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Conifer species influence *Heterobasidion irregulare* sporulation on stem discs exposed to natural inoculum and root wood decay in soil-block tests

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Summary

The fungal pathogen *Heterobasidion irregulare* has caused substantial damage to pine species in much of the south-eastern United States, but the potential for damage from Heterobasidion root disease to forest tree species of the Great Lakes Region has not been thoroughly investigated. In each of three trials in two infested stands, fresh stem discs of six native conifer species were exposed to natural inoculum and examined for the asexual *Spiniger* stage of *H. irregulare* after incubation in the laboratory. In the first trial, the fungus was infrequently observed on discs, and differences among species were not found. But in the other two trials, detection of the pathogen was much more frequent on discs of tamarack (*Larix laricina*), red pine (*Pinus resinosa*) and white spruce (*Picea glauca*) than those of northern white cedar (*Thuja occidentalis*), eastern hemlock (*Tsuga canadensis*) and balsam fir (*Abies balsamea*). In a second study, the ability of an isolate of *H. irregulare* to decay root sapwood of these same species was compared using a soil-block decay test. After 16 weeks of incubation, weight loss due to decay was greatest for red pine (23.2%) and larch (19.1%) and least for balsam fir (1.3%). Differences in the ability of *H. irregulare* to infect and saprophytically utilize substrate of these important tree species should prompt additional investigation of the relative risk posed to forests of the Great Lakes Region.

1 | INTRODUCTION

The fungus *Heterobasidion irregulare* Garbelotto & Otrosina is a recently described North American species in the former *H. annosum* (Fr.) Bref. *sensu lato* (*s.l.*) species complex (Otrosina & Garbelotto, 2010). Long present in both eastern and western North America, *H. irregulare* has more recently been discovered to have been introduced into Italy (Gonthier, Nicolotti, Linzer, Guglielmo, & Garbelotto, 2007). *H. irregulare* is closely related to its European sister species *H. annosum sensu stricto* (*s.s.*) (Dalman, Olson, & Stenlid, 2010; Linzer et al., 2008). With *H. annosum s.s*, *H. irregulare* comprised the P intersterility group (ISG) of the former species complex, so designated due to the frequent occurrence on *Pinus* hosts (Otrosina & Garbelotto, 2010). In describing *H. irregulare*, Otrosina and Garbelotto (2010) distinguished it from the western North American *Heterobasidion* species, *Heterobasidion occidentale* Otrosina & Garbelotto. Linzer et al. (2008) analysed DNA sequence information from portions of two nuclear and two mitochondrial loci of a large number of North American *Heterobasidion* isolates. All isolates originating from locations in the eastern United States and Canada were characterized as P ISG, and these can now be referred to as *H. irregulare*.

In a disease cycle similar to those of other *Heterobasidion* species (Hodges, 1969), *H. irregulare* frequently infects fresh conifer stump surfaces or wounds following deposition of airborne basidiospores (and perhaps conidia), or infects roots following percolation of spores through soil. It subsequently spreads to adjacent trees through root grafts, root contact or growth for limited distances through soil. High incidence of *H. irregulare* and severe damage that can include growth loss and mortality historically has been reported following thinning of commercial pine plantations in the south-eastern United States (e.g., Bradford, Alexander, & Skelly, 1978; Mason, 1969; Powers & Boyce, 1961; Powers & Hodges, 1970).

