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A Laboratory Assay to Determine Resistance in Butternut to Butternut Canker Disease

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ABSTRACT

Determining disease resistance of large butternut trees in the field is very difficult. In order to assess such trees in the laboratory, four experiments were done with dormant stems of butternut trees (*Juglans cinerea*) cut in Pennsylvania, enclosed in plastic bags, and shipped by next-day air to Connecticut. Five stems from each tree sampled were inoculated with four strains of the butternut canker fungus (*Ophiognomonia clavigignenti-juglandacearum*). Inoculated stems were incubated in plastic boxes at 20C in four different experiments, for 12, 13, 14, or 23 days, to determine the best duration. Canker assessments were made by peeling the bark back, and measuring canker diameters. Differences in mean canker growth suggest that this method may be

useful as a first determination of putative resistance of butternut trees to the butternut canker fungus.

Keywords: Juglans, *Sirococcus clavigignenti-juglandacearum*, *Ophiognomonia clavigignenti-juglandacearum*

Butternut (*Juglans cinerea* L.) is native to North America, but populations are declining throughout the native range due to an introduced fungal pathogen that causes lethal cankers. The fungus is *Ophiognomonia clavigignenti-juglandacearum* Broders & Boland (formerly *Sirococcus clavigignenti-juglandacearum* Nair, Kostichka and Kuntz) (Nair et al. 1979, Broders and Boland 2011). The pathogen was probably introduced recently into the United States (Furnier et al. 1999). Loss of this timber and nut tree will remove valuable lumber and wildlife mast species from the mixed hardwood forests of the Eastern U.S. and a valuable source of edible nuts for local and hobbyist nut growers who prize them (Ostry and Pijut 2000, Ostry 2001). Spread of the canker disease throughout Eastern forest stands and nut plantations and its effect on other species increases concerns about loss of local sources of genetic material of both timber and nut trees, which may have potential for genetic resistance to select disease agents (Ostry and Moore 2007, Ostry and Moore 2008, Tisserat and Kuntz 1981, Tisserat and Kuntz 1984). Inoculation tests for resistance to butternut canker have been successful only on mature trees in the field (Ostry and Moore 2008).

Jaynes and Elliston (1980) inoculated dormant stems of chestnut (*Castanea dentata*) to asses levels of virulence in the chestnut blight fungus. This suggested a

similar test with dormant stems of butternut inoculated with strains of the butternut canker fungus to assess differences in the amount of growth in the laboratory.

Materials and Methods

The sampled trees were all located in Pennsylvania (*table 1*), and were previously confirmed as *J. cinerea* by Jeanne Romero-Severson, University of Notre Dame (personal communication) using DNA markers (Hoban et al. 2008). During the winter months, a tree climber collected scion material and representative smooth-barked branch sections 1.5 to 3 cm in diameter and 30 to 35 cm long. Collections were harvested from the upper crown of trees to obtain healthy dormant material for both pathogenicity tests and grafting projects to establish seed orchard plantings of representative parent trees (*figure 1*). Dormant stem sections from each tree were enclosed in plastic bags, labeled to retain precise site location data, and shipped by next-day air to Connecticut. Branch sections were kept in their plastic bags at 3C until used. Prior to being inoculated, stems were washed in a solution of mild detergent, rinsed thoroughly with tap water, and then rinsed with demineralized water.

Four strains of *Ophiognomonia clavigignenti-juglandacearum* (*O. clav.-jug*.) were used. CT1 was isolated in 2005 from a tree growing in Chester, CT, which is a hybrid (*J. ailantifolia* crossed with *J. cinerea*) crossed with *J. cinerea* [(*Ja* x *Jc*) x *Jc*]. Strain CT11 was isolated in 2006 from a tree growing in Guilford, CT which is a butternut, *J. cinerea*. Strains 1343-1, isolated from Forest Co., WI and 1344-4, isolated from Walworth Co., WI, were provided by Earline Holmes, USDA/FS. All four fungal strains were confirmed as *O. clav.-jug.* by D. Walker, USDA-ARS, Beltsville, MD. Ten cultures

of each of the four strains were grown on potato dextrose agar (PDA) (Difco) at temperatures from 5C to 30C to determine their optimum temperature for growth (*figure 2*). All four strains grew well between 14^{o}C and 25^{o}C, and we chose 20^{o}C for incubating the cultures for inoculation.

