five percent of this area can be found in two regions; Estrie and Chaudière-Appalaches (Statistics Canada 2016). The two most cultivated species used as Christmas trees in Québec's province are Fraser fir (*Abies fraseri*) and balsam fir (*Abies balsamea*). While Canadian producers had past PRR occurrences in their plantations, the data available on Phytophthora species diversity is insufficient to tackle tasks such as systematic detection and prevention of disease spreading for this host.

In this study, we sampled Christmas tree plantations in Estrie and Chaudière-Appalaches to get an overview of the *Phytophthora* species diversity associated with PRR

symptomatic trees in Quebec main producing regions. We also demonstrated the pathogenic potential of *P. europaea*-like isolates obtained from sampling and found in association with both PRR symptomatic *A. balsamea* and *A. fraseri*. Finally, this study constitutes a first documented report for *P. europaea* in Canada.

Material and Methods

Christmas tree plantations sampling. Soils were sampled during the fall of 2019, 2020, and the spring of 2021 from 40 different sites in Chaudière-Appalaches and Estrie (20 sites each, Supplementary Table S1). All of the sites were harboring trees showing *Phytophthora* root rot symptoms. Twelve soil samples were taken in each site from trees aged 4-12 years: four showing signs of PRR and four healthy in appearance, and four from apparently healthy trees from a natural forest near the production site. Using a 5 cm diameter soil probe, three soil sub-samples, 120° apart, were taken at the crown limit of the trees up to 17 cm in depth. After removing the organic horizon, the three soil cores were cut in half and the three halves were pooled together in a plastic bag (38x19 cm Whirl-Pak). The probe was rinsed with water and heat-disinfected with a propane hand torch between samples. Samples were kept at 4°C until being processed.

Soil samples processing and baiting. Each soil sample was mixed and sieved through a 4 mm mesh basket. Root fragments retained in the sieve were transferred at 4°C for oomycetes isolation. Two hundred and fifty milligrams of homogenized soil was transferred to a microtube for subsequent DNA extraction. Two hundred and fifty milliliters of homogenized soil were put into a clean 1 L plastic container (IPL plastics, Canada) to which 500 mL of distilled water was added. Four to five healthy rhododendron

'Cunningham's White' leaves without any visible injury were rinsed with sterile distilled water and laid upon the water surface of each soil container. Five rhododendron leaves were also placed in a container filled with 500 mL of distilled water to serve as a control. The containers were incubated in the dark at 20°C until the apparition of necrosis on the rhododendron leaf tissue (4-7 days).

Following the emergence of necrosis, leaves were washed with running tap water, dried for 10 minutes on absorbing paper, and taken under a laminar flow hood. Necrotic tissues were sampled using a paper punch sterilized with ethanol and flame to obtain leaf disks (6 mm in diameter). Ten disks were taken at the margin of leaf necrotic tissues for each soil sample: five were frozen in 2 mL safe-lock tubes (Eppendorf, Hamburg, Germany) for subsequent DNA extraction and the other five were inserted into solid V8-PARPNH (clarified V8 50 mL/L, agar 15 g/L, PCNB 25 µg/mL, Ampicillin 250 µg/mL, Rifampicin 10 µg/mL, Pimaricin 10 µg/mL, Nystatin 25 µg/mL, and Hymexazol 25 µg/mL) medium (100 mm × 15 mm Petri dishes). Leaf disks were inserted at a 45° angle keeping half of the disc above the agar. Petri dishes were incubated in the dark at 20°C and were examined daily for growth. After 2 to 10 days, morphologically distinct colonies were purified by transferring hyphal tips to fresh V8-PARPH. The cultures were incubated in the dark at 20°C. All cultures not resembling a *Phytophthora* colony were excluded from further investigation. Grown cultures were preserved at 12°C.

Oomycetes isolation from roots. Lateral roots gathered from soil samples were transferred to a fine mesh strainer that was first immersed in a beaker containing distilled water and agitated to remove most of the soil. The strainer was then transferred to a beaker containing a 0.5% sodium hypochlorite solution and soaked for 30 seconds,

followed by two rinses in distilled water. Surface-sterilized root segments were dried into a laminar flow cabinet for 5-10 minutes and cut into 5-10 mm sections with a sterile scalpel. Five root sections were inserted halfway into a V8-PARPNH agar plate. The remaining roots were put into a 2 mL safe-lock tube and stored at −20°C. Media were incubated in the dark at 20°C. Morphotype colonies were purified as described above.

DNA extractions. Purified cultures were grown on V8-agar (1.5% agar, clarified V8 50 mL/L) overlayed with a sterile cellophane membrane. Mycelium was scraped with a scalpel blade and transferred to a 2 mL safelock tube (Eppendorf, Hamburg, Germany). A tungsten carbide bead (Qiagen, Hilden, Germany) was added to each tube and the bottom of the tubes was immersed in liquid nitrogen for 1 minute. The tubes were then quickly transferred to a Mixer mill adapter (Retsch, Haan, Germany) previously cooled at −80°C. Tubes were shaken for 1 minute at a frequency of 26 Hz after which a second cycle of freezing and shaking was performed. DNA was extracted following the protocol developed in Penouilh-Suzette et al. (2020) with minor modifications. In short, 800µL of Lysis buffer (0.1 M Tris-HCl pH 8, 10 mM EDTA pH 8, 1 M NaCl and 1% SDS) were added to up to 50 mg of ground mycelium along with 2 µL of Proteinase K (20 mg/mL). Samples were mixed by inversion and incubated at 50℃ for 10 minutes, while mixing by inversion twice during the incubation. 270 µL of potassium acetate 5 M was added, and tubes were mixed by inversion before incubating on ice for 10 minutes. Samples were centrifuged for 10 minutes (5,000 g), the supernatant was transferred to a new 2 mL tube, and 2 µL of RNaseA (100 mg/mL) was added. The samples were incubated for 30 minutes at 37℃ and mixed by inversion every 10 minutes. Next, 1 mL of Isopropanol was added and tubes were mixed by inversion. The samples were incubated at RT for 30 minutes.

