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SCREENING BUTTERNUT AND BUTTERNUT HYBRIDS FOR RESISTANCE TO BUTTERNUT CANKER

J.R. McKenna, M.E. Ostry, and K. Woeste[1]

Abstract.—Butternut (*Juglans cinerea*) is being killed throughout its native range by the fungus *Sirococcus clavigignenti-juglandacearum* (*Scj*). In recent years, many disease-free trees have been determined to be complex hybrids with an admixture of Japanese walnut (*J. ailantifolia*). We challenged 5-year-old trees from two progeny tests with *Scj* in 2008. The first test (northern Indiana), planted in 2003, had 37 diverse families (n=319). Thirty-two of these seedling families were derived from a grafted orchard. Five additional families were collected from hybrid trees. The second test (southern Indiana), planted in 2004, had 12 pure butternut half-sib families collected from a woodlot with: 4 resistant, 4 moderately resistant, 4 susceptible, and 1 resistant hybrid families (n=213). Resistance ratings were based on the disease status of the mother trees when the seed was harvested in the fall of 2002. Eleven black walnut (*J. nigra*) trees were also included. In early fall of 2008, trees were inoculated with two strains of *Scj* obtained from branch cankers on trees in two locations in Indiana. The trees were scored 8 months after inoculation for canker incidence and severity. Some trees in the first test were naturally infected by *Scj* and resulting canker incidence and severity were recorded. Butternut hybrid families were more resistant to natural infection than the pure butternut families. Eight months after inoculation, canker incidence and severity varied significantly among butternut hybrid families and *Scj* strain but not among pure butternut families.

INTRODUCTION

Butternut (*Juglans cinerea*) was not historically a dominant species in eastern deciduous forests, or a species that produced a large volume of timber. However, it has been locally important and very valuable for its edible nuts and its wood (Kellogg 1919). Currently, butternut is being killed throughout its native range by the butternut canker disease first reported from Wisconsin more than 40 years ago (Renlund 1971). The causal agent of the disease is a fungus, *Sirococcus clavigignenti-juglandacearum* (*Scj*), which was described as a new species by Nair and others in 1979. The knowledge we have at present indicates it began causing disease in the southern part of butternut's range (Anderson and LaMadeleine 1978). A 1976 survey of butternut in Wisconsin revealed that 31 percent were diseased and 9 percent were dead, and, by 1992, 92 percent of the trees were diseased and 27 percent were dead (Carlson and Guthmiller 1993).

Sticky conidia (asexual spores) of *Scj* can be carried by insects (Katovich and Ostry 1998, Halik and Bergdahl 2002), birds, and, presumably, small mammals into the crowns of trees, where spore germination and infection of branches occur. Infection of boles and buttress roots results from spores spread in rainsplash from the diseased branches (Tisserat and Kuntz 1983). Trees are killed as multiple cankers coalesce and girdle the

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bole. Cankers caused by *Scj* are easily recognized and the pathogen is easy to culture. A number of factors strongly indicate that *Scj* is an exotic pathogen. Detection reports and dissections of trees have shown the disease was not present in North America much more than 60 years ago; no sexual stage of the fungus has been identified, and randomly amplified polymorphic DNA (RAPD) markers of the fungus revealed no appreciable genetic variation (Furnier and others 1999). Natural resistance is rare as most native butternut has died or is dying across its range, further suggesting that the pathogen has been recently introduced. Additionally, *Scj* has not been reported as a pathogen elsewhere in the world. When the fungus was introduced and from where are still not known.

Resistance to *Scj* in butternut is fundamental for the conservation of the species (Michler and others 2006). No specific mechanism of resistance has been identified, and we do not know the number and type of genes that may be involved. However, it has been observed repeatedly that various butternut hybrids and other *Juglans* species across the landscape are less affected by the disease compared to pure butternut (Ostry 1997). Apparent differences between yard-grown butternut hybrids and forest-grown pure butternuts may be the result of hybrid vigor, reduced competition for light and water, or *Scj* resistance.

Orchard and others (1982) screened a variety of *Juglans* species and hybrids with *Scj* using artificial stem inoculations. They observed that all *Juglans* species and hybrids were susceptible and mean canker lengths for Japanese walnut (*J. ailantifolia*) were smaller than *J.* × *bixbyi* (butternut × Japanese walnut, known as a "buart" F1 hybrid), Persian walnut (*J. regia*), and black walnut (*J. nigra*). These authors and others have noted that stem inoculations through artificial wounds may bypass natural resistance mechanisms, as black walnut was only rarely infected under natural conditions (Orchard and others 1982, Ostry and Moore 2007, and others). It is possible that *Scj* infects black walnut more commonly than previously believed (Ostry and others 1997), but that colonization is usually limited to small twigs, which has a negligible effect on tree health (K. Broders, personal communication).