Four experiments were done, as the stems arrived from PA. There were not enough stems to repeat the tests on the same tree material for the four different incubation times, so comparisons between trees could only be done within single experiments. The first test was run for 12 days, the second for 13 days, the third for 14 days, and the fourth for 23 days. Holes were punched in the stems through the bark to the wood with a cork borer. Inoculations were made using plugs taken from the margins of cultures grown on PDA at 20C. The plugs were placed in the holes and the inoculation sites were covered with paper tape. The inoculated stems were placed in clear plastic storage boxes with paper towels in the bottom, and placed in an incubator at a constant temperature of 20C. Incubation times of 12, 13, 14, and 23 days were used, and at the end of these times the bark was peeled away so that the canker growth at the cambium could be clearly seen. Diameters of the cankers were measured in mm. The means within each test time were compared using an analysis of variance with multiple comparisons using least mean squares and the Bonferroni adjustment.

Results

The 23 day incubation experiment allowed the cankers to grow enough for clear distinctions to be made, without girdling the stems. Since there were not enough stems available for repeated inoculations, no comparisons can be made between experiments.

Stems from most of the trees allowed very little canker expansion (*figure 3*). Stems from a few of the trees allowed much more growth of some or all four strains of *O. clav.-jug*. used in this test (*figure 4*). Even after 23 days of growth, most of the cankers were about 6 mm in diameter (*figure 5*). Based on our data, some strains may be more pathogenic on some of the trees than others, but in most of the tests the difference is not statistically significant. However, in the 14 day test, CT1 and CT 11 grew more than 1343-1 or 1344-4 on PA 53-3 and PA 59-9; in the 23 day test, CT1 and CT11 grew more on stems from PA 59-5 and 59-6. When means of canker size were examined, the 23 day test had the greatest differences in canker size, and the cankers had still not girdled the stems. Therefore, incubation times of 15-20 days would probably be optimum for making these assessments.

Discussion

This study was initiated to see whether we could use inoculations of dormant stems from mature butternut trees to assess their resistance to butternut canker disease. If we could evaluate the resistance of mature butternut trees to *O. clav.-jug.,* it would be easier to make choices about which trees were valuable enough to propagate by grafting. This dormant stem method shows promise as a preliminary test of resistance of butternut to infection by *O. clav.-jug*.

Ostry and Moore (2008) found that the WI strain 1344 was significantly more virulent than the strain from MN used in their tests. In order to eliminate the effect of

pathogen virulence from consideration of tree resistance, we used four different strains of *O. clav.-jug*. in our experiments.

Scion wood from all the trees sampled was sent to M. Coggeshall at the University of Missouri for grafting onto black walnut root stocks. These grafted trees are intended to provide a repository for further resistance testing and possible crossing to provide seed for future out-planting. This preliminary work suggests that trees PA 33-3, PA12-6, PA 53-9, PA 59-9, PA 50-5, and PA 59-6 are probably not worth grafting for further testing or crossing, because the dormant stems inoculated allowed measurable growth of the butternut pathogen *O. clav.-jug*. *in vitro*.

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Table 1. Location of butternut (*Juglans cinerea*) trees sampled for resistance testing.

