Importance of local and long-distance *Heterobasidion irregulare* aerial basidiospore dispersal for future infection centres in thinned red pine plantations in Quebec

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ABSTRACT

Heterobasidion irregulare is a basidiomyceteous forest pathogenic fungus which causes a root disease capable of killing large trees. Infection probability is proportional to the density of aerial basidiospores infecting freshly cut stumps. The purpose of this study was to quantify for the first time *H. irregulare* aerial basidiospore density in southern Québec and to determine the importance of a local basidiospore load from a nearby infested plantation. Spore counts from automated rotary arm spore collectors were assayed using a ribosomal ITS TaqMan real-time PCR detection assay. Cumulative basidiospore deposits on a 30-cm stump were estimated to be highest in the infected plantation with 2.37 basidiospores per stump, with decreasing count of 0.52 basidiospores at a 500-m distance and 0.46 basidiospores at a 1.5-km distance. At 5 km from the infected plantation, the number of basidiospores deposited on a stump was 0.22, a value similar to those at distances of 25 and 140 km. Since a single *Heterobasidion* basidiospore may travel as far as 500 km, the level detected at 5 km may represent the background basidiospore load resulting from long-distance dispersal, and basidiospores from a nearby infected plantation are unlikely to be an added risk of infection to plantations located more than 5 km away. Long distance aerial basidiospore dispersal will remain for now the most important constant source of infectious *H. irregulare* in Québec. Despite the very low risk level per stump in this study, the large number of trees thinned annually warrants that stump treatment with Rotstop® C is still the best strategy, especially in valuable plantations.

Key words: basidiomycetes, basidiospore deposition, biological control, forest pathology, ribosomal ITS, root rot, Rotstop

RÉSUMÉ

Heterobasidion irregulare est un champignon pathogène forestier capable de tuer des arbres de grand diamètre et le risque d'infection est proportionnel à la quantité de basidiospores aériennes qui se déposeront sur les souches des pins rouges fraîchement coupées. Le but de cette étude était de quantifier pour la première fois la densité de basidiospores aériennes dans le sud du Québec et déterminer l'importance de basidiospores locales provenant d'une plantation infectée située à proximité. Le comptage des basidiospores capturées par un collecteur à bras rotatif s'est fait à l'aide de PCR en temps réel avec des sondes TaqMan visant l'ITS de l'ADN ribosomique. Le compte cumulatif de basidiospores sur une souche de 30 cm était maximal, soit 2,37 basidiospores par souche, dans la plantation infectée, suivi de 0,52 basidiospores à 500 m de distance et de 0,46 basidiospores à 1,5 km. À 5 km de la plantation infectée, le nombre cumulatif de basidiospores était de 0,22, une valeur semblable à celles se trouvant à 25 et 140 km. Puisque les basidiospores de *Heterobasidion* sont reconnues pour voyager sur des distances allant jusqu'à 500 km, le nombre cumulatif de basidiospores à 5 km peut être considéré comme le niveau basal résultant de la propagation sur une longue distance et les basidiospores produites localement ont peu de chance d'induire un risque supplémentaire d'infection à une distance de plus de 5 km. La propagation des basidiospores à longue distance restera dans un avenir rapproché la principale source de basidiospores infectieuses de *H. irregulare* dans la province de Québec. Malgré les faibles taux cumulatifs relevés, le grand nombre d'arbres coupés annuellement lors d'éclaircies porte à croire que le traitement des souches avec Rotstop® C reste la meilleure stratégie, surtout dans les plantations de grandes valeurs.

Mots-clés: basidiomycètes, dépôt de basidiospores, ITS ribosomique, carie racinaire, pathologie forestière, lutte biologique, Rotstop

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and to determine the importance of local basidiospore load from nearby infested plantations.

Methods and Materials

Five automated rotary arm spore collectors were deployed in the field in late September 2014 from the most easterly known infected plantation located in Sainte-Clotilde-de-Horton (45° 59' 35.65" N, 72° 11' 35.56" W).

The plantation was heavily damaged during the 1998 ice storm and a damaged tree removal protocol was carried out the following summer. *Heterobasidion irregulare*-related mortality was first detected in 2009 and a mortality centre continues to expand. Infected trees have been removed periodically; however, infected stumps were left on site. Spore collectors (Compagnie de Recherches Phytodata Inc., St-Edouard, QC) were installed at the end of September 2014 in an eastward transect beginning in the plantation, then at 500 m, 1.5 km, 5 km, 25 km and 140 km from the plantation. Spore collectors were programmed to spin their rotary arm for 5 min every 90 min, and sticky rods (1.5 mm X 1.5 mm X 3 cm) were collected every week from September 29 to November 15, for a total of 560 min (9.33 h) of exposure for each rod/week.