Following incubation, samples were centrifuged for 2 minutes at 10,000 g, the supernatant was removed, and the DNA pellets were rinsed with fresh 70% ethanol. Samples were centrifuged for 1 minute (10,000 g) and the supernatant was removed. Pellets were air dried for 1 to 5 minutes under a lamp and were resuspended with 50 µL PCR grade water.

Molecular identification. To identify the isolated organisms we amplified five loci: the ITS1-5.8S-ITS2 region of the 45S ribosomal RNA (ITS), the Beta tubulin, Heat Shock Protein 90 (HSP90), and the mitochondrial genes Cytochrome oxidase I (cox1) and NADH dehydrogenase (nad1). We used primer pairs already described in the literature (Supplementary Table S2). The PCR cycles were: 94℃ 2 min; 35 cycles of 94℃ 30s; Primer specific Tm 30s; 72℃ with an amplicon specific time; 72℃ 10 min. First, PCR products of expected length for the ITS (Supplementary Table S2) were sent to Sanger sequencing at the CHUL sequencing platform on an ABI 3730xl Data Analyzer (Applied Biosystems, Waltham, USA). Then, for every strain confirmed as *Phytophthora*, the other four loci were amplified and sent for sequencing. For each of the isolated strains, the sequences were used as a query against the nt/nr collection of the NCBI using BLAST (Altschul et al. 1990). All of the sequences generated for this study were deposited into Genbank under accession numbers ON008003-ON008178 (for cox1, nad1, Beta-tubulin and HSP90), OM984685-OM984728 (ITS) and OP676073 (Cox1 sequence for the RC2017-1 *P. abietivora* isolate).

Phylogenetic analysis. For each of the sequenced locus, sequences identical for more than one isolate were collapsed into one sequence. Sequences of the 5 loci from strains of 69 of *Phytophthora* species belonging to the different kown clades were

gathered from GenBank and used as references (Supplementary Table S3). Sequences obtained from our isolates and GenBank-downloaded sequences of the reference strains were aligned with Clustal W and trimmed using the MEGA X software (Kumar et al. 2018). These alignments were concatenated using a custom script in R v4.1.1 with the *seqinr* v1.0-2 package (Charif and Lobry 2007; R Core Team 2013). Phylogenetic analysis employed maximum-likelihood and the Tamura-Nei model (Tamura and Nei 1993) in MEGA X. The dataset comprised 80 concatenated sequences, 69 from the reference strains of the *Phytophthora* species and 11 distinct concatenated sequences covering the 44 isolates of *Phytophthora* recovered in this study. Positions with less than 90% site coverage were eliminated resulting in a total of 3372 positions in the final dataset. *Elongisporangium undulatum* was designated as an outgroup. Node confidence was estimated with 1000 bootstrap replicates.

Inoculum and seedlings preparation. Three *Phytophthora europaea*-like isolates obtained from the sampling were selected to test Koch's postulates. Isolates were inoculated on a V8-agar medium and incubated for one week at 20℃. Ten mycelium plugs (5 mm × 5 mm) were taken at the margin of the actively growing cultures and inoculated into a sterile mixture of vermiculite, overnight water-soaked steel-cut oat, and clarified V8 vegetable juice in a 1 : 1 : 1 ratio. Controls were prepared the same way using plugs from an uninoculated V8-agar medium. These inocula were incubated for two weeks at 20°C in the dark.

Dormant two-year-old *Abies balsamea* and *Abies fraseri* seedlings were obtained from a local nursery (Productions Resinex inc., La Durantaye, Canada) and kept in dormancy at −2℃ for two months. They were prepared for the experiment by transferring

them at 4°C for a week, followed by a week at 10°C. Fraser and balsam fir seedlings were respectively re-potted in individual 1.5L and 1L containers filled with an autoclaved soilless mix containing Pro-mix LP15 peat moss (Premier Tech, Rivière-du-Loup, Canada), vermiculite (Perlite Canada Inc., Lachine, Canada) and silica sand (Atlantic silica inc., Poodiac, Canada) in a 2:1:1 ratio amended with 4 kg/m^3 of 18-6-8 NPK Nutricote™ fertilizer (Chisso-Asahi Fertilizer Co., Tokyo, Japan). Seedlings were maintained in a greenhouse with a 16-hour photoperiod under high-pressure sodium lighting (OSRAM Sylvania Inc., Wilmington, USA), with temperatures of 20°C during the day and 18°C during the night. Seedlings were irrigated for 10 minutes periods, four times a week (≈400 mL per irrigation period). These conditions were maintained until bud burst.

Koch's postulates. After bud burst, 100 mL of the top layer of the potting medium was replaced with a fresh potting mix inoculated with 0.1% (V/V) of the *P. europaea* inoculum. Trials followed a randomized block design where three blocks containing five replicates of each inoculum/tree essence combinations were placed in different areas of the greenhouse (three blocks × two essence × (three isolates + one control) × five replicates = 120 seedlings). Growth conditions were the same as stated above except that every two weeks, the seedlings were flooded (1 cm of water above the potting medium) for a period of 48 hours to create conditions favorable to zoospores production and dissemination. The height (soil surface to the tip of the seedling rounded to the closest 5 mm) and the collar diameter of the seedlings were recorded right before inoculation (time point "0"), every other week through the first month (time points 2 and 4), and every week between week five and twelve (time points 5-12). Symptoms of PRR were assessed by visual examination of the seedlings looking for stem and branch wilting or chlorotic and

dead needles. An estimated percentage of the seedling tissues affected by these symptoms was recorded for each seedling at each time point. Seedlings were considered dead once symptoms affected 100% of their tissues.

