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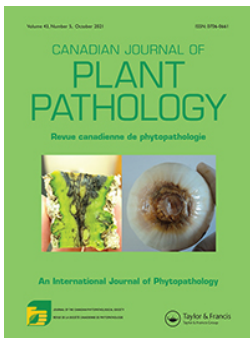
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Determining the novel pathogen *Neodothiora populina* as the causal agent of the aspen running canker disease in Alaska

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Abstract: *Neodothiora populina* Crous, G.C. Adams & Winton was determined to be a new pathogen of trembling aspen (*Populus tremuloides*) growing in Alaska, based on completion of Koch's Postulates in replicated forest and growth chamber inoculation trials. The pathogen is responsible for severe damage and widespread rapid mortality of sapling to mature aspen (≥ 80 years) in the boreal forests of interior Alaska, due to large diffuse annual (1–2 years) cankers. Isolation of the pathogen was challenging, and identification based on cultural characters was difficult. Fruiting bodies were not found on wild diseased trees, but erumpent pycnidia were found in bark overlying cankers on several stems inoculated with pure cultures.

Keywords: boreal forest, canker, forest disease, forest pathology, *Populus tremuloides*

Résumé: En se basant sur la vérification des postulats de Koch dans le cadre d'essais d'inoculation répétés en forêt et en chambre de culture, on a établi que *Neodothiora populina* Crous, G. C. Adams & Winton était un nouvel agent pathogène qui s'attaquait au peuplier faux-tremble (*Populus tremuloides*) en Alaska. L'agent pathogène est responsable de graves dommages et de la mort rapide et répandue de gaules et de peupliers matures (≥ 80 ans) dans les forêts boréales de l'intérieur de l'Alaska, et ce, à cause de gros chancre annuels (1 à 2 ans) de forme diffuse. L'isolement de l'agent pathogène s'est révélé un défi et l'identification basée sur les caractéristiques culturelles s'est avérée difficile. Aucun organe de fructification n'a été trouvé sur les arbres sauvages malades, en revanche, des pycnides éruptifs ont été trouvés dans l'écorce recouvrant les chancre sur plusieurs tiges inoculées avec des cultures pures.

Mots clés: chancre, forêt boréale, maladie des arbres forestiers, pathologie forestière, *Populus tremuloides*

Introduction

Aspen running canker is a novel, aggressive, diffuse canker disease of trembling aspen (*Populus tremuloides* Michx.) in the boreal forest of Alaska. The disease rapidly kills the phloem and cambium as the lesion spreads along the tree bole (Fig. 1), and the canker becomes slightly sunken as dead tissue collapses and live tissue adds diameter growth. The canker can appear as subtle discolouration or distinctly orange, but its

margin is typically readily discernible on thin, smooth bark. The result of the canker is whole-tree mortality with neither noticeable branch dieback nor slowly declining health. A recent study of 16 576 aspen trees found the canker in 82% of 88 plots within six Alaskan ecoregions (Ruess et al. 2021). Canker incidence was higher in smaller trees, particularly those within intermediate-aged to mature stands, wherein lesions can girdle and kill trees within a single season. A total of 2857

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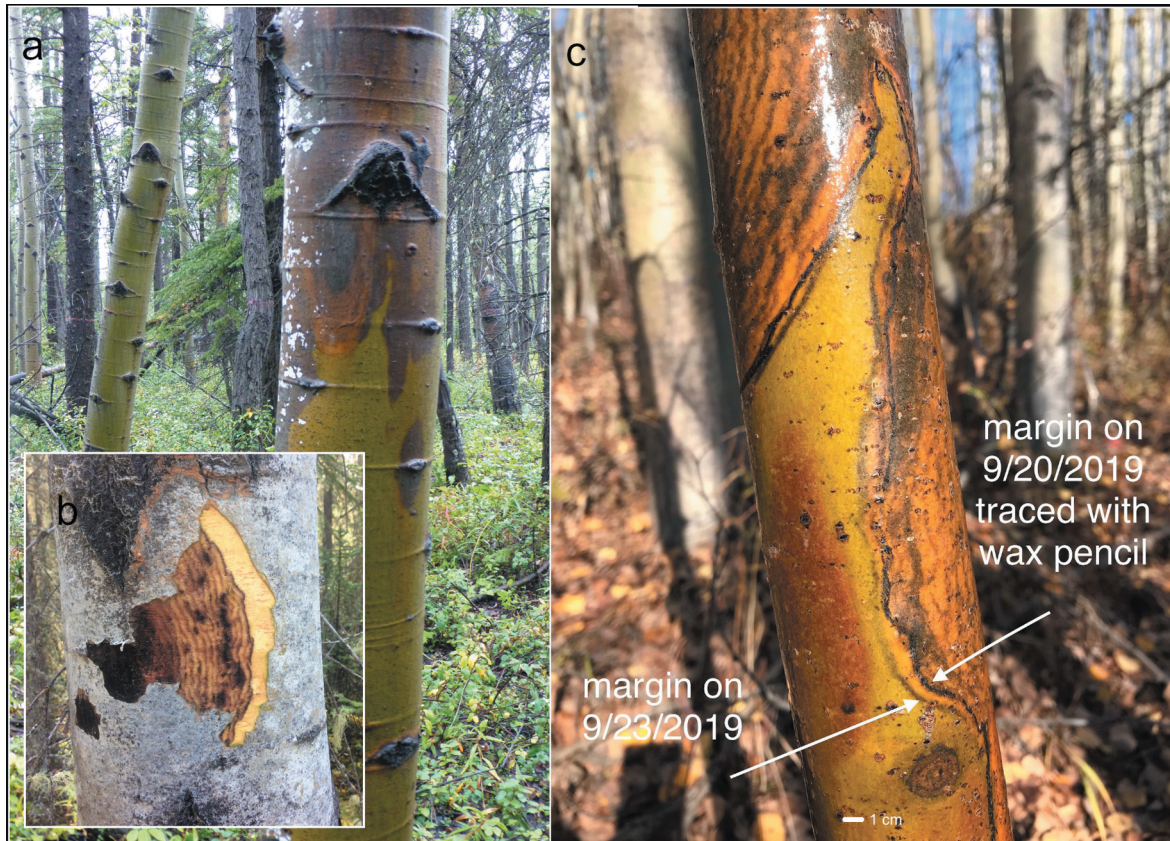


