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Widespread Distribution of Fungivorus *Aphelenchoides* spp. in Blight Cankers on American Chestnut Trees

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Abstract: Previously we showed in laboratory studies that the fungivorus nematode, *Aphelenchoides hylurgi,* was attracted to and fed upon the chestnut blight fungus, *Cryphonectria parasitica,* from American chestnut bark cankers and was a carrier of biocontrol, white hypovirulent *C. parasitica* strains. In the present field study, we recovered *Aphelenchoides* spp. in almost all (97.0 %) of 133 blight canker tissue assays (three 5-g samples each) from four eastern states. High mean population densities (227 to 474 nematodes per 5 g tissue) of *Aphelenchoides* spp. were recovered from cankers in Virginia, West Virginia, and Tennessee but not from New Hampshire (mean = 75 nematodes per 5 g tissue). Overall, most canker assays yielded population densities less than 200 nematodes per 5 g tissue. All of 12 very small or young cankers yielded a few to many *Aphelenchoides* spp. Regression analysis indicated greatest recovery of *Aphelenchoides* spp. occurred in the month of May (r = 0.94). The results indicate that *Aphelenchoides* spp. appear to be widespread in blight cankers on American chestnut trees and could play a role in biocontrol of chestnut blight.

Key words: biocontrol, chestnut blight, fungivorus nematodes, hypovirulence.

Aphelenchoides spp. are fungivorus and may be attracted to and feed upon a variety of plant pathogenic and saprophytic fungi including *Aspergillus, Botrytis, Cladosporium, Diaporthe, Fusarium, Gleosporium, Monilia, Penicillium, Pestalogia, Pyrenocheata, Trichoderma* and *Verticillium*, as well as beneficial arbuscular mycorrhizal fungi, such as *Gigaspora* and *Glomus* (Bakhtiar et al 201; Hasna et al. 2007; Ikonen 2001; Perper and Petielllo 1977; Ruess et al. 2000). In addition, an *Aphelenchoides* spp. feeding on the biocontrol fungus, *T. harzianum* Rifai, has been demonstrated to have a significant biotic constraint on the biocontrol activity of this fungus on the plant pathogen, *Sclerotinia sclerotiorum* (Lib.) de Bary (Bae and Knudsen 2001).

However, fungivorus *Aphelenchoides* spp. may also play an important positive role in the biocontrol of plant pathogenic fungi. For the chestnut blight fungus [*Cryphonectria parasitica* (Murr.) Barr] on American chestnut trees [*Castanea dentata* (Marsh.) Borkh.], the fungivorus nematode, *Aphelenchoides hylurgi* Massey, 1964, may not only feed on the chestnut blight fungus but also has the potential of spreading biocontrol strains of the blight fungus called hypovirulent strains (Griffin et al. 2009). These hypovirulent strains are infected with a virus, *Cryphonectria hypovirus 1* (CHV1), that originated in hypovirulent strains from Italy (Elliston 1985; Griffin et al. 2004). The CHV1 virus has been shown to spread in the *C. parasitca* population on American chestnut trees and contributed significantly to the control of the disease on individual American chestnut trees for over 27 years (Griffin et al. 2006). Hypovirulent strains infected with CHV1 must be inoculated on American chestnut trees, but in our previous studies the fungivorus nematode, *A. hylurgi*, occurred naturally in blight cankers from one area of Virginia (Griffin et al. 2009). If fungivorus nematodes, such as *A. hylurgi*, occur frequently in natural populations of American chestnut trees, they may be important natural biocontrol agents, both directly as fungal feeders and indirectly as agents that spread of hypovirulent strains.

While almost all canopy American chestnut trees were killed by the chestnut blight pandemic (Heald 1926; Griffin 1986), there are tens of thousands of small (< 3 cm. diameter) understory American chestnut trees in eastern forests that may grow to larger size when the forest canopy is opened up; a blight epidemic typically follows on American chestnut stems as they grow larger in this environment and almost all stems become infected by the blight fungus (Griffin et al. 1991). A similar situation exists when a plantation of American chestnuts is established. Many stems may die when the phloem is colonized to the vascular cambium. When biological control measures using hypovirulence can be implemented, American chestnuts have the potential to survive for long periods if the mycelium in a canker is infected with the CHV1 hypovirus (Griffin et al. 2006), as the vascular cambium is not killed. However, it is not known if *Aphelenchoides* spp., which could contribute to this blight control by carrying propagules of hypovirulent strains among blight cankers, are widespread in occurrence in natural blight cankers on American chestnut trees. The research reported here was undertaken to determine how common *Aphelenchoides* spp. are in blight cankers in four eastern states where American chestnut is naturally present.