Heterobasidion root disease has been infrequently reported and little studied in conifer forests of the Great Lakes Region of the United States. Although known in southern Michigan for decades (Strong & Lemmien, 1964), the known distribution of *H. irregulare* in that state is discontinuous and until recently was thought to be quite limited (Michigan Department of Natural Resources, 2015). Heterobasidion root disease in Minnesota was first reported in 2015, at a single red pine plantation in the south-eastern part of that state (Blanchette, Held, Mollov, Blake, & D'Amato, 2015). In contrast, although first detected in Wisconsin in 1993 (Stanosz, Guthmiller, & Kearby, 1995), *H. irregulare* by 2015 was known to be present in 24 of 72 counties of that state (Wisconsin Department of Natural Resources, 2015). In Wisconsin, Heterobasidion root disease has been found most frequently in plantations of red pine (*Pinus resinosa* Aiton) and white pine (*P. strobus* L.) (Wisconsin Department of Natural Resources, 2015). Red pine is commercially important and is the most-planted tree species in the region. Periodic thinnings during typical 55–90 year rotations provide stump surfaces for infection. Red pines in plantations are highly root-grafted (Armson, 1959), facilitating belowground spread. The relative susceptibility of mature red pines to Heterobasidion root disease is manifested by mortality in radially expanding pockets of dying and dead trees in these thinned plantations.

Sinclair (1964) compiled a list of conifer species reported as hosts or substrates of *Heterobasidion annosum sensu lato (s.l.)*. In addition to pines, included are species of several other genera of common economically and ecologically important conifers of the Great Lakes Region and boreal forests of the north-eastern United States and eastern Canada (Eyre, 1980). Cited reports are often anecdotal, however, and do not provide information regarding frequency of infection or relative host susceptibility. Obtaining such knowledge is difficult in areas such as the Great Lakes Region of the United States where *H. irregulare* is not yet ubiquitous or uniformly distributed or because the diversity of potential host tree species may not occur in mixtures at a single site.

Excised portions of tree stems, branches and roots have been utilized in numerous studies involving *Heterobasidion* species. Among the many examples too numerous to completely list here, are use of stem discs for pathogen detection (Jorgensen, 1961; Rishbeth & Meredith, 1957), population studies (Gonthier, Garbelotto, Varese, & Nicolotti, 2001), investigation of environmental influences on basidiospore viability (Rishbeth, 1951) and diurnal and seasonal availability of airborne inoculum (Gonthier, Garbelotto, & Nicolotti, 2005; Kallio, 1970; Sinclair, 1964). Pieces of stems, branches or roots have been inoculated to study colonization by *Heterobasidion* isolates, individually or as these interacted with other fungal species, including potential biocontrol agents (Boyce, 1963; Rishbeth, 1950, 1951; Woods, Woodward, & Redfern, 2005). The abilities of *Heterobasidion* isolates to saprophytically decay wood and the relative resistance of woods of different conifer species to decay have been assessed using standardized methods in soil-block decay tests (Daniel, Asiegbu, & Johansson, 1998; Platt, Cowling, & Hodges, 1965). Stem or branch segments have also been used in studies exploring influences of both *Heterobasidion* species and conifer substrates on sexual and asexual reproductive potential (Giordano, Gonthier, Lione, Capretti, & Garbelotto, 2014; Hsiang & Edmonds, 1989).

The Wisconsin Department of Natural Resources (2013) has prepared a "risk-based guide" for fungicide treatment to prevent Heterobasidion root disease. This guide refers to stump treatments in stands in which more than 50% of the basal area is of native pine species, and in which the future desired stand is to be primarily pines. Although this guide addresses the threat to regional tree species with which *H. irregulare* has been most frequently associated to date, our current knowledge of the potential risk to other conifers is limited. Research has been constrained, not only by a paucity of funding, but also due to the very incomplete knowledge of distribution of *H. irregulare* in the Great Lakes Region. The pathogen cannot be introduced for studies into areas occupied by stands of other important conifer species where it has not yet been detected. In addition, because several of the variety of conifers that are possible hosts rarely co-occur, comparative field studies using intact, established trees are not possible. Given these limitations, two studies were conducted to obtain information that might be useful in very preliminary attempts to estimate risk from Heterobasidion root disease to several economically and ecologically important conifers in the Great Lakes Region. In the first, fresh conifer stem discs were exposed to natural inoculum of *H. irregulare* in infested stands. In the second study, ability of *H. irregulare* to decay conifer root sapwood was compared using a soil-block decay test.