Fig 1 (Colour online) Symptoms of naturally infected aspen running canker lesions on trembling aspen in Interior Alaska. **(a)** An obvious and large lesion and a small, elliptical, discrete lesion; **(b)** debarked lesion showing successive advances of brown fungal-killed tissue; and **(c)** rapid growth and advancing canker margin during late September and a small, nearly round lesion.

trees was recorded with symptoms of canker and of those, 85% (2428) were dead and 15% (429) were still alive. The remaining 2652 dead trees (5080 total) died of unknown causes and/or had been dead too long to confidently identify canker symptoms. Between the years 2000 and 2018, canker incidence and mortality steadily increased. To date, the disease has been mapped throughout much of the boreal forest of Interior and Southcentral Alaska since it was first noticed in 2014 (Fig. 2).

Unlike other canker pathogens of aspen (e.g. *Cytospora* spp., *Ceratocystis fimbriata* Ellis & Halst., *Entoleuca mammata* (Wahlenb.) J.D. Rogers & Y.M. Ju), the organism that causes aspen running canker has not displayed diagnostic signs on wild (naturally infected) cankers. For example, *Cytospora* species on aspen regularly form fruiting bodies on the canker face and margins, *C. fimbriata* regularly emits a fruity odour, *E. mammata* (*Hypoxyton*) regularly forms black stromatic layers of fungus exposed by sluffing bark, and bacterial cankers have distinctive odours and oozing exudations or gummosis on canker

margins (Sinclair and Lyon 2005). Despite repeated sampling and microscopic examination, no evidence of bacteria, nematodes, insects (including stem borers) or fungal fruiting bodies have been found. Additionally, the disease differs from the ‘sudden aspen decline’ syndrome seen in the southern Rocky Mountains (Worrall et al. 2008; Marchetti et al. 2011; Worrall et al. 2013, 2015) by the consistent presence of large, rapidly expanding diffuse cankers, the absence of extensive branch dieback, and the lack of direct involvement of secondary insect pests and pathogens.

This paper documents the isolation of potential pathogens of aspen running canker from diseased aspen trees. We then artificially inoculated healthy aspen trees and logs with candidate pathogens and completed Koch’s Postulates. We have identified the novel pathogenic fungus *Neodothiora populina* Crous, G.C. Adams, & Winton (Crous et al. 2020) as the cause of aspen running canker and discuss difficulties with identifying causal agents of new diseases.

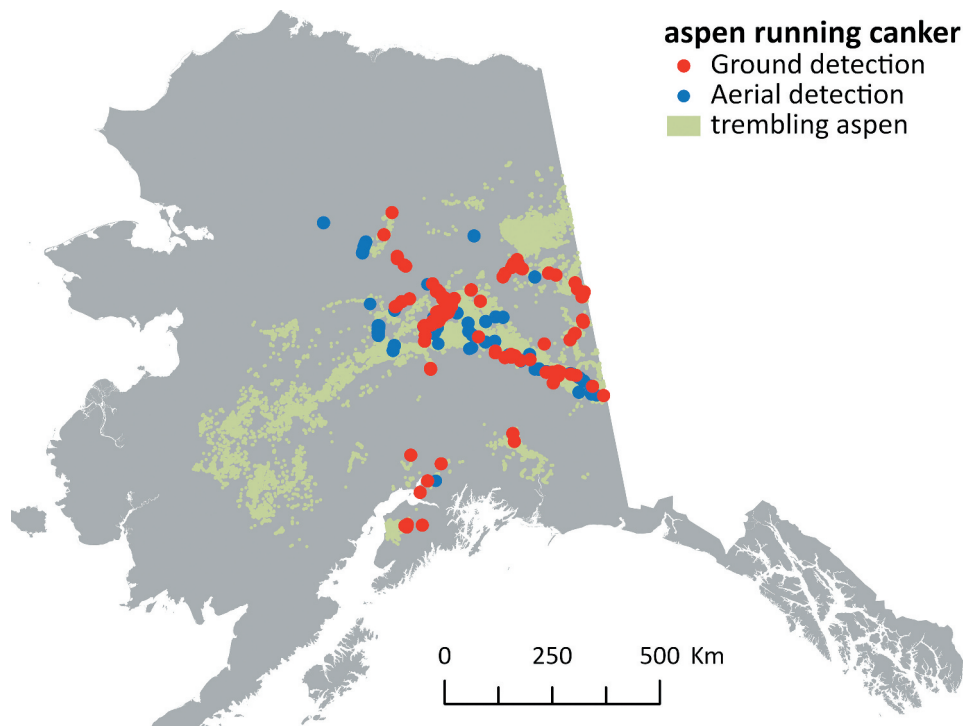


Fig. 2 (Colour online) Aspen running canker locations in Alaska based on ground and aerial survey observations as of 2019 and modelled aspen distribution. Most of these locations were found during US Forest Service Forest Health Protection (FHP) ground surveys, but in 2018, FHP also began mapping this disease in the annual aerial detection survey.