Materials And Methods

Trees studied: American chestnut trees with natural blight cankers were located during 2009 to 2011 in five counties of Virginia (Nelson, Giles, Montgomery, Craig and Botetourt) four counties in West Virginia (Greenbriar, Monroe, Raleigh, and Nicholas) one county in Tennessee (Johnson), and one county in New Hampshire (Rockingham). Stem diameters of evaluated American chestnut trees at breast height ranged

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from 5 cm to 25 cm. The American chestnut stems located during the study had one or more blight cankers; only a few had no blight cankers, which is consistent with results of earlier investigations (Griffin et al. 1991).

Excision of cankers: Stem sections of American chestnut with cankers were removed by cutting the stems on the trees in the field and they were transported to the laboratory in plastic bags to retain moisture. Generally, to collect canker tissue in the laboratory, cuts were made vertically into the canker/bark surface in the middle and at the edge of the canker and layers of canker tissue were stripped from the canker with a knife to the depth of the vascular cambium until about 15-20 g of canker tissue was obtained. Cankers that had 50 to 100% of the vascular cambium necrotic at the time of dissection, evidenced by brown discoloration, were labeled non-superficial or killing-type cankers, whereas cankers that had less than 50% of the cambium necrotic were labeled superficial cankers. The strips of tissue from the cankers were then cut horizontally into smaller pieces to facilitate weighing and storage. Plastic bags containing canker pieces were usually stored for 1 to 2 days at 6C until assayed for nematodes. In some instances, long continuous cankers were present on American chestnut stems collected in West Virginia and New Hampshire in which case several 15-20 g samples for canker assays were obtained per canker. Typically, one or two cankers were obtained from a single American chestnut stem, but some stems had several cankers.

Population assay for nematodes in canker tissue: The Baermann funnel technique was used to assay nematode populations in canker tissue. Each funnel contained 5 g of canker tissue and three replicate funnels were prepared for each canker tissue assay, unless otherwise indicated. Following extraction, nematodes were quantified with a counting chamber and an inverted bright-field light microscope. Representative nematodes were examined for characteristics of *Aphelenchoides* spp.

Results

Frequency and range of Aphelenchoides *spp. population densities recovered from chestnut blight cankers:* For 133 15 g-canker assays processed for Virginia, West Virginia, Tennessee, and New Hampshire , 129 or 97.0 % yielded *Aphelenchoides* spp. These assays were made from 80 cankers. The four assays not yielding *Aphelenchoides* spp. were from single cankers on four different American chestnut trees. Among the cankers assayed from all counties in the four states, there was a wide range of *Aphelenchoides* spp. recovered per 5 g of canker tissue with most 15 g-canker assays (73 of 133) yielding less than 200 nematodes per 5 g of canker tissue, but many canker assays yielded high population densities (Fig. 1). Overall, high mean population densities (227 to 474 nematodes per 5 g tissue) were recovered from cankers in Virginia, West Virginia, and Tennessee (Table 1) but not from New Hampshire (mean = 75 nematodes per 5 g tissue). Five of the 133 canker assays yielded very high population densities (data not shown in Fig. 1) ranging from more than 1,225 to 9, 250 nematodes per 5g of canker tissue.

Most (83.6%) 15 g-canker assays were of predominantly non-superficial cankers for which most of the