One sticky rod per sample was placed in a separate extraction tube. DNA extraction was then performed following the protocol developed by Lamarche *et al.* (2016). Briefly, 1 ml of hexane was added to each tube, vortexed for 30 seconds and rods removed. Tubes were centrifuged for 2 min at 20 000 x g and the supernatant was discarded. The spore pellet was rinsed with 1 ml of hexane and dried using a Speedvac concentrator. Samples were kept at -20°C until DNA extraction was performed.

Samples were lysed by adding a 3-mm tungsten bead, 180 µl of Buffer ATL, and 1 µl of Reagent DX (Qiagen, Valencia, CA, USA) to each sample and ground in a MixerMill 300. Samples were centrifuged and 20 µl of Proteinase K added. Samples were incubated at 56 °C overnight and DNA extraction was done the following day using the QIAamp DNA Micro kit (Qiagen).

Basidiospore counts were done using a ribosomal ITS TaqMan assay as described in Lamarche *et al.* (2016). Briefly, real-time PCR was performed using 1X QuantiTect™ Multiplex PCR NoROX Master Mix (Qiagen), 0.6 µM of each primer, 0.1 µM of TaqMan probe and 1 µl of template DNA, for a total volume of 10 µl. All reactions were on an Applied Biosystems® 7500 Fast Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). Basidiospore equivalents per rod were calculated by translating Ct values using standard curve equations. As conidia can be multinucleate (Hsiang *et al.* 1989), a selected mono-basidiospore isolate of *H. irregulare* (#98-008 confirmed with 1-2 nuclei/conidia), was used to generate standard curves with a correlation coefficient (r^2) of 0.99 (Lamarche *et al.* 2016).

Introduction

Heterobasidion irregulare (Underw.) Garbel. & Otrrosina is a basidiomyceteous pathogen that produces sporophores decomposing stumps and dead trees. This fungus causes a root disease capable of killing large trees. The primary mode of infection of this aggressive pathogen is through aerial basidiospores and successful infections occur when the basidiospores are deposited on freshly-cut stump surfaces. Once *H. irregulare* is established, it will colonize the roots and spread between infected and non-infected roots via root grafts to further its spread among uninfected trees within a plantation.

In Canada, Jorgensen (1956) was the first to report the discovery of *H. irregulare* on red pine (*Pinus resinosa* Ait.) at St. Williams in Ontario. By 1968, the pathogen had spread further north near Ottawa (Sippell *et al.* 1968, Punter 1970). This disease has since been found in Québec in 1989 (Laflamme and Blais 1995) and new infection centres have been found in 14 plantations at eight locations in different southern regions of Québec (MFFP 2016). In eastern Canada, the pathogen is found mainly in red pine plantations in southern Ontario and Québec and is progressing northward and eastward.

Red pine plantations occupy a large area of the productive forested land in southern Québec where more than 10 000 ha have been planted since the 1970s (MFFP 2015). Periodic thinning of plantations is a silvicultural prescription recommended to increase stand quality and volume. However, thinning makes these plantations vulnerable to *H. irregulare* infection as it produces fresh stumps that are infection courts for the disease in valuable plantations.

Eastern Canada is still at the beginning of a possible regional epidemic and the probability of new infections would be described as proportional to the density of aerial basidiospores (Stambaugh *et al.* 1962, Gonthier *et al.* 2005). In Europe, the probability of infection on a freshly cut stump can be as high as 100% in many countries (Vollbrecht *et al.* 1995). While the basidiospores responsible for past infections in Québec were most likely caused by long-distance dispersal (up to 500 km; Kallio (1970), basidiospores from local infected plantations most probably play an important role in future infections.

The purpose of this study was to quantify for the first time *H. irregulare* aerial basidiospore density in southern Québec

Total amount of basidiospores sampled per m^3 was calculated using: number of basidiospores per rod $\times 1000 \text{ L}/\text{m}^3/\text{h} / 21.6 \text{ L}/\text{min}$ per rod $\times 60 \text{ min}/\text{h} \times 9.3 \text{ h}$ (Fall *et al.* 2015). The amount of basidiospores $\text{m}^{-2} \text{ h}^{-1}$ for each week was calculated by dividing the weekly amount of basidiospores per m^3 by 9.3 hours, assuming total basidiospores falling on the m^2 cross-section was obtained by summing all spores in the $\text{m}^{-3} \text{ h}^{-1}$ above (Pfender *et al.* 2007). Total amount of basidiospores deposited on a 30-cm stump was calculated using: total cumulative amount of basidiospores m^{-3} on site X (stump radius² $\times \pi$), again assuming total basidiospores falling on the m^2 cross-section was obtained by summing all spores in the m^{-3} above.