For several decades, numerous healthy butternut trees have been identified in the wild where other butternuts have been killed by butternut canker disease. Many such healthy trees have been selected and have been clonally propagated to provide potentially resistant germplasm for future breeding and reintroduction. At present, a robust and effective screening method is necessary to select and sort out *Scj*-resistant sources from those susceptible to *Scj* (Michler and others 2006). Ostry and Moore (2008) investigated artificial inoculation methods with grafted ramets of a number of these selected trees with the goal of developing a satisfactory screening method to facilitate breeding canker-resistant butternut. Similar to Orchard and others (1982), Ostry and Moore (2008) were able to infect every clonal accession they tested. However, significant differences in canker incidence and mean canker length among the clones were obtained.

Although hybrids of butternut with Japanese walnut have been known to exist for some time (Bixby 1919), we are just beginning to understand how common hybridization has become. Complicating the matter further is that *Juglans* species are self-compatible (Rink and others 1989, Busov and others 2002, Bai and others 2006), and that buart hybrids are highly fruitful, making them unique among *Juglans* F1 interspecific hybrids, which otherwise yield less than each parental species or may be almost completely sterile. It is uncertain how many healthy "butternuts" surviving today are instead complex hybrids such as F2 hybrids, F1 buart backcrossed to butternut (Hoban and others 2008, Ross-Davis and others 2008, Hoban and others 2009), or even F2 hybrids backcrossed to butternut. If sufficient resistance to *Scj* is not present within butternut, fertile

hybrids would be useful for back-cross breeding, not unlike the breeding program under way with American chestnut (Orchard and others 1982, Schlarbaum 1997, Michler and others 2006).

We present preliminary results of two tests designed to screen the relative resistance of selected open-pollinated seedling families derived from an orchard of putative resistant clones and families from a natural stand where healthy trees have persisted to maturity. We also compared the relative resistance of hybrid butternut families.

MATERIAL AND METHODS

PLANT MATERIAL AND PLOT DESIGN

Seeds for both tests were collected in the fall of 2002 and grown in two test plantations. The first test was established at Purdue University's Martell Forest, West Lafayette, IN. For this plantation (Breeding Block), seed was collected from 6- to 10-year-old grafted trees in an orchard composed of putative resistant and susceptible butternuts from across the species' range (Table 1). Several offspring of the mother trees later proved to be butternut hybrids as were several open-pollinated half-sib families from highly resistant mother trees in northern Indiana (see Hybrid Determination). Seed was stratified in a cooler over the winter, sprouted in the greenhouse in April 2003, and planted in the field the following month. Ten seeds per family were planted as two five-tree plots at a spacing of 3.7 m between rows and 1.8 m within rows.

For the second test plantation (Resistance Block), seeds were collected from 12 mother trees in a 16-ha woodlot in southern Wisconsin near the town of Whitewater. This mixed hardwood stand is a natural population unique in that butternut regenerated here 50-60 years ago with a density approaching 250 trees per ha in areas, and trees exhibit both a wide range of bark color and a wide range of disease severity (Ostry and Woeste, 2004). We selected four trees from each of three categories—resistant, moderately resistant, and susceptible—based on their health status in the field (Table 1). The "resistant" female parents had been monitored and rated as canker-free and healthy for more than 20 years. As a resistant check lot, we included seeds from the ortet of a patented putative butternut, 'Bountiful,' which was growing in Vera, MO. At the time the seeds were collected, we suspected that 'Bountiful' was a hybrid, and confirmed our suspicion with DNA markers (described below) soon afterward. The seeds for this test were stratified and seedlings were grown in 7.6-liter containers in 2003 and then were planted as dormant seedlings in the spring of 2004 at the Southeastern Purdue Agricultural Center in Butlerville, IN. Trees in the plantation were planted on a 2.4-m × 2.4-m spacing as a randomized complete block. There were 18 blocks with a single tree per family in each block. For several butternut families, fewer than 18 seedlings of acceptable quality were alive by the time of planting. To achieve a full stocking rate in the plantation, black walnut seedlings were planted as filler trees and were subsequently inoculated along with the butternut.