2 | MATERIALS AND METHODS

2.1 | Field exposure of stem discs

To obtain stem discs for each of three separate trials, trees were felled on the Menominee Indian Tribe of Wisconsin's reservation in mid-August, late September and early November 2011. In addition to red pine, species were northern white cedar (*Thuja occidentalis* L.), eastern hemlock (*Tsuga canadensis* (L.) Carrière), balsam fir (*Abies balsamea* (L.) Mill.), tamarack (*Larix laricina* (Du Roi) K. Koch) and white spruce (*Picea glauca* (Du Roi) K. Koch). Stem discs approximately 15 cm in diameter and 2.5 cm thick were cut with a chain saw. A black alcohol-soluble marker (Sharpie, Sanford, Newell Rubbermaid Office Products, Oak Brook, IL, USA) was used to make eight dots, each approximately 3 mm in diameter, equally spaced on the sapwood about 1 cm in from the bark. Discs were deployed within 24 hr in two *H. irregulare* -infested red pine plantations. One site was in Waushara Co., WI, and the other in Marquette Co., WI. Four stumps bearing *H. irregulare* basidiocarps were selected, and one disc of each species was placed in a circle at a radial distance of approximately 1 m around each stump. Each disc was placed on an individual plastic bag labelled as to conifer species, plantation location and stump number which was laid on the soil.

After 3 days, discs were picked up and placed into their respective plastic bags which were sealed and brought to the laboratory. The discs were removed from bags, wrapped in paper towels moistened with 20 ml of water, replaced in the bags and incubated at approximately 22 ± 1°C. Periodically during the next 2 weeks (usually after 7 days, and again if necessary after 10 days and for the last time after 14 days), a circular area approximately 16 mm in diameter (subsequently referred to as a spot) centred on each dot was examined using a dissecting microscope at 30 × magnification for the presence of conidiophores and conidia of the *Spiniger* Stalpers state of *H. irregulare* (Brefeld, 1889; Stalpers, 1974). In addition, two similarly prepared negative control discs of each species were incubated without exposure in the field and subsequently examined for the pathogen. The number of discs on which at least one spot was positive and the cumulative number of positive spots per disc were recorded. Results for the various species were compared using the Kruskal–Wallis test of equality of medians using Minitab for Windows version 14 (Minitab Inc., State College, PA, USA).

2.2 | Soil-block decay test

On the Menominee Reservation, a large root was collected from one mature, living tree of each of the six conifer species used for the field exposure of stem discs (above). Bark was removed, and outer sapwood blocks 1 cm × 1 cm × 1 cm were cut, kiln-dried to 10% moisture content and weighed. A standard method (American Wood Protection Association, 2015) for testing wood preservatives was then employed with slight modifications. Briefly, blocks were conditioned for 1 week at 27°C and 30% relative humidity, and then sterilized in an autoclave. An isolate obtained from a diseased red pine root (confirmed as *H. irregulare* by ITS sequence comparison with sequences of well-characterized isolates in GenBank, data not shown) was used to inoculate a southern yellow pine starter strip in each of 16 soil bottles for each conifer species tested. Bottles were incubated at 27°C and 70% relative humidity for approximately 2 weeks to allow establishment of the fungus. Then four wood blocks (subsamples) of one conifer species were placed into each bottle and incubation continued. After 4, 8, 12 and 16 weeks, wood blocks were removed from four bottles (replicates) of each species, mycelium brushed off, blocks oven-dried overnight, reconditioned (as above) for 1 week and reweighed. The percentage weight loss for each block was calculated, and these data pooled to determine the percentage weight loss for the four blocks in each bottle. Percentages were converted to proportions and analysed after applying the arcsine of the square root transformation. A two-way analysis of variance was used to test for the effect of tree species, incubation period and their interaction on weight loss, and means were compared using Tukey's simultaneous tests with the software mentioned above.