The relationship between weekly basidiospore counts and distance from the infected plantation was examined using a generalized linear model in R (R Core Team 2016) with a negative binomial error structure (R function `glm.nb`, Venables and Ripley 2002).

Results

Basidiospore counts peaked during the fourth week of October 2014 (Table 1) with a total of 443 collected on the rods for that week. Highest total basidiospore count at one location was 405 in the infected plantation, which decreased to 88 basidiospores 500 m away, then to 80 basidiospores at 1.5 km, down to 14 basidiospores 140 km from the infected plantation (Table 1). Total basidiospore density cumulated over six weeks was highest in the plantation with 33.4 basidiospores m^{-3} , then 7.3 basidiospores m^{-3} at 500 m, down to 1.2 basidiospore m^{-3} at 140 km. In order to estimate the risk of infecting a stump, we calculated the cumulated basidiospore deposits on a 30-cm stump. It was highest in the infected plantation with 2.4 basidiospores per stump, followed by 0.5 at 500 m, 0.5 at 1.5 km, and 0.1 at 140 km.

The analysis conducted using the generalized linear model in R showed that there was a significant interaction between

the effects of collection date and distance, i.e., the decline in basidiospore count with increasing distance was less pronounced at dates earlier and later than the peak during the week of October 24th. The dispersion parameter (theta value) of the model was 1.16, indicating a good fit.

Discussion

The cumulative aerial basidiospore density (33.4) was highest in the infected plantation and rapidly declined to 6.6 basidiospores m^{-3} at the site located 1.5 km away (Table 1). When translated in number of basidiospores deposited on a 30-cm stump during that 6-week period, values fell steeply below 1.0 basidiospore (0.52) per stump at 500 m from the infected plantation. Edmonds and Driver (1974) also used an automated rotary arm spore collector and found deposition rates between 3 to 70 basidiospore $\text{m}^{-2} \text{ h}^{-1}$ in an infected Washington State forest, values that can be compared to our highest weekly values of $3.1 \text{ m}^{-2} \text{ h}^{-1}$ in the infected plantation and $0.4 \text{ m}^{-2} \text{ h}^{-1}$ 1.5 km away in the fourth week of October. Similarly, Gonthier *et al.* (2005) found deposit rates on fresh wood disks ranged from 169 to $15\,550 \text{ m}^{-2} \text{ h}^{-1}$ in Italian alpine forests where disease incidence ranged from 30% to 50%. Punter (1970) measurements on fresh wood disks in Ontario found values between 0.1-2.1 basidiospores $\text{m}^{-2} \text{ h}^{-1}$. Most of the weekly values we observed were under $0.2 \text{ m}^{-2} \text{ h}^{-1}$, indicating that we were clearly at the early stages of the epidemic in Québec.

The rotary arm spore collector and the wood disk method measure different phenomena. Rotary arm collectors give the number of basidiospores per m^3 , whereas fresh wood disks indicate the actual number of viable infectious basidiospores per m^2 . The number of infections on a stump is lower than the actual number of basidiospores landing on the stump for various reasons (Gonthier *et al.* 2005), including environmental factors, virulence of fungal isolates, competition with other fungi, and the ability of the host to fence off the colo-

Table 1. Number of *H. irregulare* spores collected weekly in the Sainte-Clotilde-de-Horton plantation at 0.5 km, 1.5 km, 5 km, 25 km and 140 km.