HYBRID DETERMINATION

The phenotype of each seedling was rated based on morphological characteristics. Two independent observers rated each seedling on a three-point scale: 2 = pure *J. cinerea*; 1 = pure and hybrid traits mixed; and 0 = hybrid using the methods described by Woeste and others (2009). F1 hybrid accessions were easily recognized by their shell characteristics and were excluded from the block. Thus, any remaining hybrids could be more complex F2's or backcrossed hybrids. In the former case, we were able to confirm that some of the families were hybrids using a chloroplast DNA marker based on a sequence polymorphism in the TRNF-L intergenic

Table 1.—Geographic origin of source material for the Breeding Block and Resistance Block (bold) screening.

Type	HTIRC Acc. #[2]	Ostry Acc #[3]	Origin/Location[4]	Canker Rating[5]	Observed[6]
Hybrid Butternut					
	# 696	**-**	**Vera, MO**	**None (R)**	**2003/2005**
	# 702	-	New Paris, IN	None	2003/2009
	# 704	-	Plymouth, IN	None	2002
	# 706	-	New Paris, IN	2 cankers	2003/2009
	# 707	-	Brimfield, IN	None	2003/2009
	# 708	-	Steuben Co., IN	None	2002
	# 710	OS-# 7	Madison, WI	None	1990
	# 711	OS-# 8	Madison, WI	None	1990
	# 731	OS-# 91	Clover Lick, WV	None	1996
	# 732	OS-# 92	Loudon, NH	None	1996
	# 734	OS-#128	Sanford, ME	None	1995
	# 735	OS-#129	Sanford, ME	None	1995
	# 748	OS-#184	Chequam., NF, WI	-	1996
	# 750	OS-#191	Ankeny, IA	Healthy	1996
Butternut					
	# 709	OS-# 6	Caledonia, MN	None	
	# 712	OS-# 10	Arlington, WI	Branch cankers	1993
	# 713	OS-# 14	Rochester, MN	Branch cankers	1994
	# 714	OS-# 16	Rochester, MN	2 cankers	1993
	# 715	OS-# 17	Rochester, MN	1 branch canker	1995
	# 716	OS-# 19	Rochester, MN	None	1997
	# 717	**OS-# 20**	**Whitewater, WI**	**None (R)**	**1997/2009**
	# 718	**OS-# 22**	**Whitewater, WI**	**None (R)**	**1997/2009**
	# 722	OS-# 54	Nicolet NF, WI	None	1997
	# 723	OS-# 60	Whitewater, WI	Healthy	1994/2009
	# 724	**OS-# 61**	**Whitewater, WI**	**Healthy (MR)**	**1994/2009**
	# 725	**OS-# 63**	**Whitewater, WI**	**Healthy (R)**	**1994/2009**
	# 726	OS-# 67	Mazaska Lake, MN	Healthy	1994
	# 727	OS-# 78	Rochester, MN	Cankered	1991
	# 728	OS-# 85	M. Twain NF,MO	-	1995
	# 730	OS-# 87	M. Twain NF,MO	-	1995
	# 733	OS-# 97	Perch River, NY	Healthy	1994
	# 736	OS-#132	Berlin, VT	-	1995
	# 738	OS-#141	Trade Lake, WI	Healthy	1995
	# 740	**OS-#147**	**Whitewater, WI**	**Healthy (MR)**	**1995/2009**
	# 741	OS-#148	Whitewater, WI	Healthy	1997/2009
	# 742	OS-#149	Stratford, NH	Healthy	1995
	# 743	OS-#157	PA	-	1995
	# 744	OS-#159	IA	-	1995
	# 746	OS-#171	NY	-	1995
	# 747	OS-#181	Bark River, MI	Healthy	1996
	# 751	**OS-#401**	**Whitewater, WI**	**Cankered/dead(S)**	**2002/2009**
	# 752	**OS-#403**	**Whitewater, WI**	**Cankered/dead(S)**	**2002/2009**
	# 757	**OS-#414**	**Whitewater, WI**	**Healthy**	**2002/2009**
	# 767	**OS-#423**	**Whitewater, WI**	**Cankered/dead(S)**	**2002/2009**
	# 769	**OS-#426**	**Whitewater, WI**	**Cankered (MR)**	**2002/2009**
	# 772	**OS-#430**	**Whitewater, WI**	**Cankered/dead(S)**	**2002/2009**
	# 773	**OS-#431**	**Whitewater, WI**	**Cankered (MR)**	**2002/2009**

[2] Hardwood Tree Improvement & Regeneration Center, USDA FS Northern Research/Purdue University, accession number.

[3] Ostry, USDA Forest Service North Central accession number.

[4] Location where the original scion material (plain) or seed (bold) originated.

[5] Canker rating of the original ortet or seed tree (bold) in the field. S = susceptible, MR = moderately resistant, and R = resistant selections for the resistance test.

