10.37045/aslh-2007-0016 *Acta Silv. Lign. Hung., Spec. Edition (2007) 71-74*

Sporulation and Identity of Tar Spot of Maple in Canada

Tom HSIANG* – Xiuling Lynn TIAN

Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada

Abstract – Tar spot of maple has been increasing in incidence and severity in the Great Lakes region of eastern North America since the 1990's. The purpose of this work was to examine tar spot on native and imported maple species to determine the fungal species involved. By extracting and sequencing DNA from tar spot samples obtained from Europe and across Canada, we have found that giant tar spot on Norway maple (*Acer platanoides*) is caused by *Rhytisma acerinum*, the same fungus found in Europe, whereas native maple species in North America have the large tar spot caused by *R. americanum* (e.g. on silver maple, *A. saccharinum*) or the speckled tar spot caused by *R. punctatum* (on big-leaf maple, *A. macrophyllum*). We also found that ascospore release from tar spots on Norway maple in southern Ontario occurred over a three-week period, the start of which coincided with full leaf expansion in Norway maple.

Rhytisma* / fungi / *Acer* / *acerinum* / *americanum* / *punctatum

Kivonat – A juharlevél szurokfoltosság spóraszórása és azonossága Kanadában. A juharlevél szurokfoltosságának gyakorisága és súlyossága az 1990-es évek óta növekszik a Nagytavak vidékén, Észak-Amerika keleti részén. Munkánk célja volt megvizsgálni és meghatározni a szurokfoltosságot okozó gombafajt az őshonos és behozott juhar fajokon. Európából és Kanadából származó szurokfolt mintákból kivont DNS szekvenciák alapján megállapítottuk, hogy a korai juhar (*Acer platanoides*) nagy szurokfoltjait a *Rhytisma acerinum* okozza, ugyanaz a gomba, mint Európában, míg az észak amerikai őshonos juharok nagy szurokfoltjait a *R. americanum* (például az ezüst juharon, *A. saccharinum*), a pettyes szurokfoltokat pedig a *R. punctatum* (a nagylevelű juharon, *A. macrophyllum*) okozza. Továbbá megállapítottuk, hogy a korai juharon Dél-Ontarióban az aszkospórák szórása a szurokfoltokból három hétig tartott, kezdete a korai juhar teljes kilombosodásával esett egybe.

Rhytisma* / gombák / *Acer* / *acerinum* / *americanu* / *punctatum

1 INTRODUCTION

Tar spot of maple is caused by species of the ascomycete genus *Rhytisma*, and has a worldwide distribution wherever maples are found (Table 1). Tar spot was particularly abundant in 2006 across eastern North America with most leaves of Norway maple (*Acer platanoides*) bearing multiple black spots. There has been relatively little research done on tar spot in North America. The only scientific reports have come from Connecticut (Waterman 1941) and New York (Hudler et al. 1987 & 1998). The most recent research report is one from New York (Hudler et al. 1998), which found that the fungus *Rhytisma acerinum* is the

* Corresponding author: thsiang@uoguelph.ca; Guelph, Ontario, Canada, N1G 2W1

cause of tar spot on Norway maple, both of which (host and pathogen) are immigrant species, while a native fungal species, *R. americanum*, occurs on the native red and silver maples (*A. rubrum and A. saccharinum*). This is probably the reason that a Norway maple may be heavily infected with tar spot while an adjacent red maple (*A. rubrum*) or silver maple (*A. saccharinum*) may have no spots. The purpose of this work was to examine the epidemiology of this disease, by gathering overwintered maple leaves from multiple locations in southern Ontario weekly from March through August 2006, and inspecting the asci for the presence of filiform ascospores, which initiate infections. Another objective of this research was to confirm the genetic identity of the organism causing giant tar spot on Norway maple in Ontario, as well as its relationship to tar spot on other European maples and North American maples.

Table 1. Maple species, their native range, and susceptibility to tar spot caused by Rhytisma species.