	Distance from infected plantation (in km)						Weekly total
	0	0.5	1.5	5	25	140	
Sampling week							
Oct. 3, 2014	2780*	3 (0.03) ¹	6 (0.06)	4 (0.04)	0	1(0.01)	14
Oct. 10, 2014	1584*	7 (0.06)	n/a	8(0.07)	3 (0.03)	n/a	18
Oct. 17, 2014	n/a ²	3 (0.03)	5 (0.044)	4 (0.04)	11 (0.09)	6 (0.06)	29
Oct. 24, 2014	345 (3.05)	33 (0.29)	45 (0.39)	2 (0.02)	11 (0.09)	7 (0.06)	443
Oct. 31, 2014	47 (0.42)	30 (0.27)	19 (0.17)	16 (0.01)	2 (0.02)	n/a	114
Nov. 7, 2014	13 (0.12)	12 (0.11)	5 (0.04)	3 (0.03)	1 (0.01)	n/a	34
Total spore count	405	88	80	37	28	14	
Cumulative count m^{-3}	33.41	7.26	6.60	3.05	2.31	1.16	
Cumulative spores deposited on a 30-cm stump	2.37	0.52	0.46	0.22	0.16	0.08	

¹Values in brackets indicate spore deposition per $\text{m}^{-2} \text{ h}^{-1}$ for the 7-day period.

²n/a: not available.

*Values from deposition inside the infected plantation that were interpreted as *H. irregulare* conidia and were not considered in analysis.

nization process. Rishbeth (1959) reported that aerial basidiospore viability varied between 1% and 5%. It is not known how many basidiospores are needed to actually initiate an infection on a fresh stump but extrapolation of Gonthier *et al.* (2005) data suggests that a deposition rate of 5 basidiospores $\text{m}^{-2} \text{h}^{-1}$ is needed to initiate infection on stumps. Applied to our experimental design, this would represent approximately 356 basidiospores per 30-cm stump over six weeks. In Ontario, Punter (1970) observed that successful stump infection was rare when basidiospores were trapped on wood disks at a rate lower than 100 basidiospores $\text{m}^{-2} \text{h}^{-1}$. Compared to the rotary arm spore collector method, wood disk trapping has the advantage of providing actual infection per $\text{m}^{-2} \text{h}^{-1}$ but it is very labour intensive. Rotary arm spore collectors are efficient at collecting spores from any fungal species that can be identified using next generation sequencing methods, thus making it possible to study multiple tree disease fungi simultaneously.

Kallio (1970) demonstrated that 99.9% of *Heterobasidion* basidiospores were deposited within 100 m from the fruiting body. Our model shows a similar decrease in the number of basidiospores with an increase in distance from the infected plantation. At 5 km, the number of basidiospores deposited on a 30-cm stump was 0.22, a value similar to those found at 25 km and 140 km. Since a single *Heterobasidion* basidiospore might travel as far as 500 km (Kallio 1970), this may represent the background basidiospore load resulting from long-distance dispersal. While it is not known the basidiospore threshold required to trigger an infection on a freshly cut stump, it is unlikely that local basidiospores can be an added risk of infection more than 5 km away. However, the low basidiospore density observed as the background basidiospore load is still high enough to be responsible for past infections in Québec.

Early readings of 2780 and 1584 spores per week in the plantation during October 3 and October 10, 2014, were interpreted as *Heterobasidion* conidia and were not considered in the analysis since spore count numbers at 500 m and 1.5 km from the plantation were low during those weeks. Only very small sporophores were visible in the plantation at that time. Even though conidia were reported to be more resistant than basidiospores to ultraviolet light and high temperatures (Kallio 1970), the aerial dissemination range and seasonal production of conidia is unknown.

Eastward winds are dominant in Québec and we hypothesized that an eastern transect would best capture basidiospore dispersal from an infected plantation. Due to shifting winds and the absence of meteorological data for the site, we do not know how effective we were at catching basidiospores from the infected plantation, therefore a freshly thinned plantation located directly downwind from an infected site may be more at risk than what was estimated here.

As the number of known infected plantations is presently low in the province and basidiospore density rapidly decreases with distance, long-distance aerial basidiospore dispersal will remain for a while the most important constant source of infectious *H. irregulare* basidiospores in the province. Literature indicates freshly cut stumps are infection courts in the first two weeks (Cobb and Schmidt 1964, Ross

1968) and for this reason, thinning is not recommended during summer and fall when spores may be present. This two-week threshold is explained by the crust of resin that forms after that period, possibly inhibiting spore penetration. However, cold temperatures during late fall and winter thinning can reduce resin formation and lead to infection during warmer winter periods, suggesting that late fall and winter thinning is not an absolute control method.

Despite the very low risk level associated with the calculated number of basidiospores deposited on a 30-cm stump in this study, the large number of trees thinned annually warrants that stump treatment (Dumas and Laflamme 2013) with the biological control agent Rotstop® C (BioForest Technologies Inc., Sault Ste. Marie, ON) is still the best strategy, especially in valuable plantations. In future experiments we will deploy across the southern province of Québec new rotary arm spore collectors to achieve year-long weekly monitoring.

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