After 12 weeks, all seedlings were carefully removed from their containers and the root systems were sampled by separating them from the stem at the height of the root collar. The root systems were washed to remove potting media, taking care to retain as much root tissue as possible. The clean root systems were successively plunged into a beaker containing clean distilled water for 1 minute, then surface sterilized into a beaker containing a 0.5% sodium hypochlorite solution for 30 seconds. Finally, the roots were rinsed twice in clean distilled water for 30 seconds. Surface-sterilized root systems were dried for 10-15 minutes in a laminar flow cabinet and then 5 to 10 roots were sampled and cut into 5 to 10-mm segments. Two aliquots of 10 root segments were taken for each seedling. The remainder of the surface sterilized root systems were oven-dried for 48 hours at 70℃. The dried root systems were weighed to assess the severity of root degradation. To satisfy the fourth postulate, we used the two aliquots of root segments. One was used for oomycete isolation as previously described. The other was used for DNA extraction using the DNeasy Powersoil Pro Kit (QIAGEN, Hilden, Germany) and molecular detection of Phytophthora using the nad1 PCR also as previously described.

Statistical analyses. All statistical analyses were performed with the R software v4.1.1 (R Core Team 2013) using custom scripts and the FSA package v0.9.3 for the Dunn's Test function (Ogle 2017). Unless stated otherwise, the significance level used for all statistical tests is $\alpha = 0.05$. The area under the disease progression curves (AUDPC) was calculated as described (Madden et al. 2007). Data representations, survival

analyses and statistical comparisons were made using the beanplot v1.3.1 (Kampstra 2008), beeswarm v0.4.0 (Eklund 2021), survival v3.2-11 (Therneau and Grambsch 2000) and survminer v0.4.9 (Kassambra 2021) packages.

Results

Phytophthora isolations. In 2019, 41 *Phytophthora* isolates were recovered from sampling, while the 2020 sampling yielded three isolates (Supplementary Table S4). Among them, 40 were recovered by baiting soil samples with Rhododendron leaves. These isolates were obtained from 22 unique soil samples with some samples yielding up to four isolates. DNA sequencing of the Beta-tubulin, ITS, HSP90, cox1 and nad1 genes coupled with BLAST analyses revealed that the isolates recovered belong to at least seven different species. Based on the ITS sequences, we obtained 18 *P. chlamydospora* isolates, 8 *P. europaea*-like isolates, 7 *P. gonapodyides* isolates, 3 *P. gregata* isolates, 6 *P. megasperma* isolates, and 2 *P. sp. Kelmania* isolates. Among the *P. chlamydospora* isolates, 10 isolates had BLAST hit results for the sequence of the other loci that did not correspond to *P. chlamydospora*. Excepted for 6 isolates of *P. gonapodyides* and 2 isolates of *P. chlamydospora* that were obtained from soil samples taken in natural fir stands in the vicinity of christmas tree plantations, all other isolates were from soil samples under firs showing PRR symptoms. Despite the fact that 160 soil samples from healthy trees in plantations were analysed, no *Phytophthora* was recovered from these samples. The fact that few isolates were obtained from healthy trees coupled with the low numbers of *Phytophthora* isolates recovered from the 2019, 2020 and 2021 samplings precluded statistical analysis. However, we can observe that *P. europaea*-like isolates were recovered from the highest number of independent

samples (8 isolates from independent samples) which could imply a role for this organism in the development of PRR.

Phylogenetics analysis. Our phylogenetic analysis revealed that the isolates obtained from Christmas tree plantations primarily come from clades 6 and 7 (Figure 1). Most of the isolates obtained from our survey are related to known *Phytophthora* species, except for a group of 10 isolates (from 7 independent soil samples) that seem to be related among themselves but not to any other species used as reference. For those sequences, BLAST analyses would not return consistent identity results. Our phylogeny placed those isolates in clade 6b as a monophyletic taxa sister to the *P. chlamydospora* and *P. gonapodyides* group.

While we identified *P. europaea*-like isolates as potential pathogen candidates, our phylogeny indicates that these isolates would be more closely related to *P. abietivora*. From our concatenated sequence data (3884 bp), we identified 19 SNPs between our *P. europaea* reference and the *P. abietivora* type strain. Half of these SNP (9) were located in the coxI mitochondrial locus. From those 19 SNPs, 17 were shared between our *P. europaea*-like isolates and *P. abietivora*, only one was shared between our *P. europaea*-like isolates and *P. europaea* (in the ITS locus) and one seemed to be private to our *P. europaea*-like isolates while *P. europaea* and *P. abietivora* shared the same nucleotide (in the coxI locus).

Progression of PRR and survival in seedlings. Visual examination of the seedlings allowed the observation of a rapid onset of PRR symptoms after the second flooding period (four weeks post-inoculation, Figure 2a, b). The statistical comparison of

AUDPC values indicates a difference between treatments (Kruskal-Wallis test, $H(7) = 118.53$, $P < 0.001$). Post-hoc tests showed that, in Fraser firs, the AUDPC of inoculated seedlings was significantly different from the uninoculated controls for all the tested isolates (Two-sided Dunn's test $P < 0.001$, Benjamini-Hochberg corrected). In balsam firs, the results show that two out of three seedling groups are significantly different from the uninoculated controls (Two-sided Dunn's test P: 170_B = 0.049, 62_E < 0.001, and 97_A = 0.301, Benjamini-Hochberg corrected). The comparison between inoculated Fraser and balsam seedlings also shows a significant difference in AUDPC (Wilcoxon rank sum test, $W=595$, $P < 0.001$).