3 | RESULTS

3.1 | Exposure of stem discs

The *Spiniger* state of *H. irregulare* was rarely detected during examination of discs exposed during the first (mid-August) trial, and thus, no differences among species were found (data not shown). However, results from the two later trials in which the pathogen was more frequently observed indicated that conifer species strongly influenced both numbers of positive discs and numbers of positive spots per disc (for spots per disc values of $p \le .01$, Table 1). In these two later trials, the pathogen was frequently observed on tamarack, red pine and white spruce discs. In contrast, the *Spiniger* state was not seen or only rarely seen on discs of northern white cedar, eastern hemlock and balsam fir. Although mycelium and conidiophores of various other fungi often proliferated on discs, these did not appear to frequently obscure spots to interfere with detection of the *Spiniger* state. The pathogen was never detected on negative control discs.

3.2 | Soil-block decay test

Length of the incubation period, conifer species and their interaction (values of p for each ≤.01, Table 2) strongly influenced root sapwood weight loss due to decay by *H. irregulare* in the soil-block decay test. For each species except balsam fir, weight loss was greater with longer periods of incubation. Percentage weight loss did not differ significantly among species after 4 weeks of incubation. However, after

TABLE 1 Number of discs (of four) and mean[a] (and range) in number of spots per disc (of eight) of six conifer species on which the *Spiniger* state of *Heterobasidion irregulare* was observed after exposure to natural inoculum at each of two sites followed by incubation in the laboratory

		No. of positive discs, mean no. (range) of positive spots per disc						
Trialdate	Site[b]	*Thuja occidentalis*	*Tsuga canadensis*	*Abies balsamea*	*Larix laricina*	*Pinus resinosa*	*Picea glauca*	p-value[c]
Late September	W	0, **0.00** (0–0)	0, **0.00** (0–0)	1, **0.25** (0–1)	2, **1.75** (0–4)	3, **3.75** (0–7)	4, **8.00** (8–8)	<.01
	M	0, **0.00** (0–0)	0, **0.00** (0–0)	1, **0.50** (0–2)	4, **4.25** (1–7)	4, **4.25** (2–8)	4, **7.00** (6–8)	<.01
Early November	W	0, **0.00** (0–0)	0, **0.00** (0–0)	1, **0.50** (0–2)	4, **6.50** (5–8)	4, **6.25** (4–8)	4, **5.50** (1–8)	<.01
	M	0, **0.00** (0–0)	0, **0.00** (0–0)	0, **0.00** (0–0)	4, **4.50** (1–7)	4, **6.50** (2–8)	2, **2.25** (0–6)	.01

[a]Mean values are represented in bold.
[b]W, Waushara County, WI; M, Marquette County, WI.
[c]Values of p for comparisons among species of no. of positive spots per disc at one location in one trial for Kruskal–Wallis test of the equality of medians, corrected for ties.

TABLE 2 Percentage weight loss for root sapwood blocks of six conifer species incubated for various periods with *Heterobasidion irregulare* in a soil-block test. Each value is a mean[a] of four replicate soil bottles, with four blocks (subsamples) incubated in the same bottle

Incubation period (weeks)	Mean weight loss (%)					
	Thuja occidentalis	*Tsuga canadensis*	*Abies balsamea*	*Larix laricina*	*Pinus resinosa*	*Picea glauca*
4	1.9 a A	2.2 a A	2.0 a A	4.5 a A	4.7 a A	3.1 a A
8	1.9 a AB	1.0 a A	0.3 a A	6.5 ab BC	13.4 b C	9.5 b C
12	5.3 b AB	2.9 ab AB	1.6 a A	13.2 bc CD	18.6 bc D	6.7 ab BC
16	9.7 b B	7.8 b B	1.3 a A	19.1 c CD	23.2 c D	12.3 b BC

[a]Data were analysed after applying the arcsine of the square root transformation to proportions. Two-way analysis of variance indicated effects of conifer species, incubation period and their interaction (each at $p \leq .01$). Transformed data for means within a column that are followed by the same lower-case letter were not significantly different at $p \leq .05$ using Tukey's simultaneous tests. Transformed data for means within a row that are followed by the same upper-case letter were not significantly different at $p \leq .05$ using Tukey's simultaneous tests.