Common name	*Acer* species	Native range	*Rhytisma* species		
			R. acerinum	*R. americanum*	*R. punctatum*
Norway maple	*A. platanoides*	Europe	+		
Sycamore maple	*A. pseudoplatanus*	Europe	+		
Field maple	*A. campestre*	Europe	+		
Red maple	*A. rubrum*	Eastern N. America	?	+	
Sugar maple	*A. saccharum*	Great Lakes	?	+	
Silver maple	*A. saccharinum*	Eastern N. America	?	+	
Mountain maple	*A. spicatum*	Eastern N. America	?	+	
Big-leaf maple	*A. macrophyllum*	Western N. America			+
Vine maple	*A. circinatum*	Western N. America			+

2 METHODS

2.1 Sporulation

After snowmelt, overwintered leaves of Norway maple bearing tar spots caused by *Rhytisma acerinum* were collected from a copse of maples at the Guelph Turfgrass Institute, Guelph, Ontario every week from March through August in 2006. Samples were also taken from other locations in southern Ontario, such as the Niagara Dufferin Park (Niagara Falls), the Royal Botanical Gardens (Burlington) and the Queen's Royal Park (Niagara-on-the-Lake) from May to August, 2006. Diseased Norway maple leaves were soaked in distilled water for 24 h to allow the apothecia to open, and many spots were examined from each location, with several cross-sections per spot. The percent asci that were empty was estimated. Maple phenology and weather conditions were also recorded at each sampling.

2.2 Genetic identity

Many attempts were made to isolate the fungus from dried and fresh maple tissues. as well as from ascospores and conidia. Several putative isolates were subjected to DNA sequencing (methods below), but none proved to be *Rhytisma* species. Because we were unable to obtain pure cultures, we attempted to extract DNA directly from tar spot samples. We collected local samples of infected maple (*A. platanoides* and *A. saccharinum*), and obtained infected specimens of *A. pseudoplatanus* (courtesy of Dr. Roland Weber, Biotechnology, University of

Kaiserslautern, Kaiserslautern, Germany), and *A. macrophyllum* (courtesy of Dr. Brenda Callan, Canadian Forestry Service, Victoria, B.C., Canada). We also obtained a specimen of Amur maple (*A. ginnala*) as well as a specimen of tulip tree (*Liriodendron tulipfera*) with tar spot, but we were unable to extract *Rhytisma* from these old samples.

We used the Qiagen DNAeasy kit (Qiagen Inc., Mississauga, Ontario, Canada), to extract DNA. This DNA was then amplified with conserved ITS primers which target the internal transcribed spacer region of ribosomal DNA spanning the 3' end of the 18S gene to the 5' end of the 28S gene. The primer pair, ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) were from White et al. (1991). The 12 5 ul reaction mixture for PCR amplification contained the following : 10 ng DNA, 1 DNA polymerase buffer, 0 5 ìm of each primer, and 1 U Tsg DNA polymerase (Biobasic, Scarborough, Ontario, Canada). Amplifications were performed in a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA), with an initial denaturation step of 94 for 2 min, followed by 35 cycles of 94 for 30 s, 55 for 1 min, and 72 for 2 min, and a final extension at 72 for 7 min. These PCR reactions were diluted 10-fold in water, and sent for sequencing at the Laboratory Services Division, University of Guelph with both forward and reverse primers. At least two tar spot sequences from each maple species were used for analyses.

In addition to sequences downloaded from GenBank for *Lophodermium pinastri* (GenBank Accession AF462434), and *Rhytisma salicinum* (GenBank Accession AY465516), the sequences obtained from ITS sequencing mentioned above were subjected to phylogenetic analysis by multiple alignment of the sequences in CLUSTALX (Thompson et al. 1997), and generation of a dendrogram depicting relationships between the sequences.

3 RESULTS & DISCUSSION

In southern Ontario, the first symptoms of tar spot on Norway maple appeared in late June as small, round, light green, chlorotic spots, 2 mm across. Spots enlarged to 15 mm by mid-August, and developed small black tar-like raised structures on the adaxial surface with a yellow margin. Conidia, which are considered non-infective and possibly spermatizing, appeared as a shiny layer on the black stroma at this time. By early September, the individual spots merged into a circular black spot up to 2 cm across.

Overwintered Norway maple leaves collected in March 2006, had stroma, paraphyses and asci (56-80 µm × 8.5-10.6 µm), but no ascospores were visible. By the middle of April, the asci were still undifferentiated, but were found to contain globular vacuoles or bodies. The asci became swollen as spores developed, and filiform ascospores were first observed in early May, averaging 55 × 2.0 µm. By late May, after soaking in water, slits in the hysterothecia (modified apothecia) on the leaf surface opened, and contained a grayish milky substance. At this time, Norway maples were abundantly producing and shedding pollen, and small samaras were formed, with leaf sizes averaging 10 cm × 15 cm. By the end of May, a few partially filled or empty asci were observed (5.3%), with ascospore release through the tips, and paraphyses becoming curled beside asci after spore release. In early June, Norway maple leaves reached their full size (20 cm × 24 cm), and 10% of the asci had fully discharged their spores. By the end of June, nearly all the asci were empty. The practical implication is that fungicide protection against tar spot, if necessary, needs only to be applied during a very short period, which begins near the end of full leaf expansion in Norway maple.