First seedling deaths were recorded as soon as five weeks post-inoculation. While uninoculated controls showed only little losses (Fraser 13.333%, 2 replicates out of 15 and balsam 6.666%, 1 replicate out of 15), the inoculated seedlings showed a large proportion of dead seedlings (Fraser 97.7%, 44 replicates out of 45 and balsam 60%, 27 replicates out of 45) (Figure 2c). Accordingly, there is a significant difference in survival probability between treatments (log-rank test, $\chi^2(7) = 95$, $P < 0.001$). Pairwise comparisons show that all isolates inoculated onto Fraser fir seedlings lead to a significantly different survival probability compared to the uninoculated control (Figure 2c, log-rank test $P < 0.001$ for all isolates, Benjamini-Hochberg corrected). In balsam fir survival probability was significantly different in two isolates when compared to the uninoculated control (Figure 2c, log-rank test P: 170_B = 0.012, 62_E < 0.001, and 97_A = 0.054 Benjamini-Hochberg corrected). The comparison between inoculation Fraser and balsam seedlings shows a significant difference in survival probability. (log-rank test, $\chi^2(1) = 37.3$, $P < 0.001$).

Impacts of *P. europaea*-like isolates on fir seedlings growth. We used the height, stem diameter and root system mass collected during the experiment to assess the effect of the inoculation with *P. europaea*-like isolates on the growth of balsam and Fraser firs seedlings.

We calculated height increment as the difference in height between the final (t_{12}) and initial (t_0) time points measurements. Statistical comparisons indicate significant differences in height growth between treatments (Kruskal-Wallis test, $H(7) = 67.182$, $P < 0.001$). Post-hoc tests show that inoculated Fraser firs had height increment significantly different than the uninoculated trees (Figure 3a, Two-sided Dunn's test P: 170_B = 0.040, 62_E = 0.013, and 97_A = 0.004, Benjamini-Hochberg corrected) while such observation could only be made with isolate 62_E in balsam firs (Figure 3b, Two-sided Dunn's test P: 170_B = 0.310, 62_E = 0.04, and 97_A = 0.989, Benjamini-Hochberg corrected).

Root collar diameter increment of the seedling was calculated the same way as height increment by subtracting the final and initial time points measurements. Statistical comparisons indicate significant differences in root collar diameter increment between treatments (Kruskal-Wallis test, $H(7) = 60.946$, $P < 0.001$). Post-hoc tests show that inoculated Fraser firs had significantly lower diameter increment than the uninoculated trees (Figure 3c, Two-sided Dunn's test P: 170_B = 0.003, 62_E < 0.001, and 97_A < 0.001, Benjamini-Hochberg corrected). In balsam fir, only the seedlings inoculated with the 170_B and 62_E *P. europaea*-like isolates had significantly different root collar diameter increments than the controls. (Figure 3d, Two-sided Dunn's test P: 170_B = 0.0233, 62_E < 0.001, and 97_A = 0.3189, Benjamini-Hochberg corrected).

Dry-root weight at the final time point also showed significant differences between treatments Kruskal-Wallis test, $H(7) = 61.244$, $P < 0.001$). Post-hoc tests show that both inoculated Fraser firs (Figure 3e, Two-sided Dunn's test Ps: 170_B < 0.001, 62_E < 0.001, and 97_A = 0.002, Benjamini-Hochberg corrected) and inoculated balsam firs (Figure 3f, Two-sided Dunn's test P: 170_B = 0.003, 62_E < 0.001, and 97_A = 0.004, Benjamini-Hochberg corrected) had significantly lower dry-root weight than their respective uninoculated control.

Re-isolation of the *P. europaea*-like isolates on diseased seedlings. None of the necrotic root sections used for reisolation of the *P. europaea*-like isolates yielded live oomycetes after inoculation on a fresh medium. Using a PCR approach based on the amplification and sequencing of the nad1 locus allowed the detection of the *P. europaea*-like isolates in DNA extracted from root samples. While the isolates could be detected in more than the third of our symptomatic or dead seedlings (16/45 for Fraser fir and 18/45 for balsam fir), they were not detected in any of our uninoculated controls regardless of their health status. Statistical analyses indicate a significant relationship between positivity of the nad1 PCR and both the status (healthy or symptomatic/dead) of the seedling at the end of the experiment (Pearson's Chi-squared test with Yates' continuity correction, $\chi^2(1, 120) = 16.860$, $P = < 0.001$) and its inoculation status (Pearson's Chi-squared test with Yates' continuity correction, $\chi^2(1, 120) = 14.008$, $P < 0.001$).

Discussion

Phytophthora root rot has been reported throughout the Christmas fir cultivation range. The problem has been well documented in the USA where both the diversity of the

Phytophthora species (Chastagner and Benson 2000; McKeever and Chastagner 2016) and the severity (Benson and Grand 2000) of the PRR were described. Surveys were also conducted in European countries to identify Phytophthora species associated with PRR on Christmas trees (Pettersson et al. 2019; Shafizadeh and Kavanagh 2005; Talgø et al. 2006; Talgø et al. 2007). While PRR is present in Canadian Christmas tree plantations, details about the cohorts *Phytophthora* species and the intensity of the disease are lacking in Canada. To fulfill this gap, we surveyed PRR in Christmas tree plantations in Quebec, the province with the largest production in Canada.

Despite extensive surveying over three years, only a small number of *Phytophthora* isolates were recovered. In total, 44 *Phytophthora* isolates belonging to seven different taxa were recovered. Of the 480 soil samples processed, 22 (4.58%) yielded at least one *Phytophthora* isolates. This is low compared to other studies that reported *Phytophthora* recovery rates of 21% from forest soil samples of the Eastern and North-Central U. S. oak ecosystems (Balci et al. 2007), 50% for the Fraser fir plantations in the Southern Appalachians (Pettersson et al. 2017), 9% from the total sampled trees in North Carolina (Benson and Grand 2000). Our *Phytophthora* spp. recovery rate is still lower when isolation from PRR symptomatic trees is considered. Fifteen soil samples (12.55%) yielded *Phytophthora* isolates compared to 54% and 20% respectively reported by Pettersson et al. (2017) and Benson and Grand (2000). Direct isolation from roots was generally less successful than baiting, with less than 10% (4 out of 44) of the isolates obtained from root samples. Similar results were obtained by (Aghighi et al. 2016), where most of the *Phytophthora* isolates associated with declining *Rubus anglocandicans* were recovered by baiting rather than direct isolation from roots.