[6] Most recent year(s) when canker status of each ortet or seed tree was last evaluated.

spacer (Aradhya and others 2006, Woeste, unpublished data). The Japanese walnut haplotype of the TRNF-L sequence polymorphism is recognized by the restriction enzyme *Mbo*II. In practice, most butternut hybrids can be identified using this cleaved amplified polymorphic sequence (CAPS) marker to determine whether a suspect tree carries the chloroplast of Japanese walnut or butternut. Because *Juglans* chloroplasts are inherited maternally, maternal siblings of a seedling containing a Japanese walnut chloroplast will contain the same chloroplast, irrespective of their male parent. A second marker (ITS/*Bs*IE) is a nuclear (CAPS) marker based on polymorphism in the ITS sequence. This marker is useful for detecting F1 hybrids or cases where a hybrid has pollinated a pure butternut (Woeste, unpublished data).

FUNGAL STRAINS AND CULTURE

We had no information regarding different levels of aggressiveness among Indiana *Scj* strains in our collection. We used a strain isolated from the Breeding Block at the Martell Forest designated as *Scj* IN-1375-4A and a second strain from more than 100 km away in the Hoosier National Forest of southern Indiana, designated *Scj* IN-1378-3 and collected in August 2008. Inoculum was prepared from sporulating 2-month-old cultures of these two *Scj* strains grown on malt agar at 20 °C in the dark.

INOCULATION

In late September and early October 2008, when the trees are most susceptible to infection (Ostry and Moore 2007, 2008), all trees with at least a 1.0-m tall, relatively clear trunk and with a minimum diameter at breast height (d.b.h.) of 17 mm, were inoculated in both plantations. Four 6-mm diameter holes were drilled through the bark and just into the cambium of the main trunk of each tree and each of the four wounds was separated by at least 20 cm analogous to the method developed for screening chestnut to the chestnut blight (*Cryphonectria parasitica*) fungus (Anagnostakis 1992). A 6-mm diameter plug of fungus and agar was inserted into the hole, with the fungal hyphae facing the cambium. Each inoculated wound was wrapped with one layer of masking tape. *Scj* IN-1375-4A was consistently inoculated into the upper two holes and *Scj* IN-1378-3 into the bottom two holes, following the method for chestnut screening (Anagnostakis 1992). This systematic approach is useful to keep track of each strain easily over time. Previous studies with different *Scj* strains randomly inoculated along branches in the greenhouse showed differences in aggressiveness (Ostry, unpublished data), suggesting strain rather than branch position was a significant factor in canker development. For trees with less than 1.0 m of clear trunk and a d.b.h less than 17 mm, only two wounds were made, each with a single inoculation of each strain.

CANKER EVALUATION

In the Breeding Block, natural canker infection began in 2006, when the seedlings were in their third growing season. *Scj* was isolated from several samples of natural cankers in August 2008, confirming *Scj* as the causal agent. Each tree was evaluated and rated for cumulative natural canker incidence and severity in November of 2009 using a subjective scale. Incidence was rated as 0 to 3, where 0 = no natural cankers; 1 = 1 or 2 cankers; 2 = 3 to 5 cankers, and 3 = 6 or more. Severity was assigned to each tree based on the average relative size of the cankers, where 1 = small ~30 × 10 mm (length × width); 2 = medium ~60 × 20 mm; and 3 = large ~100 × 25 mm sized cankers.

Responses to inoculations were evaluated after 8 months in May 2009. The external vertical length and maximum horizontal width of each canker were recorded. Each individual artificial inoculation site was treated as an experimental unit. To achieve balanced data for statistical evaluations, compensating for dead,

missing, or small suppressed trees, we considered all of the upper and lower inoculations per *Scj* strain as two replications. Incidence data were determined simply as the number of inoculations per family that produced a measurable canker beyond non-inoculated wounded controls. Because half or fewer of our inoculations produced a canker, instances where no canker resulted were removed from the dataset for comparison of canker size among genotypes and *Scj* strains. Hence, for both tests, both canker incidence and canker size were associated with the three main fixed effects: *Scj* strain, family, and butternut type—hybrid or pure. Canker incidences and severity (external length) were subject to 2-way ANOVA (Excel 2007, Microsoft Corp., Seattle, WA). Least squares means separation tests for canker size on hybrid families were performed using Fisher's least significant difference procedure. Differences between butternut hybrids and pure butternut in canker development from natural infection were analyzed using Chi-square (SAS Version 9.2, SAS Institute, Cary, NC, 2008).