Materials and methods

Study sites

The two study sites and inoculation trials were within the Bonanza Creek Experimental Forest, approximately 30 km SW of Fairbanks, Alaska. The Rosie Creek fire burned large sections of this upland mature white spruce (*Picea glauca* (Moench) Voss) landscape in 1983, leaving a patchwork of stands which now are developing through mid-succession with varying densities of white spruce, Alaska paper birch (*Betula neoalaskana* Sarg.) and trembling aspen. The two sites (64.7418 N, -148.31614 W and 64.73243 N, -148.33106 W) were separated by 1.6 km and located on gradual south-facing slopes within dense stands of nearly pure aspen ranging between 6 and 25 cm DBH (diameter at breast height).

Wild cankers

Large, naturally occurring cankers (10–700 cm) on live trees are widespread (Ruess et al. 2021) and can be difficult to detect as the lesions are frequently high in the tree and often display little discernible bark

discolouration as the inner bark is killed (Fig. 1b). These subtle cankers are best found by touch when the lesions are slightly sunken as dead tissue collapses and live tissue adds diameter growth in rapidly growing trees. Upon debarking, these subtle canker lesions have distinct margins between diseased and healthy tissue (Fig. 1b). Occasionally cankers on live trees are colourfully orange to brown (Fig. 1a,c); and the lesion margins are readily apparent on the outer bark. Small lesions (<10 cm) are almost always undetectable unless they are significantly discoloured; they can be round, or vertically (e.g. lower lesion in Fig. 1a) or horizontally (e.g. lower lesion in Fig. 1c) elliptical. Inventory data show that lesions can girdle and kill trees within a single season (Ruess et al. 2021); however, the latency period of wild cankers is unknown. The disease is called ‘aspen running canker’ because cambium and phloem of aspen trees are rapidly killed as the diffuse lesions expand along nearly the entire bole and eventually girdle the tree (Fig. 1a). Multiple small lesions have not been observed to coalesce. Lesion expansion is rapid and successive margins are occasionally discernible with (Fig. 1b) or without debarking (Fig. 1c). We have

measured nearly 1 cm growth within a 3-day period (Fig. 1c). Fruiting bodies or other pathogen signs on wild cankers have not been found and host defences such as callusing are not apparent.

Pathogen diagnostics

Cursory metabarcoding assays were completed for eukaryotes (18S rDNA), fungi (ITS rDNA) and bacteria (16S rDNA) present in the advancing margins of infected cambium of four samples from one diseased tree. Four samples of cambium from a healthy tree were used as control. Cambium was sampled aseptically in the field with a surface-sterilized 4-mm diameter Haglöf Increment Hammer (Forestry Suppliers, Jackson, MS), stored frozen until processed, and submitted to RTLGenomics (<https://rtlgenomics.com/>) for the three Illumina assay protocols utilizing primers from the Earth Microbiome Project. These methods are described in detail at <https://earthmicrobiome.org> and in Thompson et al. (2017). Routine bioinformatics analysis was performed by RTLGenomics.

Isolations of putative causal agents were attempted from recently collected wild cankers using standard methods. Isolations were attempted both after ~3 day shipment to the University of Nebraska-Lincoln and immediately after collection at the University of Alaska Fairbanks. The outer bark was wiped with ethanol, briefly flamed, and cut away. Thin strips of the inner bark, cambium, and outer sapwood were excised from the margin of necrotic tissues and again surface disinfected by soaking for 2 min in 3% hydrogen peroxide, then blotted dry on sterile paper towels. Small tissue samples (~2 mm × 10 mm) were cut from these pieces with a scalpel, then placed aseptically on selective media and incubated.

Selective isolation of Pythiaceae was attempted on PARPH-cV8 medium (Ferguson and Jeffers 1999) and incubated in the dark at 18°C. Isolation of true fungi from tissue samples was attempted on MEA (2% malt extract agar) with 200 ppm of the antibacterial streptomycin sulphate and incubated at 5°C, 13°C and 25°C for 14 days. Fungal cultures, including yeasts, were subcultured to obtain single-cell derived colonies, maintained on MEA, and stored at -80°C in 15% glycerol. Leonian agar amended with 200 ppm streptomycin sulphate was used to promote pycnidium formation (Leonian 1924) which aids identification, particularly of *Cytospora* species (Adams et al. 2005). Methods for isolating and identifying yeasts were chosen following revelation that the microbiome of diseased aspen canker was

predominantly yeasts (see Results). Sabouraud agar amended with 100 ppm chloramphenicol or 100 ppm cycloheximide were used because the Sabouraud medium favours cultivation and enumeration of yeasts from material containing numerous other fungi (Sandven and Lassen 1999). Addition of the antibiotic cycloheximide was used for differentiating tolerant from intolerant yeasts. Difco potato dextrose agar (PDA) amended with 200 ppm streptomycin sulphate was used after a serendipitous observation that some yeast-like cultures formed melanin pigment on PDA but not on the Leonian medium. CHROMagar Candida agar (Fisher Scientific Q93241972) was used because the proprietary metals promote differentiating colours among species. Inoculated plates were incubated at 25°C for 3–5 days in darkness.