16 weeks of incubation, mean weight loss ranged from only 1.3% for balsam fir to 19.1% for tamarack and 23.2% for red pine.

4 | DISCUSSION

Estimation of risk posed by Heterobasidion root disease and the need for treatment or potential benefits of treatment to prevent stump infection has been hindered by a lack of fundamental information regarding the interaction of *H. irregulare* with the variety of economically and ecologically important conifers of Great Lakes Region forests. As is true for herbarium records and anecdotal lists of tree species as substrates from which basidiocarps have been collected, extrapolations from studies utilizing stem discs and autoclaved wood blocks concerning likely disease incidence or severity should be made with caution. However, results of these two studies support the current concern about impacts to the area's conifer resource.

The observation of the ability of *H. irregulare* to infect red pine discs is consistent with its history as a well-established pathogen of the many pine species native to the south-eastern United States. Although lacking in detail, a brief report from that region also established the potential for frequent infection of red pine. In a thinned red pine plantation in North Carolina, well south of the natural occurrence and commercial cultivation of this tree species, a loss of 25–30 per cent of residual stems to Heterobasidion root disease was reported (USDA Forest Service, 1965). Red pine also is the species most often mentioned in both peer-reviewed literature and state or provincial reports of Heterobasidion root disease in conifer forests of New England, New York, Michigan and eastern Canada (e.g., Laflamme & Blais, 1995; McLaughlin, Hsiang, Halicki Hayden, & Greifenhagen, 2010; Miller, 1960; Sinclair, 1964).

In contrast to these reports of the occurrence of Heterobasidion root disease in red pine plantations, experimental quantification of the ability of *H. irregulare* to decay red pine wood has not been frequently reported. Platt et al. (1965), however, included red pine with a variety of southern pine species in similar soil-block assays of decay caused by *H. irregulare*. Platt and others reported that after 12 weeks incubation, two isolates of *H. irregulare* obtained from red pine caused an average of 21.9% weight loss of red pine root wood, comparable to the result obtained in the current study. In that study, the average percentage weight loss caused by the two isolates from red pine was actually greater than average weight losses produced by isolates from shortleaf, Virginia, and loblolly pines on blocks of root wood of the respective hosts from which these isolates were obtained. Thus, although future studies might reveal differences in decay abilities among a larger number of *H. irregulare* isolates from various hosts, it can be concluded that this pathogen has the potential to utilize the red pine substrate at least as effectively as wood of southern pine species it is known to frequently colonize and kill.

Past reference to *H. irregulare* as a member of the P ISG may have inadvertently led to an unjustifiably narrow consideration of potential risks. Non-pine species that predominate in northern coniferous forests of eastern North America are not found or are much less common in the commercial conifer plantations of the south-eastern United States. Among these northern conifers are tamarack and white spruce, and the frequencies of infection of discs and appreciable weight loss due to decay of these species in the soil-block assay of the current study do suggest suitability as a substrate for *H. irregulare*. Although the list of hosts or substrates of *H. annosum s.l.* in the U.S. National Fungus Collections Fungus-Host Database (Farr & Rossman, 2017) includes *Larix* sp., tamarack is not mentioned. Similarly, the Specimen Database of the same organization lists *Larix* sp. as the source of a specimen collected in Maine, but the host species name is not included. This same Fungus-Host Database does include white spruce based on collection of a basidiocarp in Minnesota (Gilbertson & Lombard, 1976). However, for both tamarack and white spruce, descriptions of incidence and severity of disease in forests and experimental evidence that might be provided by inoculation trials are lacking at present.