RESULTS

BREEDING BLOCK

The correlation between canker length and width was highly significant ($P \leq 0.0001$, r = 0.56). Given this correlation, we considered only vertical canker length (parallel with the axis of the trunk) in the analyses. Inoculated butternut hybrids and pure butternuts had equal canker lengths when the data were pooled over all families and both *Scj* strains, (ANOVA $P \leq 0.16$.) When butternut hybrid and pure butternut families were analyzed separately, butternut hybrid families differed significantly ($P \leq 0.02$) for canker size, while canker size on pure butternut families was not significantly different.

The *Scj* strain used was a highly significant source of variation for canker length (ANOVA $P \leq 0.0001$). *Scj* IN-1375-4A produced larger cankers (ANOVA $P \leq 0.007$) on both butternut hybrids and pure butternut than did *Scj* IN-1378-3 (Table 2). Inoculation with *Scj* IN-1375-4A led to nearly twice the number of cankers per family compared to *Scj* IN-1378-3 (Table 3). The canker incidence was remarkably consistent between butternut hybrids and butternut. After inoculation with *Scj* IN-1375-4A, the incidence of infection in butternut hybrids and pure butternut was 55 percent and 50 percent, respectively. Inoculations with *Scj* IN-1378-3 resulted in a canker incidence of 26 percent for both types. Family effects were not significant for canker incidence. Incidence ranged from 10 percent to 85 percent for *Scj* IN-1375-4A inoculations and 0 percent to 50 percent for *Scj* IN-1378-3 inoculations for both types.

Cankers were significantly smaller (12 percent) on hybrid families than on pure butternut families, with an overall canker length of 63 mm vs. 71 mm, respectively (Table 2). After inoculation with *Scj* IN-1375-4A, the ranges of canker lengths were 33 mm to 92 mm for hybrid families and 51 mm to 94 mm for butternut families. After inoculation with *Scj* IN-1378-3, the ranges of canker lengths were 19 mm to 92 mm for hybrids and 23 mm to 94 mm for butternut. The range data did not include two families that made no cankers when inoculated with *Scj* IN-1375-4A. Cankers produced by *Scj* IN-1378-3 were more variable in size than those produced by the other strain. The coefficients of variation (CV) for canker length for the hybrids and butternut inoculated with *Scj* IN-1375-4A were 0.28 and 0.17, respectively. By contrast, butternut hybrids and butternut inoculated with *Scj* IN-1378-3 had CV's of 0.47 and 0.41, respectively.

Table 2.—Length of cankers 8 months after inoculation of Breeding Block hybrid butternut and butternut families with two *Scj* strains, West Lafayette, IN.

				Canker Length (mm)		
				Scj Strain		
Type	Family	Sdlgs (no.)	Inoc. (no.)	IN-1375-4A	IN-1378-3	Avg.
Hybrid Butternut						
	# 706	3	6	32.6 a	18.5 ab	25.6
	# 707	8	20	46.9 ab	00.0 a	23.5
	# 750	10	20	50.2 ab	68.3 c	59.3
	# 748	7	12	58.5 b	52.0 c	55.3
	# 711	10	18	55.1 b	91.8 d	73.5
	# 735	10	20	74.9 c	59.5 c	67.2
	# 710	10	20	75.9 cd	63.3 c	69.6
	# 732	9	18	90.3 cd	32.0 b	61.2
	# 704	9	18	78.8 cd	70.1 cd	74.5
	# 702	6	12	79.3 cd	69.8 cd	74.6
	# 708	8	13	86.1 cd	71.5 cd	78.8
	# 734	10	18	93.2 cd	88.5 d	90.9
	# 731	10	20	95.3 d	27.7 b	61.5
Sum /Mean		110	215	70.5**[7]	54.8**	62.7
SD				19.9	27.5	19.4
Butternut						
	# 709	10	20	78.8	23.3	51.1
	# 712	10	16	77.2	52.5	64.8
	# 713	10	16	67.6	37.3	52.4
	# 714	9	18	62.5	93.0	77.7
	# 715	7	16	62.1	68.8	65.4
	# 716	9	18	65.7	60.9	63.3
	# 717	9	16	92.0	89.8	90.9
	# 718	10	20	89.5	88.4	89.0
	# 722	10	20	93.8	46.1	69.9
	# 723	8	16	81.4	88.8	85.1
	# 726	9	15	93.3	87.7	90.5
	# 727	10	16	57.9	102.0	80.0
	# 728	8	14	50.6	41.7	46.1
	# 730	10	20	78.7	81.5	80.1
	# 733	10	18	76.1	99.9	88.0
	# 736	10	20	68.5	67.5	68.0
	# 738	9	14	59.4	0.0	29.7
	# 741	10	20	73.3	55.5	64.4
	# 742	7	14	68.5	91.8	80.1
	# 743	10	18	93.3	56.9	75.1
	# 744	9	18	84.3	60.0	72.1
	# 746	10	20	86.7	89.7	88.2
	# 747	10	20	73.1	42.2	57.6
Sum/Mean		122	403	75.4*	66.3*	70.9
SD				12.6	26.7	16.0

[7] Scj strain significantly affected canker length, $P \leq 0.007$ for both butternut hybrid and butternut families. Among families, significant difference occurred only for hybrid families. Means followed by different letters are significant at $P \leq 0.02$ by Fisher's protected least significant difference = 19.7 mm.