After incubation, cultures were grouped by visible cultural characteristics and DNA was isolated from several representative cultures for each group. Extraction and purification of DNA was completed using the DNeasy Plant Mini Kit (Qiagen, Germantown, MD). Routine molecular identifications were performed by sequencing the rDNA inter-transcribed spacer (ITS primers ITS1F, Gardes and Bruns 1993; ITS4; White et al. 1990) and searching for sequence similarities with NCBI MegaBLAST. More rigorous identifications included segments of the translation-elongation factor 1- α (*tef1* primers 1018F/1620R, Stielow et al. 2015) and the β -tubulin genes (*tub2* primers Bt2a/Bt2b, Glass and Donaldson 1995). Species determinations as reported were based on highest percentage homology matches and microscopic examinations. Taxonomically informative partial gene sequences of relevant fungal species identified from this study were deposited as accessions in the NCBI GenBank database (Table 1).

For pathogenicity tests, the potentially pathogenic fungi (including yeasts) were grown on Leonian agar amended with 1.25 g L⁻¹ NaNO₃ and 200 ppm streptomycin sulphate and incubated for 7–14 days at 25°C in 9 cm Petri plates. In general, multiple plugs of inoculum or loop-streaking were used to inoculate the plates.

Log inoculations

Fifty asymptomatic logs of aspen (8–10 cm diameter and approximately 1.5 m length) were cut on 3 October 2019, and the ends were sealed with ANCHORSEAL 2 Green Wood Sealer (U.C. Coatings, Buffalo, NY) to delay drying. Logs were surface sterilized with Clorox wipes and wounded with an Arrow T50 Heavy Duty staple gun (without the staple) resulting

Table 1. Fungi isolated from wild cankers and used for pathogenicity inoculations (the newly identified pathogenic species *N. populina* is in bold). Isolates were preliminarily identified by MegaBLAST of rDNA ITS. Sequences of phylogenetically informative genes, ITS, LSU, *tefl* and *tub2* were accessioned in NCBI GenBank when available. Sequences from the culture ex-type specimen were submitted by Crous et al. (2020). NA = not available.

Isolate label	Nearest MegaBLAST match (% ITS similarity)	Final Taxon designation	GenBank Accession Numbers			
			ITS	LSU	<i>tefl</i>	<i>tub2</i>
A	<i>Nakazawaea wyomingensis</i> MH595318 (99.7%)	<i>Nakazawaea wyomingensis</i>	MW362329	MW362437	MW692015	MW692030
B	<i>Leptosphaerulina australis</i> KJ739515 (98.0%)	<i>Leptosphaerulina</i> sp.	MW362328	NA	NA	NA
D	<i>Valsa nivea</i> KR045638 (99.0%)	<i>Valsa nivea</i>	MW362324	MW362434	MW692011	MW692027
E	Uncultured fungus isolate GU564975 (99.6%)	<i>Neodothiora populina</i>	MW362332	MW362439	MW692018	MW692022
F	<i>Ogataea nonfermentans</i> TYPE NR_155607 (93.5%)	<i>Ogataea</i> sp.	MW362330	MW362442	MW692016	MW692031
G	<i>Coniochaeta</i> sp. AY219880 (97.9%)	<i>Coniochaeta</i> sp.	MW362327	MW362436	MW692014	MW692029
I	Uncultured fungus isolate GU564975 (99.6%)	<i>Neodothiora populina</i>	MW362333	MW362440	MW692019	MW692023
J	Uncultured fungus isolate GU564975 (99.6%)	<i>Neodothiora populina</i> (culture ex-type)	MW175365.1	MW175405.1	MW173127.1	MW173142.1
K	<i>Kalmusia longispora</i> TYPE NR_153979 (100%)	<i>Kalmusia longispora</i>	MW362325	NA	MW692012	MW692021
L	<i>Botrytis cinerea</i> MT790314 (99.8%)	<i>Botrytis</i> sp.	MW362322	MW362432	MW692009	MW692025
M	<i>Capronia munkii</i> AF050248 (100%)	<i>Capronia munkii</i>	MW362323	MW362433	MW692010	MW692026
N	<i>Knufia cryptophialidica</i> NR_121501 (100%)	<i>Knufia cryptophialidica</i>	MW362326	MW362435	MW692014	MW692028

in a 1 mm x 10 mm cut. A 3 mm thick by 4 mm diameter plug of each isolate was smeared into the wound and then sprayed with a light coating of ANCHORSEAL 2 to delay drying. Each log was inoculated with all 12 available isolates (see Table 1) and a negative control (sterile agar plug). Immediately after inoculation logs were placed in a Conviron (Winnipeg, MB) PGW36 walk-in growth chamber for 30 days at 15°C, 18 hours light (~600 µE) and 50% humidity. Canker lesion width and height were measured on vertical and horizontal axes to the nearest mm. After measurement, reisolutions from lesions on four logs were attempted on Sabouraud dextrose agar amended with 100 ppm chloramphenicol (Becton, Dickinson and Company Sparks, MD) and malt extract agar amended with 100 ppm chloramphenicol (Hardy Diagnostics, Santa Maria, CA). Reisolutions were identified by DNA sequencing of the ITS and *tef*.

Field inoculations

For culture inoculations, 10 asymptomatic, apparently healthy trees between 8 and 10 cm DBH were haphazardly selected at each of the two field sites and inoculated with the same isolates as the log inoculations (Table 1). However, isolate B was unavailable due to culture contamination. Trees at the first site were

inoculated on 16 September 2019. Trees at the second site were inoculated on 20 September 2019. Inoculation procedures were performed identically to those used on the cut logs. Canker lesions (or inoculation scars for controls and unsuccessful colonization) were measured monthly starting in April 2020 and ending in August 2020, 11 months after inoculation.