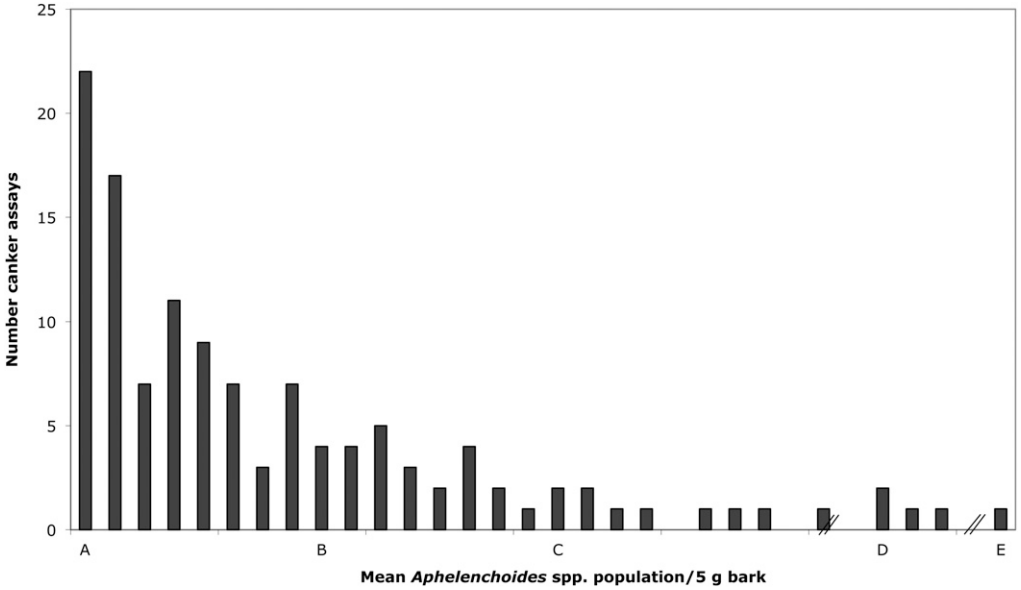

Fig. 1. Frequencies of American chestnut blight-canker assays yielding (left to right) low (A) to high (E) population densities of *Aphelenchoides* spp. from bark cankers collected over the four states of Virginia, West Virginia, Tennessee, and New Hampshire. Data bars labeled A to E represent mean numbers of *Aphelenchoides* spp. recovered from 5 g. of bark canker tissue with A = 1 - 25, B = 200 - 225, C = 400 – 425, D = 800 – 825 and E = 1100 -1125 nematodes. Unlabeled intermediate bars are shown at increasing 25-nematode intervals between A and C. Breaks (//) are shown when very high population densities were widely separated from each other. In addition, four values were 0 and five values were larger than 1125 (not shown).

TABLE 1. Mean population densities of *Aphelenchoides* spp. recovered from blight cankers on American chestnut trees (*Castanea dentata* (Marsh.) Borkh.), in Virginia, West Virginia, Tennessee, and New Hampshire.

State	No. of cankers	No. canker assays	*Aphelenchoides* spp. per 5 g. mean ± S.E
Virginia	68	91	473. 9±101.0a
West Virginia	4	23	227.3±25.7a
Tennessee	4	5	230.7±31.7a
New Hampshire	4	14	74.5±11.2a
Totals	80	133	

a Per 5 g. of canker tissue with three 5-g. replicates per canker assay. Variation expressed as standard error.

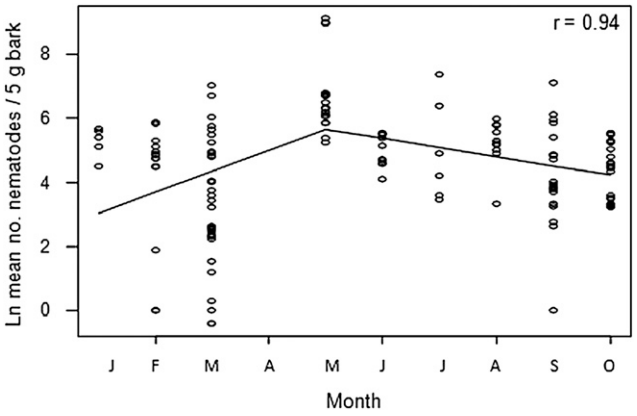

FIG. 2. Linear regression analysis of the relationship between population density of *Aphelenchoides* spp. in blight cankers and month of collection (January, J, to October, O) of blight cankers from American chestnut trees (*Castanea dentata* (Marsh.) Borkh.) from the four states of Virginia, West Virginia, Tennessee, and New Hampshire. A significant ($P < 0.01$) association was found with a high correlation coefficient ($r = 0.94$). The regression is composed of two regression lines. The slope of the ascending line is 0.726 and the slope of the descending line is -0.147.

vascular cambium was colonized by the blight fungus (infections that are most common in nature). Some of the superficial cankers came from stems that were inoculated in previous years with white hypovirulent strains of *C. parasitica*. All of 17 (5 g or less) assays from 12 small or young cankers (1.7 to 12.0 g of tissue assayed), giving only one or two assays per canker (vs. the normal three), yielded *Aphelenchoides* spp. with numbers ranging from 2 to 542 nematodes per assay and 6 to 866 nematodes per canker. In limited, supplementary trials, no *Aphelenchoides* spp. were recovered from bark tissues collected from any of the few (3) American chestnut trees found during the study that had no cankers on the stems.

Month of year when most Aphelenchoides spp. *were recovered from cankers:* Regression analysis (Draper and Smith 1981) was used to determine how recovery of *Aphelenchoides* spp. varied over the several months (January to October) of collection of cankers. The analysis (FIG. 2) indicated there was a significant ($P < 0.01$) association between month of analysis and *Aphelenchoides* spp. population density ($r = 0.94$), with the greatest mean population density recovered from cankers collected in the month of May. This analysis is composed of two regression lines; the slope of the ascending left line is 0.726 and the slope of the descending right line is – 0.147.