Table 3.—Canker incidence 8 months after inoculation of hybrid butternut and butternut families with two *Scj* strains in the Breeding Block, West Lafayette, IN.

Type	Family	Sdlgs (no.)	Canker Incidence by *Scj* Strain (%)	
			IN-1375-4A	IN-1378-3
Hybrid Butternut				
	# 702	6	58	25
	# 704	9	67	28
	# 706	3	50	17
	# 707	8	35	0
	# 708	8	77	31
	# 710	10	55	35
	# 711	10	50	22
	# 731	10	45	15
	# 732	9	50	6
	# 734	10	56	50
	# 735	10	85	35
	# 748	7	58	33
	# 750	10	30	45
Sum/mean		110	55***[8]	26***
SD			15	14
Butternut				
	# 709	10	60	25
	# 712	10	31	13
	# 713	10	44	13
	# 714	9	67	33
	# 715	7	19	19
	# 716	9	67	33
	# 717	9	56	44
	# 718	10	65	40
	# 722	10	30	20
	# 723	8	69	25
	# 726	9	47	27
	# 727	10	56	13
	# 728	8	43	43
	# 730	10	65	30
	# 733	10	61	39
	# 736	10	10	35
	# 738	9	43	0
	# 741	10	60	25
	# 742	7	43	21
	# 743	10	67	39
	# 744	9	22	22
	# 746	10	85	35
	# 747	10	35	15
Sum/mean		214	50***	26***
SD			19	11

[8] *Scj* strain significantly affected canker incidence $P \leq 1.0 \times 10^{-6}$.

NATURAL INFECTION

Butternut hybrids and pure butternut trees differed significantly (Chi-square value with 1 degree of freedom = 25.4 P ≤ 0.0001) for incidence of canker development resulting from natural infection by *Scj* (Table 4). Cankers developed on 6 out of 133 butternut hybrid trees over the last 3 years compared to 58 out of 227 pure butternut trees. No significant difference in disease severity among butternut hybrid or pure butternut families was observed. Because families were planted in only two groups of five contiguous seedlings, we did not have enough statistical power, given the low incidence of canker development resulting from natural infection, to analyze the data for family effects.

RESISTANCE BLOCK

In the Resistance Block, only one butternut hybrid family (#696) was included. *Scj* strains were significantly different in terms of canker incidence (ANOVA, $P \leq 0.0001$). *Scj* IN-1375-4A produced cankers 39 percent of the time vs. 15 percent with *Scj* IN-1378-3 (Table 5). Canker development on pure butternut families did not differ significantly. In response to inoculation with *Scj* IN-1378-3, trees among the butternut hybrid family #696 and black walnut trees had a canker incidence of 3 percent and 4 percent, respectively.

The correlation for canker length and width was similar to that in the Breeding Block and was highly significant ($P \leq 0.002$; r = 0.55). We analyzed canker severity by canker length alone. Once again, we found *Scj* strain to be a highly significant source of variation for canker size (ANOVA $P \leq 0.0006$); *Scj* IN-1375-4A produced larger cankers (Table 6). There was no difference, however, among pure butternut families in canker size. The hybrid family #696 and black walnut had canker lengths similar to pure butternut families when inoculated with *Scj* IN-1375-4A. When inoculated with *Scj* IN-1378-3, these two genotypes produced smaller canker lengths compared to pure butternut (Table 6).

DISCUSSION

Despite past reports that the *Scj* pathogen has little to no genetic variation (Furnier and others, 1999), the present studies, along with those of Ostry and Moore (2007, 2008) demonstrate that *Scj* strain significantly affects canker size. Inoculation of *Scj* IN-1375-4A induced more cankers, and in most cases, larger cankers than the other strain, and may indicate that *Scj* strains vary in aggressiveness. An elegant system utilizing two strains, one highly aggressive and the other much less aggressive, was developed for screening American chestnut for susceptibility to chestnut blight (Anagnostakis 1992). Seedlings that developed large cankers when inoculated with the weaker strain of chestnut blight are rogued out quickly as they have no resistance. Trees that show resistance to the weaker strain are then evaluated for their reaction to the more aggressive strain, retained, and evaluated more carefully. Just how much *Scj* strains vary in aggressiveness needs to be investigated further and future screening should include a highly aggressive strain.