Schweizer (1932) and Hudler et al. (1987) had earlier reported that isolation of *Rhytisma* from infected plant tissues was possible on common artificial media without special supplements. We were never able to isolate the fungus in pure culture, and in subsequent communications with Dr. George Hudler of Cornell University (Ithaca, NY, USA), he indicated that he suspected what they isolated may have been *Aureobasidium*. He said that such cultures were commonly and easily obtained by scooping out the milky contents of overwintered leaves where the apothecia had been induced to open by wetting. We confirmed his suspicions by sequencing of isolates which turned out to be have the best match with *Aureobasidium pullulans* with 99% identity for both the ITS and partial 18S sequences.

Previous work by Hudler et al. (1998) to delineate *Rhytisma* species was based on morphology and RFLP of ribosomal DNA, but there was no sequence data. The only ITS sequence available for a *Rhytisma* species at the start of this project was one for *R. salicinum* (GenBank Accession AY465516), although there was an 18S and a 28S sequence for *R. acerinum* in GenBank (AF356695 and AF356696, respectively). In this study, we were able to obtain ITS sequence for fresh samples of maple with tar spot. Based on the phylogenetic analysis of ITS sequences from tar spot of Norway maple and silver maple from Ontario, sycamore maple from Europe, and big-leaf maple from British Columbia, we found that Norway maple tar spot specimens had sequences almost identical to those from sycamore maple, and conclude that tar spot of Norway maple in Ontario is caused by *R. acerinum*. The sequences from silver maple were quite different and considered to be *R. americanum*. The big-leaf maple tar spot yield a even more divergent sequence, and *R. punctatum* was not found on any Norway or silver maples even for spots which had a similar punctate phenotype. Both *R. americanum* and *R. acerinum* can pass through an initially punctate (speckled tar spot) stage when spots are just forming, where they might be easily confused with *R. punctatum.*

In summary, we have found that ascospore dispersal from overwintered Norway maple leaves occurred during a 3-week period in June. The practical implication of this result is that fungicide protection against tar spot, if necessary, needs only to be applied during a very short period, and this period coincides with the end of full leaf expansion in Norway maple in this region. We have also confirmed that the giant tar spot on Norway maple in Ontario is caused by *R. acerinum* which is the same as the fungus found in Europe, whereas native maple species in North American have the large tar spot caused by *R. americanum*. Although we were unable to grow the isolates in pure culture, we have obtained DNA that is similar to other species in the Rhystimataceae, and we plan to continue DNA sequencing to further resolve the relationships between *Rhytisma* species and closely related genera such as *Lophodermium*.

Acknowledgements: This study was funded by Landscape Ontario and the Canada-Ontario Research and Development (CORD) Program IV.

REFERENCES

HUDLER, G.W. – BANIK, M.T. – MILLER, S.G. (1987): Unusual epidemic of tar spot on Norway Maple in upstate New York. Plant Disease 71: 65-68.

HUDLER, G.W. – JENSEN-TRACY, S. – BANIK, M.T. (1998): *Rhytisma americanum* sp. nov.: a previously undescribed species of *Rhytisma* on maples (*Acer* spp.). Mycotaxon 68: 405-416.

THOMPSON, J.D. – GIBSON, T.J. – PLEWNIAK, F. – JEANMOUGIN, F. – HIGGINS, D.G. (1997): The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research, 25: 4876-4882.

WATERMAN, A.M. (1941): Diseases of shade and ornamental trees: annotated list of specimens received in 1940 at the New Haven Office, Division of Forest Pathology. Plant Dis. Rep. 25: 181-182.

WHITE, T.J. – BRUNS, T. – LEE, S. – TAYLOR, J. (1990): Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: INNIS, M.A. - GELFAND, D.A. - SNINSKY, J.J. - WHITE, T.J. (eds): PCR Protocols: a guide to methods and applications. Academic Press, San Diego, CA, U.S.A.: 315–322