DISCUSSION

The results of this study suggest that *Aphelenchoides* spp. have a widespread geographic distribution among blight cankers on American chestnut trees in the native range of the species. This finding also suggests that they have the potential to contribute to biological control of chestnut blight in many geographic areas. The mechanisms of biological control could be two: (a) by directly feeding on *C. parasitica* in cankers and (b) by spreading propagules of hypovirulent strains among cankers, as previously demonstrated (Griffin et al. 2009). It is likely that the second mechanism may be the more important where hypovirulent strains have been inoculated on American chestnut trees and the cankers have produced sporulation structures (stromata) that produce conidia containing the hypovirus (Hogan and Griffin 2008; Robbins and Griffin 1999). The first mechanism could also contribute to control on these trees by reducing the biomass of the pathogen in phloem tissues of cankers, but further research is needed to determine the extent to which the biomass of *C. parasitica* is reduced in cankers by fungal feeding. In pure culture studies, *Aphelenchoides hylurgi* produce large numbers of nematodes on individual Petri plate pure culture colonies of *C. parasitca* (Griffin et al. 2009) and much of the mycelium appears devoid of cytoplasm. In contrast to pure fungal cultures, blight cankers on American chestnut stems are composed of host tissue and *C. parasitica* tissue (mycelium), but it is not known how much of the canker tissue assayed, or observed under a microscope, in this study or other studies is fungal tissue, as it is difficult to discern or measure.

In this field study, several of the blight cankers evaluated were from trees that were inoculated in previous years with white, hypovirulent strains and *Aphelenchoides* spp. may have contributed to the spread of these hypovirulent strains previous to our canker sampling for nematodes. At the time of nematode sampling, the cankers on these hypovirulent strain-inoculated trees were recorded as superficial, when dissected in the nematode assays. Other tests have confirmed the cankers on these trees were infected with white, hypovirulent strains (Hogan and Griffin 2002).

Our overall data strongly suggest that *Aphelenchoides* spp. are likely present in cankers before hypoviulent strain inoculation of the cankers, as 97% of the cankers evaluated here yielded *Aphelenchoides* spp. and only a small portion of these cankers were from trees that were inoculated with hypovirulent strains. The nematodes

may have spread among cankers in several ways. This could be accomplished by rainfall in which the nematodes may be washed down the surface of the stem having cankers or they move short distances in water films on the bark surface, being attracted by the blight fungus in the canker. The latter was demonstrated in our previous study (Griffin et al. 2009). Also, another agent may be a carrier of the nematode including insects, such as ants, mites, or birds attracted to the cankers or stems. Ants can be observed on American chestnut trees with careful observation but other insects are less common. The insects and mites may also aid in spread of the hypovirulent stains (Anagnostakis !982; Nanelli and Turchetti 1999; Russin et al 1984), although nematodes are more numerous in cankered bark tissues and are more closely associated with the fungal mycelium and conidia on the cankers . As discussed in earlier studies (Hogan and Griffin, 2008), the carrier, either nematode or insect, may move and carry conidia or mycelial fragments from a hypovirulent canker to a virulent canker where the hypovirulent propagule may germinate and establish a small hypovirulent canker in the phloem adjacent to the virulent canker. Subsequently, the hyphae of the two types of *C. parasitca* strains in the cankers may fuse (anastomose) resulting in movement of the hypovirulence virus, CHV1, into the mycelium of the virulent canker (Hogan and Griffin 2002).

The *Aphelenchoides* spp. population densities found in this study were quite variable ranging from 2 nematodes in one 5-g subsample and 0 in two other subsamples of a 15-g canker sample to the highest mean value of 9, 250 nematodes per 5 g for a 15-g canker sample. Also, some small assays, less than 15 g, had as many as 866 individuals. However, as indicated above, other 15-g canker samples (4 of 133) had no *Aphelenchoides* spp. It is not clear what could account for the large variations in population densities, although large variations were previously found for *A. hylurgi* growing on *C. parasitica* colonies in the laboratory (Griffin et al. 2009). The regression analysis data for monthly population densities of *Aphelenchoides* spp. recovered from cankers also showed a large variation at each month. The large number of assays done in these evaluations may have aided in obtaining a significant association between month of collection and *Aphelenchoides* spp. population density with a corresponding high r value (0.94). Differences in the *C. parasitica* biomass in individual cankers, differences in environmental conditions at the field sites for moisture and temperature at the time of sampling, and differences in timing of initial natural infestation of cankers with *Aphelenchoides* spp. all may account for some of the variation observed in population densities among the natural cankers collected in the field.

The population densities in cankers from New Hampshire were much lower than those from other states, although the number of cankers assayed was the same as for West Virginia and Tennessee. New Hampshire is on the northern fringe of the native American chestnut geographic range, with lower population densities of American chestnut in the forest understory than the other states (G. Griffin, personal observation). How this would affect the development of *Aphelenchoides* spp. is not clear, unless the low temperatures of this northern environment were an important factor in nematode reproduction, as has been found for American chestnut survival (Griffin et al. 2006). The month of May, a warm and moist month, generally, had the highest mean population densities in the present study.

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