Phytophthora chlamydospora, P. *gonapodyides*, and *P. gregata* belong to clade 6, and are considered riparian-associated species (Brasier et al. 1993). These three species have been reported worldwide in various natural ecosystems (Hansen et al. 2015; Wan et al. 2020). *Phytophthora chlamydospora* and *P. gonapodyides*, are believed to mainly act as saprophytes, with episodic fine root infections on weakened hosts (Hansen et al. 2015). Associations between *Phytophthora gonapodyides* and true fir species were previously reported (Chastagner et al. 1995; McKeever and Chastagner 2016; Pettersson et al. 2019), but to our knowledge, this study is the first to report the association between *P. gonapodyides* balsam fir (*Abies balsamea*). *Phytophthora chlamydospora* was previously found to be associated with *Abies concolor* and *Abies procera* (Brasier et al. 1993). Still, the present study is the first to report associations of this species with *Abies balsamea* and *A. fraseri*. *Phytophthora gregata* was identified as an aggressive pathogen of Australian wetland plants, being able to infect through the roots and the stem of susceptible hosts (Wan et al. 2020). Other studies have reported associations of *P. gregata* with other broadleaf forest species (see the summary table of global *Phytophthora gregata* records in Wan et al. 2020). Still, to our knowledge, this study is the first report of this species in Canada, and also the first to report the association of this species with a conifer species (*Abies balsamea*). Further studies are required to investigate the aggressivity of *P. gregata* on true firs.

Among the isolated *Phytophthora chlamydospora* there was a group of 10 isolates that did not group with *P. chlamydospora* in our phylogeny. Besides the ITS sequences, the other loci had higher nucleotide identities with other species. These strains may

represent a new species or a hybridization event which could be confirmed with more in depth genomic and phenotypic studies.

Phytophthora megasperma is a known aggressive pathogen of fir species and can affect many other plant hosts. It has been isolated from *Abies fraseri*, *A. grandis*, and *A. procera* in the USA (Chastagner et al. 1995; McKeever and Chastagner 2016), from *A. procera* in Ireland (Shafizadeh and Kavanagh 2005), from *A. lasiocarpa* in Norway (Talgø et al. 2007), and *A. nordmanniana* in Sweden (Pettersson et al. 2019). This study is the first to report associations of *P. megasperma* with *Abies balsamea* and *A. fraseri*.

True firs were shown to be susceptible to *Phytophthora sp. 'kelmania'*, but this study is the first to report the association of this *Phytophthora* species with balsam fir (*A. balsamea*). Previously, McKeever and Chastagner (2016) have isolated *P. sp. 'kelmania'* from Fraser fir (*Abies fraseri*), Canaan fir (*Abies balsamea var. phanerolepis*) and Turkish fir (*Abies bornmuelleriana*). This was the second most commonly isolated *Phytophthora* species in North Carolina on Fraser fir showing PRR symptoms, and artificial inoculations confirmed that Fraser fir was highly susceptible to *P. sp. 'kelmania'*. More recently, Kondo et al. (2022) performed artificial inoculations and showed that *P. sp. 'kelmania'* induced PRR symptoms on Sakhalin fir seedlings (*Abies sachalinensis*).

Phytophthora europaea was initially isolated from soil associated with declining oak forests (*Quercus spp.* and *Q. robur)* in France and Germany (Jung et al. 2002). It was since then isolated in Europe and associated with *Quercus* species (Balci and Halmschlager 2003; Tkaczyk et al. 2017), in North America associated with diverse deciduous tree species and riparian ecosystems (Balci et al. 2006; Reed et al. 2019;

Reeser et al. 2011; Sims et al. 2015), and in Australia associated with *Dacrydium cupressinum* and *Iris sibirica* (International Collection Of Microorganisms (ICMP) 2022). More recently, surveys of Christmas tree plantations in the US recovered anecdotal *P. europaea* isolates on *Abies fraseri* (McKeever and Chastagner 2016; Pettersson et al. 2017). *P. europaea* has already been previously reported twice in Canada, once from a diseased balsam fir imported from North Carolina (Robideau et al. 2011), and a second time from a diseased balsam fir reported in the Annual 2017 Canadian Plant Disease Survey (Elmhirst 2017). This study constitutes the first official and well documented report of its presence in Canada's Christmas tree plantations and its association with PRR symptomatic *Abies balsamea* and *A. fraseri*.

Our phylogenetic analysis revealed that our isolates were more closely related to *P. abietivora* than *P. europaea*. However, our phylogeny and the phylogeny of Li et al. (2019), which formally described *P. abietivora*, show some discordances. They show *P. abietivora* as a sister species to *P. flexuosa* and those two as a sister group to *P. europaea* (Li et al. 2019) while we show *P. europaea* and *P. abietivora* in a group sister to *P. flexuosa*. This may be the result of a difference in the resolution of both phylogenies as ours covers most of the *Phytophthora* clades but lacks multiple sequences per species ans Li's phylogeny focuses on clade 7. We decided to identify our isolates as *P. europaea*-like because *P. abietivora* is a very recently described species initially supported by a single isolate (Li et al. 2019). Given their high sequence similarity (0.5% out of 3884 base pairs sequenced), it is probable that isolates were identified as *P. europaea* in studies preceding the formal description of *P. abietivora* and now would need to be reconsidered as *P. abietivora*. Molnar et al. (2020) reported the identification of *P. abietivora* isolates