Number	Location County/Township/Ownership
PA 2-2	Allegheny / Pittsburgh North Park
PA 5-3	Bedford / Pavia / Blue Knob State Park
PA 5-4	Bedford / Pavia / Blue Knob State Park
PA 5-7	Bedford / Napier / Private
PA 5-8	Bedford / Napier / Unknown
PA 10-1	Butler / Franklin / Moraine State Park
PA 10-3	Butler / Brady / Moraine State Park
PA 10-10	Butler / Brady / Moraine State Park
PA 12-2	Cameron / Lumber / Elk State Forest
PA 12-6	Cameron / Portage / Private
PA 17-7	Clearfield / Brady / Private
PA 18-3	Clinton / Chapman/ Private
PA 18-4	Clinton / West Keating/ Sproul State Forest
PA 20-2	Crawford / Oil Creek / Private
PA 20-3	Crawford / Oil Creek / Private
PA 24-5	Elk / Millstone / Unknown
PA 25-7	Erie / Wayne / Private
PA 25-9	Erie / Wayne / Private
PA 27-1	Forest / Tionesta / Municipal Park
PA 27-2	Forest / Tionesta / Municipal Park
PA 27-5	Forest / Kingsley / U.S. Army Corp of Engineers
PA 27-7	Forest / Kingsley / U.S. Army Corp of Engineers
PA 33-2	Jefferson / Washington / Private
PA 33-3	Jefferson / Warsaw / Private
PA 37-1	Lawrence / Perry / Mc Connells Mills State Park
PA 37-7	Lawrence / Slippery Rock / Mc Connells Mills State Park
PA 38-2	Lebanon / South Londonderry /Private
PA 43-3	Mercer / Sandy Lake / Private
PA 50-4	Perry / Centre / Little Buffalo State Park
PA 53-3	Potter / Warton / Sinnemahoning State Park
PA 59-5	Tioga / Elk / Tioga State Forest
PA 59-6	Tioga / Elk / Tioga State Forest
PA 59-9	Tioga / Chatham / Private

Figure 1. Timothy Frontz sampling branches in a butternut tree in PA.

Figure 2. Growth of four strains of Ophiognomonia clavigignenti-juglandacearum at five temperatures. For each temperature, ten cultures of each strain on PDA were incubated in a dark incubator, and colony diameters measured. The bars represent the mean mm diameter increase per day for each strain.