Artificial inoculation of trees with *Scj* did not reproduce results seen in the field under natural conditions. In the Breeding Block, butternut hybrid families with a low incidence of butternut canker from natural infections were readily infected when artificially inoculated through stem wounds. In the Resistance Block, incidence of canker was similar among black walnut and the #696 butternut hybrid family. Additionally, there was no difference in canker incidence among diverse families selected in the native stand in Whitewater, WI, and only marginal differences in canker size among inoculated butternut hybrid families in the Breeding Block. This result strongly suggests that results of artificial stem inoculations do not reflect natural resistance observed over decades in the field. Such an inoculation method apparently does not allow the expression of resistance mechanism(s).

Table 4.—Incidence of butternut canker from natural infection on butternut hybrids and pure butternut families as of the sixth growing season in the Breeding Block, West Lafayette, IN.

Type	Family	Sdlgs (no.)	Canker Incidence (%)	Avg. Severity[9]	Avg. Size[10]
Hybrid Butternut					
	# 702	7	0	-	-
	# 704	10	0	-	-
	# 706	5	0	-	-
	# 707	10	0	-	-
	# 708	9	0	-	-
	# 710	10	20	1.0	3
	# 711	10	10	1.0	1
	# 731	10	0	-	-
	# 732	10	0	-	-
	# 734	10	0	-	-
	# 735	10	10	1.0	3
	# 748	10	10	1.0	1
	# 750	10	10	1.0	3
Sum/mean		121	5***[11]	1.0	2.1
SD			7	0.0	1.0
Butternut					
	# 709	10	40	1.3	2.3
	# 712	10	20	3.0	3.0
	# 713	10	20	1.5	1.0
	# 714	10	40	2.0	2.3
	# 715	8	50	2.5	2.0
	# 716	10	40	2.0	2.0
	# 717	10	30	1.3	1.3
	# 718	10	20	1.5	2.5
	# 722	10	40	1.3	1.5
	# 723	10	10	1.0	1.0
	# 726	10	10	1.0	1.0
	# 727	10	40	1.5	1.5
	# 728	10	20	1.0	2.0
	# 730	10	10	1.0	2.0
	# 733	10	0	-	-
	# 736	10	0	-	-
	# 738	9	0	-	-
	# 741	10	20	1.5	1.5
	# 742	10	20	1.0	2.5
	# 743	10	30	1.3	2.0
	# 744	10	80	2.0	2.0
	# 746	10	50	1.2	2.2
	# 747	10	10	1.0	3.0
Sum/mean		227	26	1.5	1.9

[9] Average severity based on ratings: 1 = 1 or 2 cankers; 2 = 3 to 5 cankers, and 3 = 6 or more.

[10] Average size class of cankers where 1 = small ~30 × 10 mm; 2 = medium ~60 × 20 mm; and 3 = large ~100 × 25 mm.

[11] Hybrid butternut has a highly significant lower incidence of natural infections, $P \leq 0.0001$ by chi-square.

Table 5.—Canker incidence 8 months after inoculation of butternut families in the Resistance Block, Butlerville, IN.

				Canker Incidence (%)	
				Scj Strain	
HTIRC ACC #	Type	Rating[12]	Sdlgs. Inoc. (no.)	IN-1375-4A	IN-1378-3
# 717	Butternut	R	18	43	20
# 718	Butternut	R	17	24	12
# 725	Butternut	R	16	37	20
# 757	Butternut	R	16	59	26
	Means			41***[13]	20
# 724	Butternut	MR	17	29	21
# 740	Butternut	MR	15	33	30
# 769	Butternut	MR	12	42	8
# 773	Butternut	MR	18	50	13
	Means			39	18
# 751	Butternut	S	18	32	18
# 752	Butternut	S	15	44	14
# 767	Butternut	S	11	37	15
# 772	Butternut	S	16	44	13
	Means			39	15
# 696[14]	Hybrid Butternut	R	17	46	3
BW[15]	Black walnut	R	11	32	5
	Means			39	4

[12] Rating based on the canker status and health of each seed tree in the forest stand near Whitewater, WI. Trees were designated in fall 2002 as (R) resistant; (MR) moderately resistant; (S) susceptible. Revaluation of the stand in November 2009 showed (R) trees remaining healthy, (MR's) in decline, and (S's) dead or dying.