from the collection of the Pennsylvania department of agriculture. They could track the first isolation of *P.abietivora* from *A. fraseri* and *Tsuga canadensis* as far as 1989. They also discuss the high similarity in the sequences with *P. europaea*, especially for the ITS (Molnar et al. 2020). More recently, Bily et al. (2022) isolated *P. abietivora* from multiple deciduous trees, which would fit with the *P. europaea* hosts already described. They also observed genetic variation between their strains and the *P. abietivora* type strain for the loci they sequenced (Bily et al. 2022). In California, a survey reported the isolation of *Phytophthora sp. cadmea,* baited from soil of restoration areas. This undescribed taxon has high sequence similarity with *P. europaea* and *P. abietivora* (Bourret et al. 2020). Based on those observations, we suggest caution be used when considering *P. europaea* and *P. abietivora* as different species. While we agree that these species may represent diverging lineages, more extensive population genomics and phenotypic studies may be necessary to untangle the relationship between them.

It is interesting to note that to this date *P. europaea* is not reported as associated with *Abies* species in Europe. Moreover, it is considered as a weak pathogen of deciduous trees (Jung et al. 2017). In North America, Bily et al. (2022) reported that *P. abietivora* strains recently collected on deciduous trees also seemed to be weak pathogens to the deciduous trees species they challenged with their strains. Considering this information, an interesting hypothesis could be that *P. europaea* was introduced to North America from Europe through plant nursery trade like other species from this genus (Goss et al. 2009; Moralejo et al. 2009). After spreading throughout North America, it could have invaded the Christmas tree plantation environment and started diverging, becoming the

P. abietivora that was recently described. Population genomic studies of *P. europaea /P. abietivora* strains from Europe and North America could help us confirm this scenario.

The fulfillment of Koch's postulates with three *P. europaea*-like isolates obtained from diseased trees confirmed that this species is able to infect both *A. balsamea* and *A. fraseri* seedlings. However, disease progression and survival curves suggest that the infection in *A. balsamea* progresses slower than in *A. fraseri*, suggesting that the *P. europaea*-like isolates are more aggressive on *A. fraseri*. The sensitivity of *A. fraseri* to PRR is well documented for other *Phytophthora* species (Benson and Grand 2000; Hinesley et al. 2000; Quesada-Ocampo et al. 2009) and could explain this difference in survival throughout of our greenhouse experiment. It is difficult to explain why our reisolation attempts from root tissues were unsuccessful when the isolates' DNA could be recovered in root tissues. One hypothesis could be that our surface sterilization was too harsh, but our methodology did not differ much from what is usually used in such experiments (Bily et al. 2022; Pettersson et al. 2017). Although we could not directly satisfy the criteria of the fourth postulate, the fact that we only detected the inoculated isolates' DNA in inoculated seedlings showing symptoms of PRR confirmed that the inoculated isolates did indeed cause the disease observed in our seedlings. Our experiment also showed that *P. europaea*-like isolates could infect seedlings naturally without prior damage to the trees.

This study provides a sample of the Phytophthora diversity present in the Christmas tree cultures of the province of Québec. For many of the species reported here, the association with either *A. fraseri* or *A. balsamea* is novel information and many were not reported yet in Canada. More investigation will be needed to evaluate the impact of

these potential pathogens as Koch's postulates were only tested on the *P. europaea*-like isolates. We also present strong evidence that our *P. europaea*-like isolates are root rot pathogens of balsam and Fraser firs. The information we provide will be helpful in the development of targeted detection tools and pathogen management strategies that will help producers lower the risks of PRR outbreaks in their plantations. Other complementary work with metagenomics analysis and high throughput analysis is also in process.

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-Conflict of interest

The authors declare no conflict of interest.

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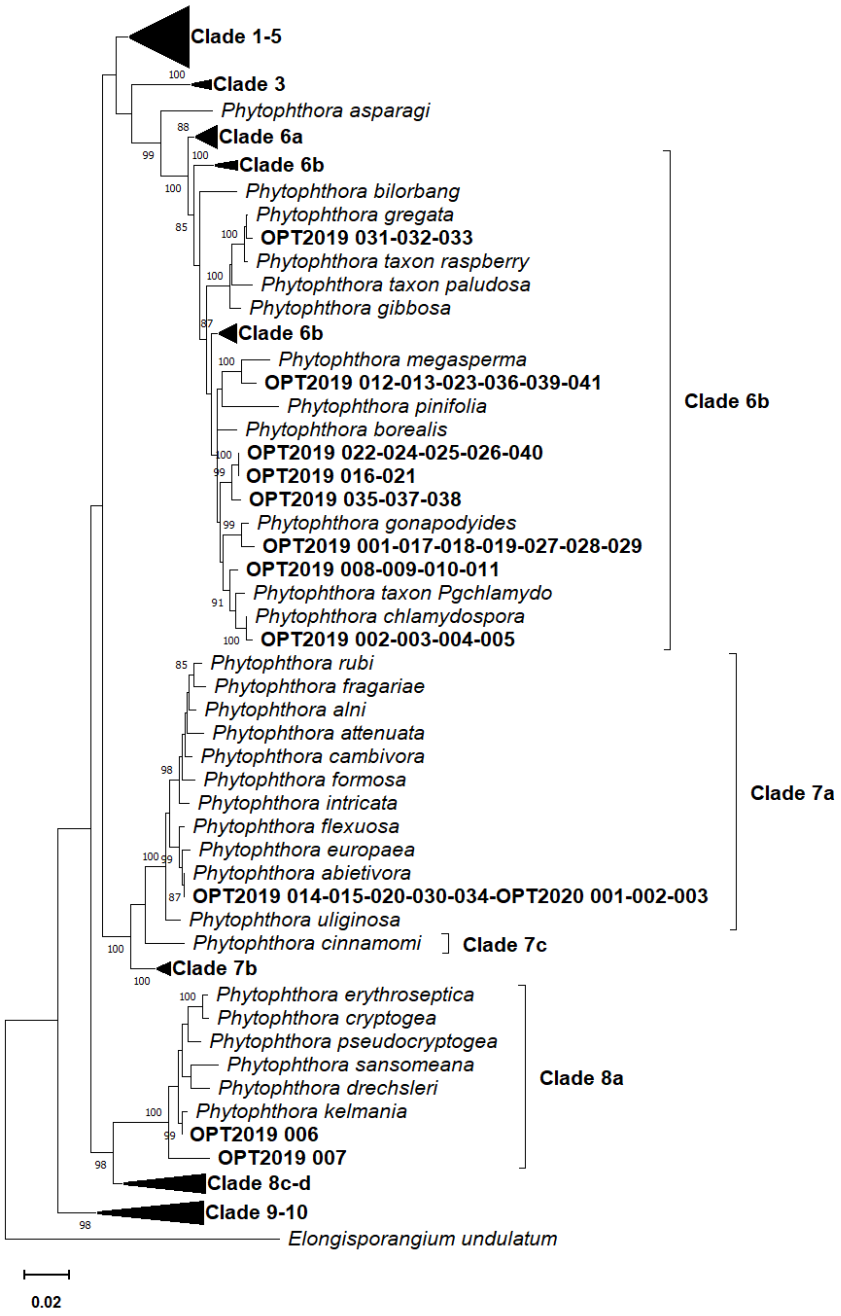