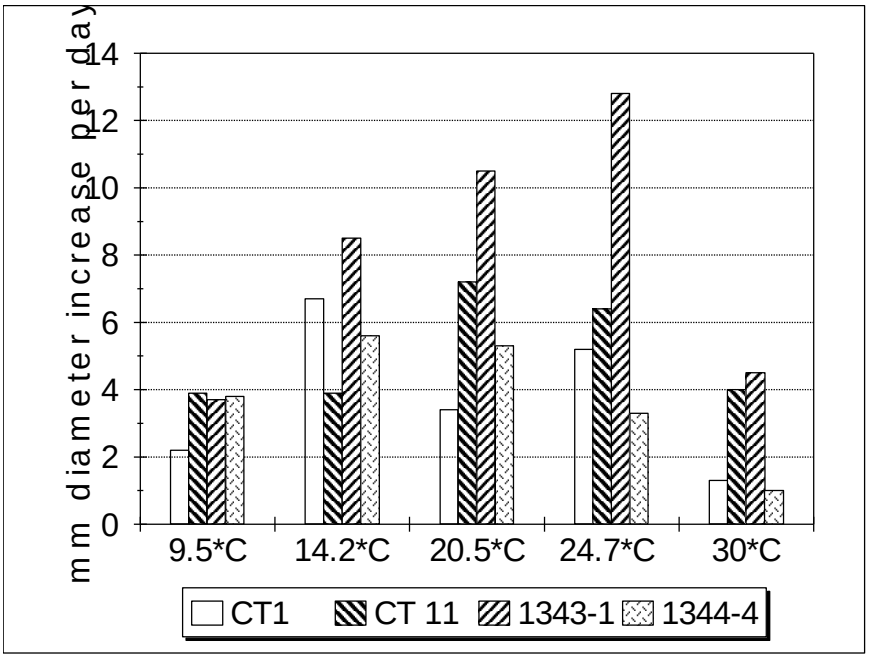
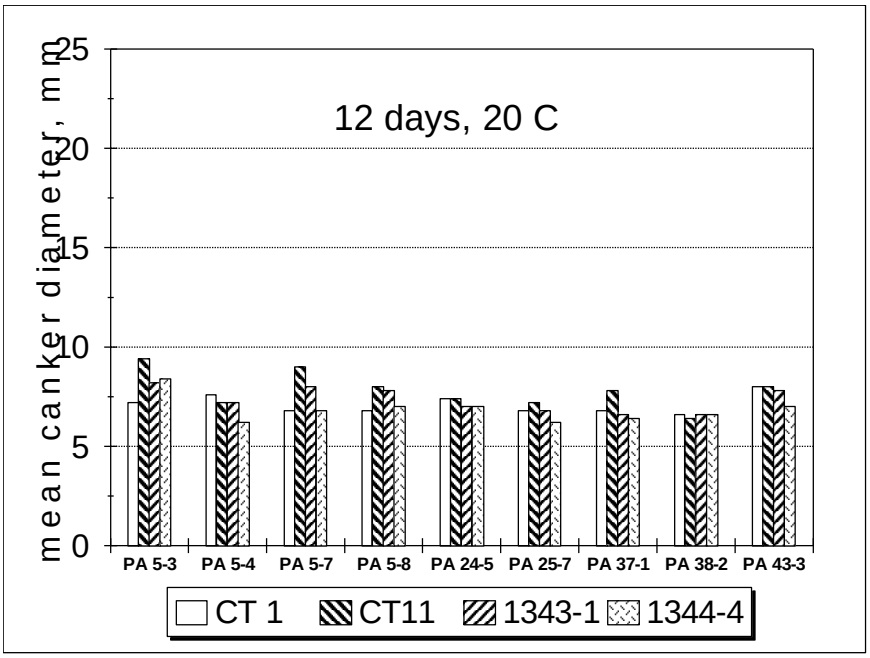
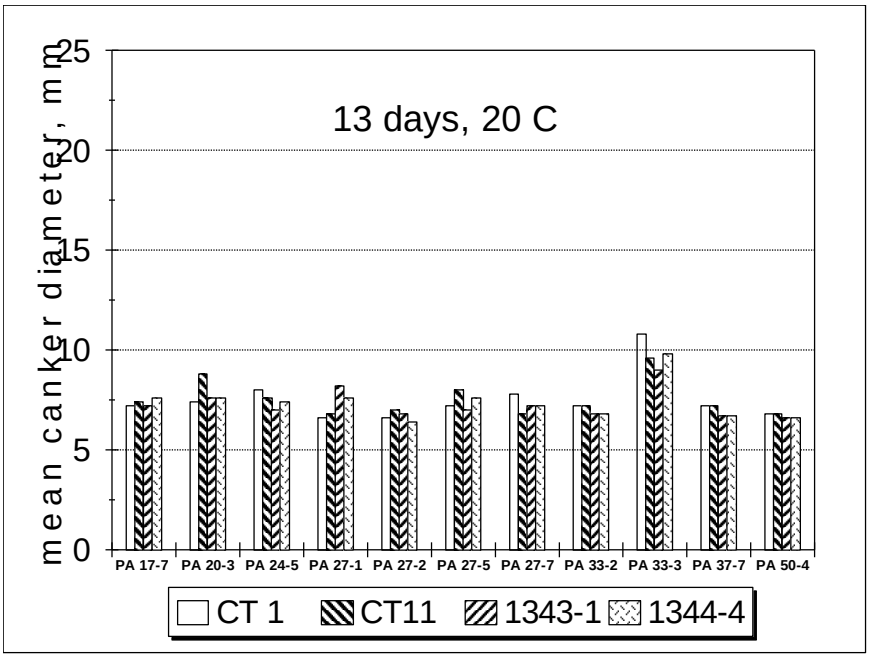


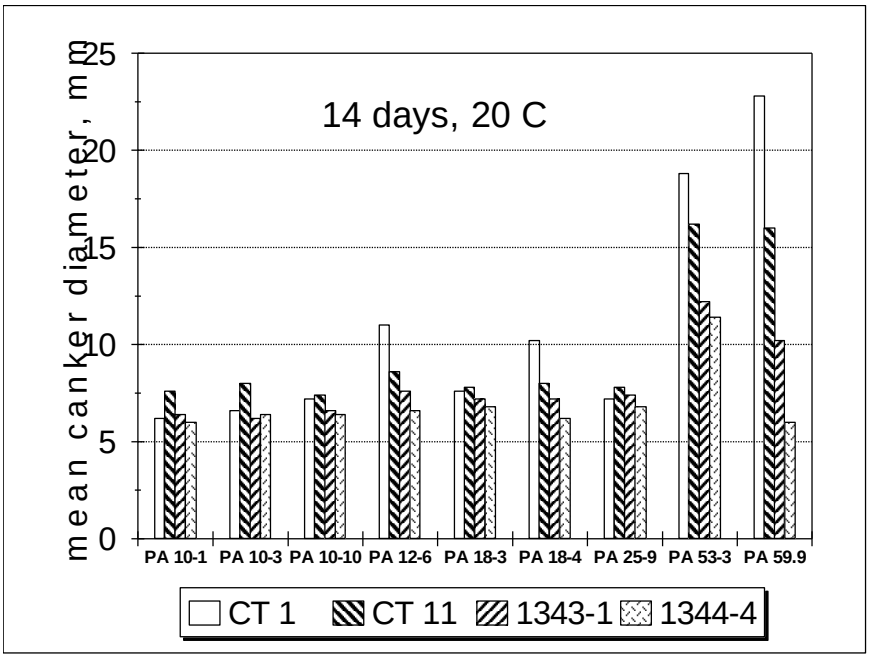
Figure 3. Stems of PA-10-10 inoculated with four strains of *Ophiognomonia clavigignenti-juglandacearum* after incubation for 14 days at 20C. From left to right strains are CT1, CT11, 1343-1, and 1344-4.