[13] *Scj* strains are significantly different, $P \leq 0.2.5 \times 10^{-5}$.

[14] Half-sib family of the hybrid butternut cultivar 'Bountiful.'

[15] Black walnut seedlings added to complete blocks where butternut families were absent at the time of planting.

The suggestion that Japanese walnut offers a source of resistance to *Scj* is supported by our results. The most compelling evidence is that butternut hybrids and pure butternuts showed a significant difference in the frequency of canker development as the result of natural infection in the Breeding Block, but not in the frequency of those produced in response to artificial inoculations.

Despite the considerable variation in these families' genetic background, the incidence and severity of canker resulting from artificial inoculations were similar. Nevertheless, absent a better inoculation method, numerical differences in canker size may still be a useful measure of relative resistance for screening breeding material. Ostry and Moore (2008) found differences in canker length among the grafted ramets they tested ($P \leq 0.05$), similar to differences among hybrid families in the breeding block that we observed ($P \leq 0.02$). Ostry and Moore (2008) also found that canker size ranged greatly within a genotype, which we also observed, and that separation of clones by mean canker length did not result in well defined resistant and susceptible classes. The check group used by Ostry and Moore (2008), which consisted of seedlings presumed to be susceptible, had the largest mean canker length. The two resistant checks in the Resistance Block had ~20-mm smaller canker lengths than all of the pure butternut families.

Table 6.—Length of cankers 8 months after inoculation of butternut families in the Resistance Block, Butlerville, IN.

				Canker Length (mm)	
				Scj Strain	
HTIRC ACC #	Type	Rating[16]	Sdlgs. Inoc. (no.)	IN-1375-4A	IN-1378-3
# 717	Butternut	R	18	100.6	44.8
# 718	Butternut	R	17	79.9	88.3
# 725	Butternut	R	16	107.6	86.0
# 757	Butternut	R	16	106.1	80.3
	Means			98.5***[17]	74.9
	SD			12.8	20.3
# 724	Butternut	MR	17	96.9	53.3
# 740	Butternut	MR	15	101.3	56.9
# 769	Butternut	MR	12	77.1	47.5
# 773	Butternut	MR	18	98.0	96.8
	Means			93.3	63.6
	SD			11.0	22.4
# 751	Butternut	S	18	103.1	46.7
# 752	Butternut	S	15	84.3	73.8
# 767	Butternut	S	11	94.8	83.0
# 772	Butternut	S	16	87.5	81.0
	Means			92.4	71.1
	SD			8.3	16.8
# 696[18]	Hybrid Butternut	R	17	43.0	32.5
BW[19]	Black walnut	R	11	104.7	50.5
	Means			73.8	41.5
	SD			47.5	21.5

[16] Rating based on the canker status and health of each seed tree in the forest stand near Whitewater, WI. Trees were designated in fall 2002 as (R) resistant; (MR) moderately resistant; (S) susceptible. Re-evaluation of the stand in November 2009 showed (R) trees remaining healthy, (MR's) in decline, and (S's) dead or dying.

[17] Scj strains are significantly different $P \leq 2.5 \times 10^{-5}$.

[18] Half-sib family of the hybrid butternut cultivar 'Bountiful.'

[19] Black walnut seedlings added to complete blocks where butternut families were absent at the time of planting.

For the Resistance Block, we selected butternut mother trees that exhibited clear differences in resistance in the field. Although we expected their progeny families also to reflect these differences, we found no differences among them despite field observations of the mother trees' level of resistance over 20 years. If our method of stem inoculations did in fact circumvent the tree's resistance mechanism(s), it may not be surprising that we found no differences among these progeny, and furthermore, there may be no basis for inferring the number of genes or gene-action involved in *Scj* resistance in butternut. Resistance to *Scj* in butternut may be a complex quantitative trait and screening more than 10 to 18 seedlings per family may be necessary. This approach needs to be investigated.

Final conclusions cannot be drawn at this time with just 8 months of data. In the present two studies, 12 months after inoculation, some cankers have callused and appear to have compartmentalized the pathogen; this response will need to be factored into future analyses. Conversely, some of the inoculations that were

scored as having no appreciable canker development relative to non-inoculated wounds have produced cankers at 12 months. Most significantly, some trees scored as resistant based upon inoculation studies have been infected by wild inoculum, with significant canker development. Further observations at 20 months and 24 months will be needed to draw a final conclusion on the fate of each inoculation. The rate of canker elongation over time, and the number of new cankers that develop from natural infections now that the disease is established in the plantings, may reveal more family differences in reaction to *Scj*.

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