Fig. 1. Multiple *Phytophthora* species were recovered in plantations and natural forests: Maximum likelihood analysis of the recovered isolates and reference taxa based on concatenated ITS, Cox1, β-Tubulin, Nad1, and HSP90 sequences. *Pythium undulatum* is used as an outgroup. The bootstrap test was conducted with 1,000 replicates bootstrap

values >80% are indicated on the nodes. The scale bar shows the number of expected substitutions per site. References are represented in italic and isolates obtained in this study are represented in bold.

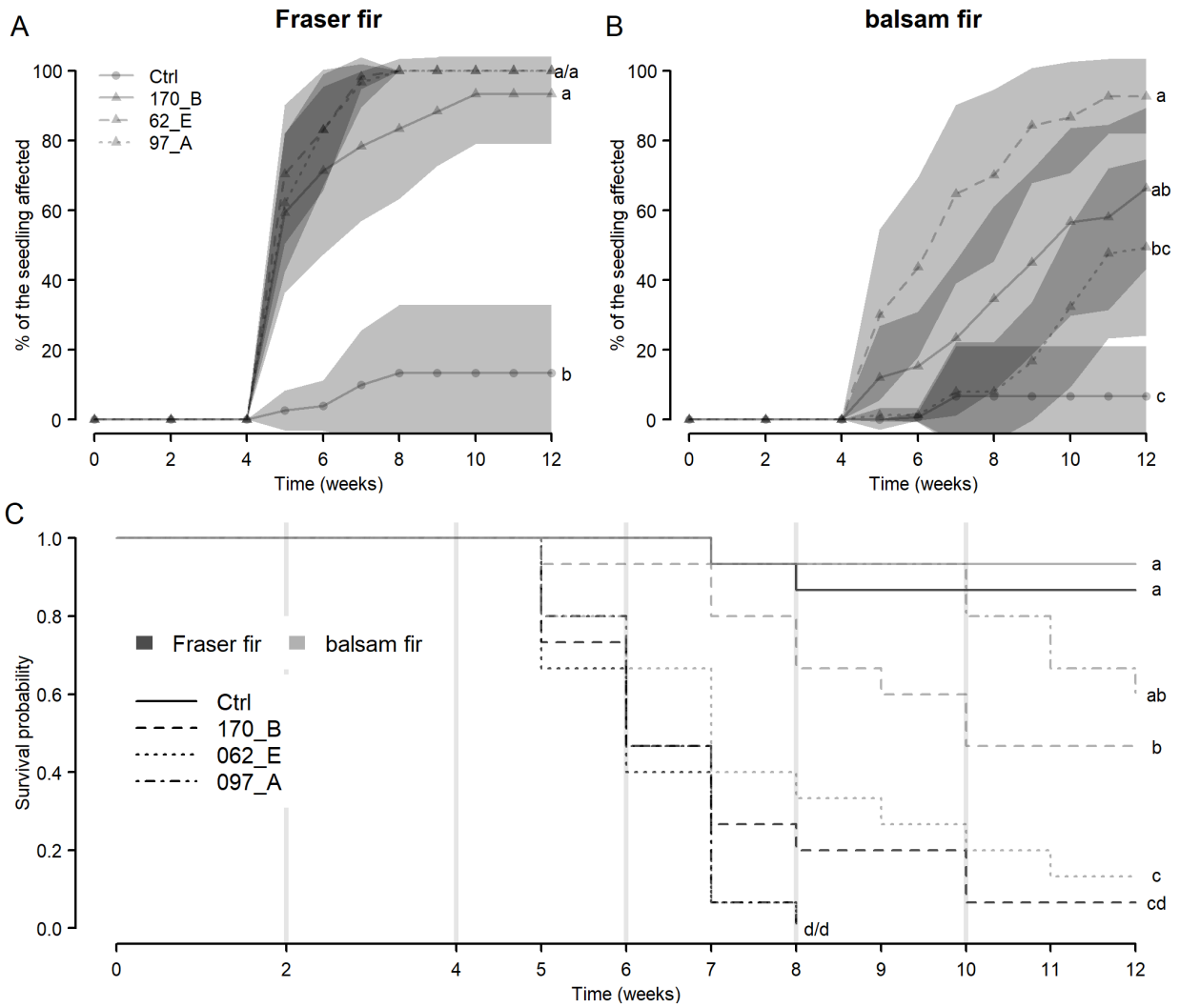
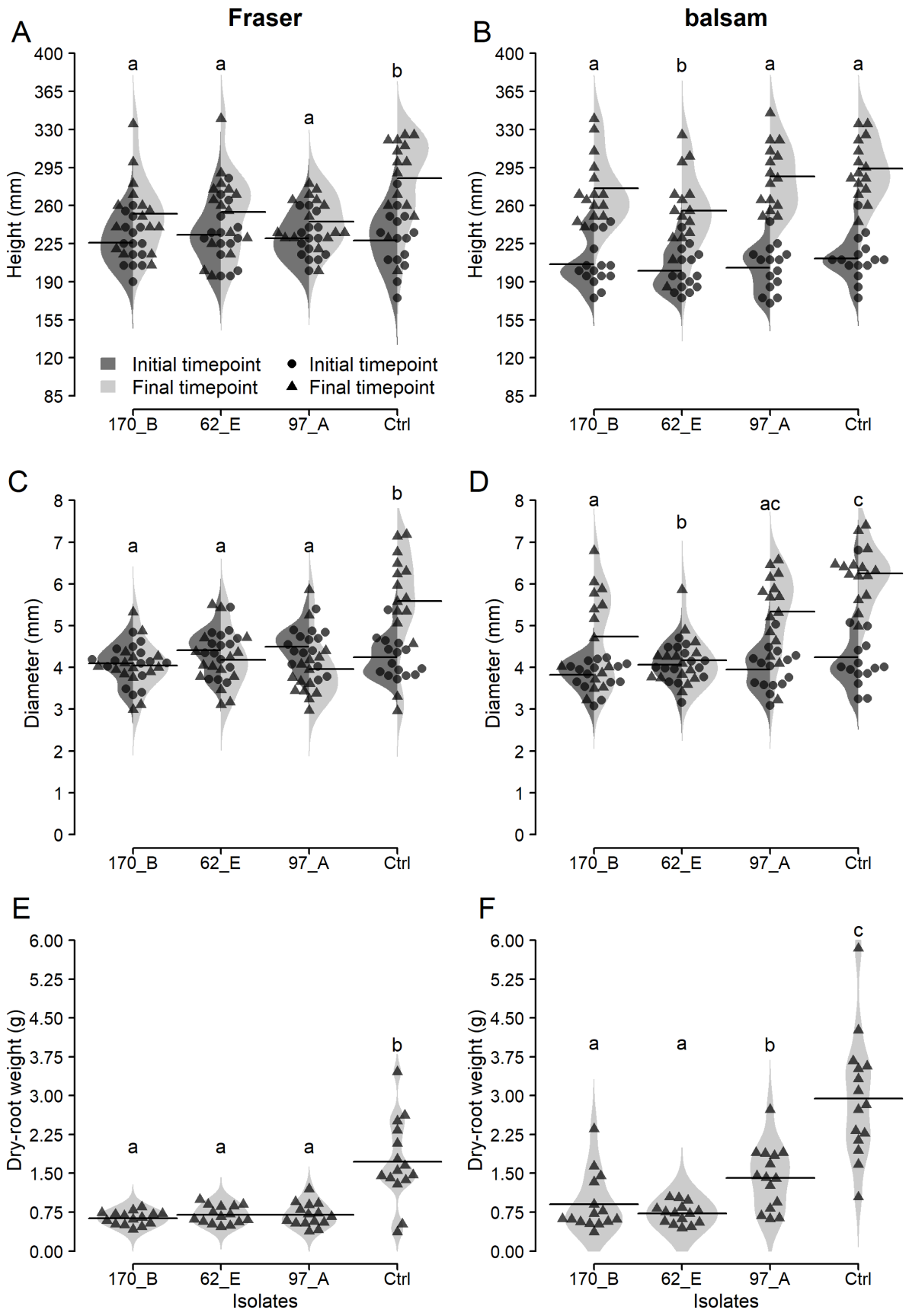


Fig. 2. Rapid onset of PRR symptoms lead to seedlings death in inoculated groups: (**A, B**) Disease progression curve in Fraser and balsam fir seedlings represented as the percentage of the plant affected by symptoms of PRR for each seedling. The replicates' data was averaged for each tested isolate and uninoculated controls (Ctrl); darkened areas represent 95% confidence interval and letters indicate significant differences in AUDPC (two-sided Dunn's test). (**C**) Survival probability varies between inoculated and uninoculated (Ctrl) seedlings; grey horizontal lines represent the timing of each 48-hours

flooding period and letters indicate significant differences in survival curves (Log-rank test).


A
Fraser
B
balsam
Height (mm)
Initial timepoint
Final timepoint
Initial timepoint
Final timepoint
170_B
62_E
97_A
Ctrl
C
D
Diameter (mm)
E
F
Dry-root weight (g)
Isolates

708

Fig. 3. Inoculation with *P. europaea*-like isolates affects growth in both Fraser and balsam fir seedlings: (**A, B**) Beanplots comparing height distributions between the initial time point (inoculation week, t_0) and the final time point (12 weeks post-inoculation, t_{12}) for each isolate inoculated and the uninoculated controls (Ctrl). Letters indicate significant differences in height increments (Dunn's test). (C, D) Beanplots comparing collar diameter distribution between t_0 and t_{12} for each isolate inoculated and the uninoculated controls (Ctrl). Letters indicate significant differences in diameter increments (Dunn's test). (E, F) Beanplots of dry root mass distribution at t_{12} for each isolate inoculated and the uninoculated controls (Ctrl). Letters indicate significant differences in dry root weight (Dunn's test). Black bars on the plots illustrate the mean of the data. Symbols represent individual data points for each time point.