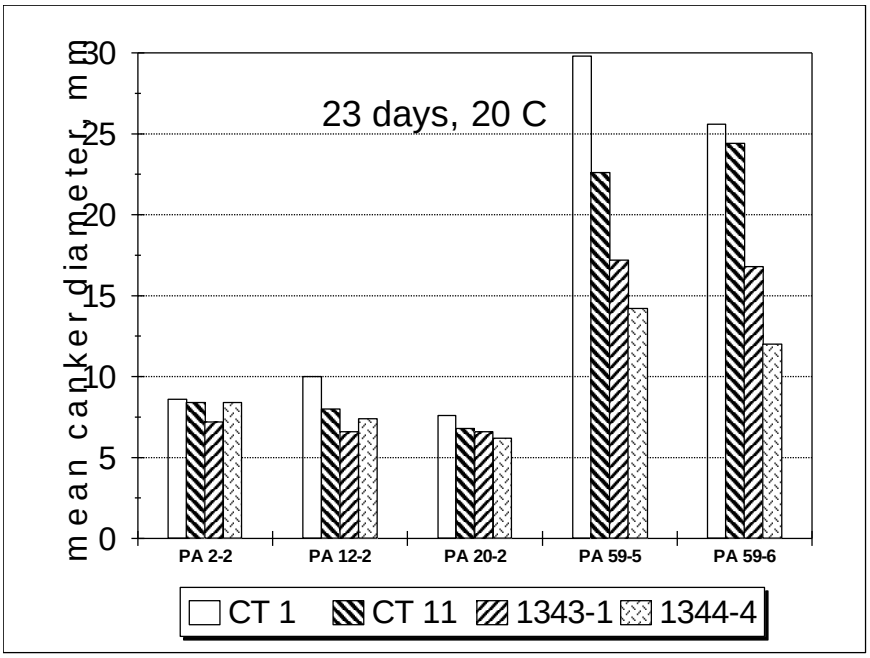
Figure 4. Stems of PA 59-9 inoculated with four strains of *Ophiognomonia clavigignenti-juglandacearum* after incubation for 14 days at 20C. From left to right strains are CT1, CT11, 1343-1, and 1344-4.

Figure 5. Four separate experiments of dormant butternut (*Juglans cinerea*) stem inoculations with four strains of *Ophiognomonia clavigignenti-juglandacearum* in the laboratory. Shown are mean canker diameters after inoculation of stems and incubation for (experiment 1) 12 days, (experiment 2) 13 days, (experiment 3) 14 days, and (experiment 4) 23 days. Each bar represents the mean diameter of five cankers.




13 days, 20 C
mean canker diameter, mm
0
5
10
15
20
25
PA 17-7
PA 20-3
PA 24-5
PA 27-1
PA 27-2
PA 27-5
PA 27-7
PA 33-2
PA 33-3
PA 37-7
PA 50-4
CT 1
CT11
1343-1
1344-4


14 days, 20 C
mean canker diameter, mm
0
5
10
15
20
25
PA 10-1
PA 10-3
PA 10-10
PA 12-6
PA 18-3
PA 18-4
PA 25-9
PA 53-3
PA 59.9
CT 1
CT 11
1343-1
1344-4


23 days, 20 C
mean canker diameter, mm
0
5
10
15
20
25
30
PA 2-2
PA 12-2
PA 20-2
PA 59-5
PA 59-6
CT 1
CT 11
1343-1
1344-4

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