Analysis of variance for cut log and standing live tree inoculations were performed separately for each measurement date on log-transformed lesion area (Area of ellipse = π (width x height)) in JMP 15.2.0 (SAS Institute, Inc.). Means comparisons with a control were accomplished with Dunnett's method. Outlier strategy was to perform analyses with and without outlying observations and leave them in if conclusions did not differ.

Results

Pathogen diagnostics

Cursory eukaryotic metabarcoding of healthy cambium revealed nearly 100% of the amplicon reads matching *P. tremuloides* with no hits of bacteria or fungi. Diseased samples yielded 63% of reads matching a species of the yeast genus *Nakazawaea*, 36% the host *P. tremuloides*

and 0.5% bacteria (putative mitochondrial sequences). For the bacterial assay, only one genus of bacteria (*Halospirulina* sp.) accounted for 74% of the identified amplicon reads of healthy trees, 22% were unknown (taxonomic classification uncertain) and 3% were unclassified (NCBI contains missing information). There were 22% *Halospirulina* sp. reads in diseased samples while the rest were either unclassified or unknown. For the fungal assay, the healthy tree yielded no amplification. However, the canker samples yielded several fungal genera including the *Nakazawaea* yeast (~99%), an unidentified Dothioraceae (0.2%, determined later as *Neodothiora*), a *Capronia* species in the Chaetothyriales (0.1%), and an unclassified species of *Knufia* (0.1%). Since yeasts were the overwhelming percentage (>99%) of the fungal microbiome in diseased cambium, we focused our attention on identifying the yeast and yeast-like cultures.

Over a three-year period, samples shipped to Nebraska repeatedly failed to yield any fungi, including general contaminants, except for isolation of one culture of *Knufia cryptophialidica* L.J. Hutchison & Unter. This fungus is a common endophyte of *P. tremuloides*; it grew slowly and produced a small, dark brown, mounded culture after 30–60 days at 25°C on Leonian agar. Successful isolations from cankers resulted only from same-day sample processing. Selective media did not yield any cultures of pythiaceus fungi.

The majority of sampled canker margins yielded several isolated fungi that were subsequently used for pathogenicity test inoculations (Table 1). Preliminary ITS MegaBLAST identifications included purported plant pathogens *Valsa nivea* (anamorph *Cytospora* sp.), a *Botrytis* species closest to *B. cinerea*, a species of *Coniochaeta* (anamorph *Lecytophora* sp.), *Leptosphaerulina australis*, known endophytes (*K. cryptophialidica* and black yeast-like *Capronia munkii* (anamorph *Exophiala* sp.)), and a saprobe (*Kalmusia longispora*). Three pale mucoid yeast or yeast-like species were also recovered: *Nakazawaea wyomingensis*, a species of *Ogataea* closest to *O. nonfermentans*, and repeated isolations of an unknown species later named *Neodothiora populina* (see below). The *Coniochaeta* species formed a moist, yeast-like colony with submerged hyphae and aggregated conidial masses. The five yeasty species were found to be generally distinguishable by using three different media. *Nakazawaea wyomingensis* and the *Ogataea* sp. grew on Sabouraud amended with 100 ppm cycloheximide. On CHROMagar® Candida (Fig. 3), *N. populina* grew as a blue colony, the *Coniochaeta* sp. formed a green-blue colony, *C. munkii* an olive blue green, and the *Ogataea* sp.

formed a magenta colony, whereas the other yeasts grew whitish with hints of pink purple. On PDA, *N. populina* formed a 1-mm wide dark brownish-grey (burnt umber) margin of hyphal growth on the very perimeter of the mucoid, whitish, yeast-like colony.

Preliminary identifications of isolates that occurred too infrequently to justify use for inoculations included *Cytospora notastroma*, *Cytospora chrysosperma*, *Septotinia populiperda*, *Sydowia polyspora* (anamorph *Hormonema dematioides*) and *Aureobasidium pullulans*. Using a range of temperatures of incubation did not yield any different fungi. Most cultures selected for DNA-identification and inoculation trials were from the 5°C and 15°C plates, upon which individual cultures were less likely to be overgrown by neighbouring colonies.

Log inoculations

After one month in the growth chamber, there was overwhelming evidence that mean lesion sizes differed among the 13 treatment groups on cut logs (F-test $p < 0.0001$). Only isolates E, I and J (later identified as *N. populina*) caused larger lesions than the control (Fig. 4, Dunnett's two-sided $p < 0.0001$ for each). These cankers were similar, but generally smaller than wild cankers (compare Fig 1 to 5a). Reisolations recovered the original fungal species used as inoculum, thus satisfying Koch's Postulates. After 3 months in the growth chamber, lesions had dried out and fruiting bodies had formed on the E, I and J lesions. Six lesions with fruiting bodies from all three isolates from four different logs were deposited in the University of Alaska Fairbanks Herbarium (ALA) as accessions H1280666-H1280671.