Although *H. irregulare* was absent or rare on discs of balsam fir, eastern hemlock and northern white cedar exposed to natural inoculum, additional research is necessary before these species are discounted as hosts that could be seriously damaged by the pathogen. Balsam fir was identified as the source of a single collection from Connecticut included in the Specimen Database of the National Fungus Collections (Farr & Rossman, 2017). We have examined recently killed balsam fir seedlings and saplings occurring naturally in the understory of a previously thinned, infested red pine plantation in central Wisconsin (observation of third and last authors). Cultures were obtained and identified by sequence analysis as *H. irregulare*. Infection of balsam roots by contact with roots of red pine stumps infected after thinning seems likely. Presence of numerous, fertile basidiocarps on these balsam firs indicates their potential to produce inoculum for further spread of the pathogen. And although weight losses due to decay of eastern hemlock and northern white cedar in the soil-block assay were initially very low, decay had increased by the end of the study. Despite a lack of literature describing disease development by *H. irregulare* on eastern hemlock and northern white cedar, both of these non-pine conifers also are listed among species that may be hosts or substrates in the databases mentioned above (Farr & Rossman, 2017).

Many examples from the literature document the broad host range of the closely related European sister species *H. annosum s.s.*, thereby providing justification for consideration of risks to non-pines as potential hosts of *H. irregulare*. Norway spruce (*Picea abies* (L.) Karst.) is an often-reported host of *H. annosum s.s.*, and spread from colonized stumps to trees has been studied (Oliva, Bendz-Hellgren, & Stenlid, 2011). In a survey of occurrence of *Heterobasidion* species in the Czech Republic, *H. annosum s.s.* was collected from Norway spruce and European larch (*Larix decidua* Mill.), as well as Scots pine (*Pinus sylvestris* L.) and several angiosperm tree species (Sedlák & Tomšovský, 2014). Additionally, a basidiocarp collected from silver fir (*Abies alba* Mill.) was identified as *H. annosum s.s.* based on sequence analysis. Doğmuş-Lehtijärvi, Erdoğan, Lehtijärvi, Woodward, and Aday Kaya (2016) demonstrated by inoculations that isolates obtained from basidiocarps on Nordmann fir (*Abies nordmanniana* (Steven) Spach) in Turkey were aggressive on pines and also on cedar of Lebanon (*Cedrus lebani* A. Rich.). In northern Spain, Mesanza and Iturritxa (2012) identified *H. annosum* s.s. from four pine species, but also from Norway spruce, Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and Port Orford cedar (*Chamaecyparis lawsoniana* (A. Murray bis) Parl.). Wang, Pålsson, Ek, and Rönnberg (2012) studied infection of hybrid larch (*Larix x eurolepis* Henry) stump surfaces in southern Sweden and found that in plantations at sites formerly occupied by Norway spruce, 61% of isolates from these stumps were *H. annosum s.s.* Incidence of Heterobasidion root disease in 5-year-old hybrid larches planted on infested sites formerly occupied by Norway spruce in Sweden was 70%, and all isolates were identified as the former P intersterility group (i.e., *H. annosum s.s.*).

The potential for Heterobasidion root disease to damage conifer forests of the Great Lakes Region is still very uncertain. While the known geographic distribution of *H. irregulare* has greatly increased, support for research on the interaction of this pathogen with the diverse conifer species of the northern forests of the Great Lakes Region has been inadequate. In addition, studies to determine the abilities of *H. irregulare* to initially infect fresh stumps and subsequently decay both sapwood and heartwood of stumps and roots to spread to adjacent extant or subsequently established trees cannot be undertaken in stands of potentially significant hosts that are not yet infested. However, preliminary indications of the ability of *H. irregulare* to utilize other Great Lakes Region conifers should prompt re-evaluation of current management guidelines focused merely on pines, especially if other likely host species are growing in proximity to currently infested pine stands.

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