Field inoculations

Eight months (May 2020) after inoculating live trees, evidence was overwhelming that mean lesion sizes differed among the 12 treatments (F-test $p < 0.0001$). Evidence was convincing that E, I and J isolates caused significantly larger lesions than the control (Dunnett's two-sided $p < 0.0001$ for each). There was also slight evidence that isolate L differed from the control (Dunnett's two-sided $p < 0.0121$). Remeasurements at monthly intervals revealed the same result over the next 11 months (August 2020), by which time only isolates E, I and J caused significantly larger lesions than the control (Fig. 4, Dunnett's two-sided $p < 0.0001$ for I and J, Dunnett's two-sided $p < 0.0007$ for E). These canker

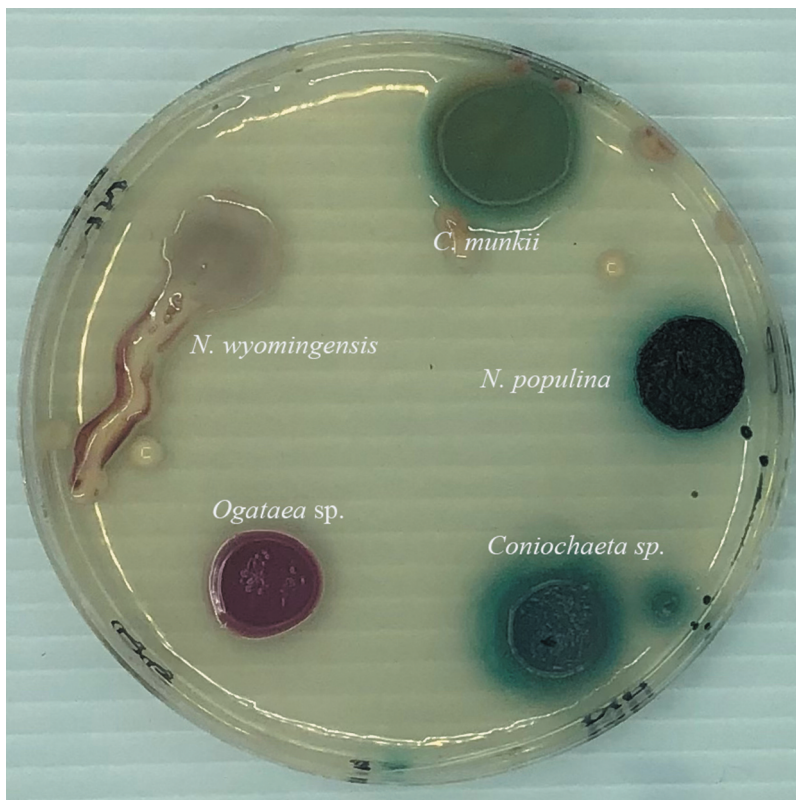


Fig. 3 (Colour online) Isolates of pale yeast-like colonies can be differentiated rapidly by colour when grown on CHROMagar® Candida incubated at 25°C for 72 h.

lesions were similar, but generally smaller than wild cankers (compare Fig. 1 with Fig. 5b). Twelve months after inoculation fruiting bodies had formed on several of the E, I and J lesions. A lesion of isolate J with fruiting bodies from one tree was deposited in the University of Alaska Fairbanks Herbarium (ALA) accession H1280665.

New fungal species

Cultures of isolates E, I and J and the seven herbarium specimens were sent to Westerdijk Fungal Biodiversity Institute (PO Box 85167, 3508 AD Utrecht, Netherlands) for formal identification. All isolates and specimens were identified and described in Crous et al. (2020) as the novel fungal genus and species *Neodothiora populina* Crous, G.C. Adams, & Winton (MycoBank MB837858).

Discussion

The new disease of aspen we refer to as aspen running canker has the visible symptom of an extensive large diffuse canker (usually only one canker) with a sharp

margin of discoloured inner bark (Fig. 1). The canker eventually girdles the tree, which often results in mortality within 1–2 years. To our knowledge, the sudden appearance, widespread distribution and high mortality on aspen has not been witnessed previously by forest pathologists. We have demonstrated the involvement of a novel pathogen in the aspen running canker disease; *N. populina* is a newly described yeast-like fungus that is challenging to isolate. Lesions resulting from artificial inoculations of *N. populina* caused statistically larger cankers than the other treatments and control, but were generally smaller than naturally occurring canker lesions. There are two plausible explanations for this difference: 1) despite repeated efforts and statistical support, we failed to isolate the real causal agent of aspen running canker from wild cankers, or 2) artificial inoculation conditions were suboptimal compared to natural infections. We believe the latter explanation is the more parsimonious; when inoculating trees with canker fungi, it is a typical experience that the size of the resulting lesion is directly dependent on the season of inoculation in relation to the period of tree dormancy (Adams et al. 2005). Inoculation of aspen with virulent *Cytospora*

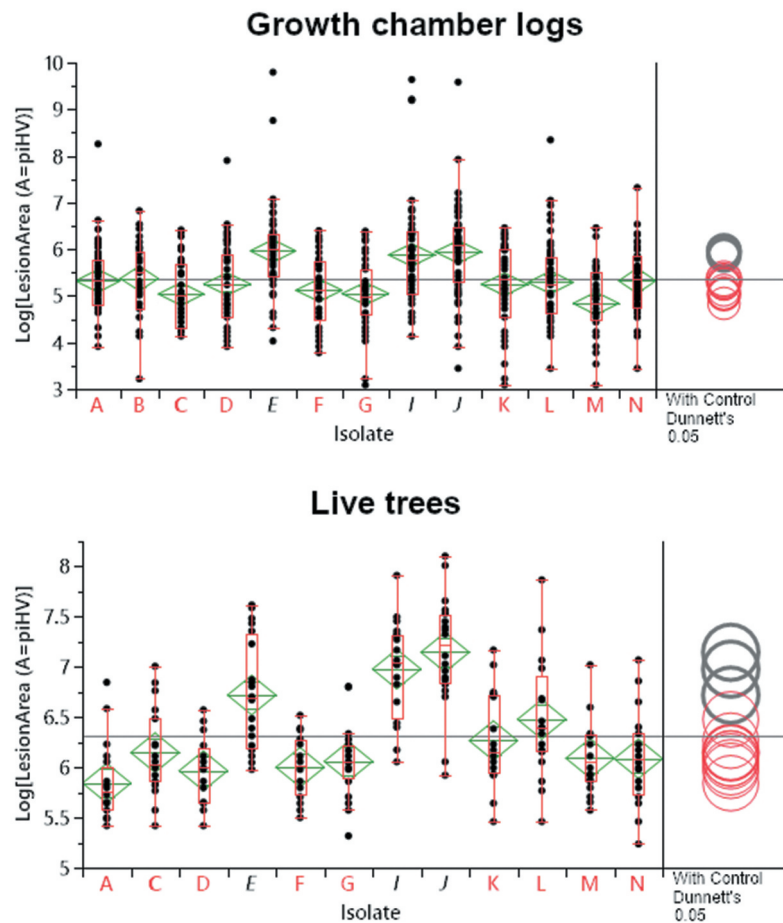


Fig. 4 (Colour online) ANOVA results of lesion area for each isolate (log transformed) with Dunnett's multiple comparisons with a control group (isolate C, sterile agar plug). Horizontal line is the grand mean of the response, points are model residuals, green triangles show estimated means with upper and lower 95% confidence intervals, and outlier box plots are red. Isolate labels and comparison circles in black/dark grey differ significantly from the control ($p < 0.05$). Isolate codes are described in Table 1.

species in summer results in small delimited lesions with copious gummosis, whereas inoculations in late fall results in metre-long running cankers which begin to expand during bud break (Adams et al. 2005). For optimal lesion size, perhaps inoculations in Alaska should have begun in October or in late-May preceding bud break. In alder, this relationship between dormant host and growing fungus was reversed when hot summer temperatures initiated canker enlargement by *Cytospora umbrina* (Worrall et al. 2010a). Symptoms of aspen running canker in the forests of Alaska are somewhat similar to milder cankers on aspen in the Rocky Mountains, which are caused by various species of *Cytospora*. However, *Cytospora* canker differs in that it frequently oozes liquid (Jacobi 2013) and produces extensive fruiting bodies on dead bark tissue over the canker lesions (Kepley and Jacobi 2000). In addition,

Cytospora species are readily isolated from *Cytospora* canker margins, whereas we isolated *Cytospora* spp. only from old cankers of previous years or canker samples stored for weeks in a cold room. We found little sign of *Cytospora* canker in Alaskan aspen stands and when it was observed, it was generally on fallen limbs and trunks that had been dead for several years.

In the Rocky Mountains from the southern ranges in the USA to Canada, sudden aspen decline (SAD) has been widely reported and studied (Worrall et al. 2008, 2010b, 2013; Marchetti et al. 2011). SAD was reported to not affect saplings (Worrall et al. 2008). The aspen running canker in Alaska is most destructive on saplings in the understory of intermediate-aged and mature stands, but is nearly absent from younger aspen growing in early-successional stands (Ruess et al. 2021). Aspen running canker has been observed primarily on live



Fig 5 (Colour online) Canker lesions resulting from inoculation of *Neodothiora populina* isolates E, I and J on (a) cut logs and (b) live trees.

standing trees with turgid bark and recently dead trees with conspicuous sunken cankers showing retention of fine terminal twigs in canopies. These symptoms represent very recent mortality (1–2 years) and rarely display the branch dieback and reduced-canopy typical of decline symptoms (personal observations, Ruess et al. 2021). In SAD, mature trees show extensive branch dieback while active cankers are generally not observed (Marchetti et al. 2011); insects and diseases were reported to be minor secondary contributors to drought-associated mortality. Insects and other diseases were rarely seen on the trees suffering from aspen running canker, other than the ubiquitous aspen leaf miner (*Phyllocnistis populiella* Cham.), which has been reported to exacerbate trees stressed by drought (Wagner et al. 2020). Moisture stress has been implicated in higher incidence of aspen running canker throughout interior Alaska (Ruess et al. 2021). Unfortunately, endophytic fungi from symptomatic tissues were not included in identification or inoculation studies in SAD affected aspen. We now believe, based on the work of this study, that the potential role of new pathogenic fungi, particularly *N. populina*, should be revisited and pursued with exhaustive isolations as pathogenicity trials might reveal or exclude

a significant fungal contributing factor. We found that cursory examination of diseased and healthy host microbiomes was especially beneficial in revealing dominant and rare microbes and in estimating the likely classification of the known and unknown microbes present. Our Illumina amplicon results served to guide our attention towards yeast and yeast-like potential pathogens.

A significant barrier to identifying the organisms involved in plant disease is that many isolated fungi are not readily identifiable because they have not previously been considered pathogens. This is especially true of the yeast and yeast-like fungi. Today, identification of fungi often begins with DNA barcoding. Cultural and microscopic morphological study then follows, rather than the historical morphological identification paradigm. The worldwide GenBank database is rapidly accumulating gene sequences of tree-inhabiting (endophytic) fungi, chiefly due to efforts by the team of Arnold (2007) and U'ren (2019) at the Robert L. Gilbertson (Forest) Mycology herbarium at University of Arizona (<http://cals.arizona.edu>). The herbarium has expanded with 30 000 accessions of endophytic fungi, and their phylogenetically informative sequences deposited in GenBank. Haroim et al. (2015) has also deposited many sequences of *P. tremula* fungal inhabitants. However, in each case,

most tree-inhabiting fungal accessions are entered as ‘unidentified’ or ‘uncultured’ endophyte. In our study, including the endophytes and presumed endophytes in Koch’s Postulates inoculation trials was important for resolving the causal agent of this new aspen running canker disease. Completing Koch’s Postulates with all of the species that were repeatedly isolated from diseased trees revealed a virulent fungus that would traditionally have been considered an endophyte, and thus not likely to have been included in pathogenicity trials.

The first challenge in isolating the pathogen was to obtain any fungi from the canker margins. The second challenge was to conceptually regard pale or whitish yeast-like cultures as potential tree pathogens. Practising forest pathologists commonly use standard forest pathology media based on malt extract agar during isolations from diseased tissues of woody plants and become familiar with the characteristic cultures of common plant-inhabiting black yeast-like endophytic fungi, such as *Aureobasidium* and *Hormonema* species (de Hoog and Yurlova 1994). Pale yeast-like cultures are typically regarded as common contaminants; therefore, such cultures are overlooked. We have discovered that placing pale yeast-like colonies obtained from diseased aspen tissues onto CHROMagar® Candida can aid in distinguishing the new pathogen based on its diagnostic blue colour. We first observed hyphal growth in *N. populina* by transferring the yeast morph onto rice extract agar, which is a standard method among yeast research scientists used for yeast identification purposes (Joshi et al. 1993). Later we discovered media of low nutrient levels, such as Difco PDA, could induce dark pigmentation and hyphal growth. In describing *N. populina*, Crous et al. (2020) succeeded in inducing pycnidial formation described as a *Dothichiza* morph, and a black yeast *Hormonema* morph. The pycnidium has a multilayered (six layers) wall and resembles the *Sclerophoma* morph commonly seen with *Hormonema dematioides*, a black yeast morph in the (generally familiar) darkly pigmented cultures of *Sydowia polyspora* (Sutton and Waterson 1970). *Hormonema* and *Aureobasidium* morphs produce conidia directly from foci on undifferentiated vegetative hyphal cells in a blastic manner with percurrent proliferation. *Hormonema* morphs produce the conidia asynchronously (as does *N. populina*), whereas *Aureobasidium* morphs synchronously form the conidia (de Hoog and Yurlova 1994). *Endoconidioma populi* is an endophyte of aspen that also forms a *Hormonema*-like morph, but is differentiated by forming the conidia endogenously (Tsuneda et al. 2004). These and other fungal species

could be confused with *N. populina* unless phylogenetically informative gene sequences are compared. In fact, GenBank #GU564975.1 ‘Uncultured fungus isolate UPSC_A12_12’ from European aspen is a close match in ITS sequence homology (99.6% similarity) to *N. populina*. Albrechtsen et al. (2010) described a diverse endophytic mycota from European aspen.

Whenever a destructive new pathogen suddenly appears on a well-studied and economically valuable timber species like trembling aspen, questions arise as to whether the new pathogen might be an invasive species or an endemic favoured by a compromised host. In Alaska, the combined stresses of drought and an outbreak of leaf miner have likely compromised trembling aspen (Ruess et al. 2021). Rapid and extreme changes in the climate in Alaska over the past decades (Chapin et al. 2014) have been stressful on numerous forest species and introductions of new insect pests have also resulted in new stresses. To our knowledge, there have been no previous reports of occurrence of such massive diffuse cankers occurring on individual stressed aspen, or aspen stressed in an unusually unfavourable environmental niche. Because common endophytes on one continent may be unknowingly transported to another continent in nursery stock, for example, with propagation cuttings of European aspen, it is possible that *P. tremula*, grown in a nursery in North America may then have been shipped to Alaska and unknowingly carried *N. populina* as a symptomless endophyte. The scenario of an endophyte becoming a highly virulent pathogen when it encounters and infects a naive host that is taxonomically related to the original host has occurred before. This scenario played out with *Discula destructiva* transferring from Asian *Cornus kousa* to the native American *Cornus florida* and *C. nuttallii*. This host shift resulted in widespread and rapid dogwood mortality induced by infection of epicormic shoots on the main stem which resulted in large rapidly running cankers (Daughtrey and Hibben 1994). Further identification and cultivation of the uncultured endophyte of *P. tremula* that corresponds to the GenBank #GU564975.1 sequence could be crucial in investigating whether it is pathogenic on *P. tremuloides* and whether this Dothioraceous fungus is *N. populina* or a very close undescribed relative.

In the past decade, we have participated in four new and sudden forest disease studies in which we have struggled to identify causal agents: 1) the present study on the aspen running canker of *P. tremuloides* in Alaska; 2) *Tsuga heterophylla* branch dieback and mortality in Alaska (unpublished data); 3) Alder canker and mortality in Alaska and the high altitude

southern Rocky Mountains (Stanosz et al. 2010; Worrall et al. 2010a); and 4) sudden aspen death on *P. tremuloides* in the southern Rocky Mountains in the USA. (Kepley et al. 2015). The first three diseases presented as undiagnosed canker diseases occurring on three different hosts. In each case, isolations resulted in the recovery of many different fungi from the rapidly advancing margins of diffuse cankers. Once identified, several of the recovered species were found to have been traditionally considered endophytes, while several others were either known pathogens or closely related to known pathogens. In each study, repeated attempts to complete Koch's Postulates with all or most recovered species revealed that the most virulent fungus would traditionally have been considered an endophyte, and thus usually not included in pathogenicity trials. Including fungi usually considered endophytes has resulted in important and crucial new information in forest pathology. We therefore emphasize the need for including host-identified endophytic fungi routinely in current and future Koch's Postulate trials. We further encourage reasonable recording of such identified fungi in publications and populating GenBank with the sequence data and natural history information.

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