2004

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Michael E. Ostry
University of Missouri

Keith E. Woeste
Purdue University, woeste@purdue.edu

Recommended Citation

Ostry, M. E. and K. Woeste. 2004 Spread of butternut canker in North America, host range, evidence of resistance within butternut populations and conservation genetics, p. 114 – 120. IN: Michler, C.H. et al., eds. Black walnut in a new century, Proceedings of the 6th Walnut Council Research Symposium; July 25-28, Lafayette, IN. Gen. Tech. Rep. NC-243. U.S.D.A. Forest Service, North Central Research Station, St. Paul, MN (refereed).

Spread of Butternut Canker in North America, Host Range, Evidence of Resistance Within Butternut Populations and Conservation Genetics

M.E. Ostry and K. Woeste[1]

Abstract—Butternut canker is killing trees throughout the range of butternut in North America and is threatening the viability of many populations in several areas. Although butternut is the primary host, other *Juglans* species and some hardwood species also are potential hosts. Evidence is building that genetic resistance within butternut populations may be exploited for conservation and restoration of the species.

INTRODUCTION

Butternut (*Juglans cinerea* L.) is being killed throughout its range by a canker caused by the fungus *Sirococcus clavigignenti-juglandacearum* Nair, Kostichka, and Kuntz, described as a new species in 1979 (Nair and others 1979). Although there are no reports of this fungus outside of North America, it is thought to be an exotic pathogen (Furnier and others 1999). Spores of the fungus develop under infected bark in sticky masses and are dispersed by rainsplash and wind during the growing season.

Butternut is valued for many uses and is important for wildlife and forest diversity, however, its infrequent occurrence within forest stands and its relatively small kernel and hard shell have, in part, limited its commercial importance as a timber or nut species (Ostry and Pijut 2000). As local supplies of healthy butternut trees become scarce the value of the wood has increased.

Butternut was listed under Category 2 on the list of Endangered and Threatened Plants under the Federal Endangered Species Act of 1973, however, this category has been eliminated and currently butternut has no official listing status. The first state to enact a measure to conserve butternut was Minnesota where in 1992 a moratorium on the harvest of healthy butternut on State lands was enacted. Butternut remains a "species of concern" or a "sensitive species" in many states and is a Regional Forester Sensitive Species in the Eastern Region on 13 of the 16 National Forests. In Canada, butternut was listed endangered by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) in November 2003.

SPREAD OF THE PATHOGEN

The first reported occurrence of butternut canker was from southwestern Wisconsin where all but two butternut trees in a 40-acre woodlot were diseased (WI Conserv. Dept. 1967). A survey of butternut in 36 Wisconsin counties in 1976 revealed that 31 and 9% of the trees were diseased and dead, respectively. In contrast, in a 1992 resurvey of 32 Wisconsin counties 92 and 27% of the trees were diseased and dead, respectively (Carlson and Guthmiller 1993).

A survey for butternut canker in the eastern United States revealed that the disease was present in at least 14 of the 16 states surveyed (Anderson and LaMadeleine 1978). In that report the authors mention the disease had essentially eliminated many populations of butternut in North and South Carolina. Early reports of butternut decline throughout the northeastern United States were attributed to the fungus *Melanconis juglandis* Ellis & Everhart Graves (Ostry 1997b) that causes branch dieback but not stem cankers. Although cankers are obvious, unless close examinations

[1]Research Plant Pathologist and Research Plant Molecular Geneticist, North Central Research Station, USDA Forest Service, 1561 Lindig Avenue, St. Paul, MN 55108 (MEO) and Hardwood Tree Improvement and Regeneration Center, Department of Forestry and Natural Resources, Forestry Building, 195 Marsteller St. West Lafayette, IN 47907 (KW). MEO is corresponding author: to contact call (651) 649-5113 or e-mail at mostry@fs.fed.us.

Citation for proceedings: Michler, C.H.; Pijut, P.M.; Van Sambeek, J.W.; Coggeshall, M.V.; Seifert, J.; Woeste, K.; Overton, R.; Ponder, F., Jr., eds. 2004. Black walnut in a new century, proceedings of the 6th Walnut Council research symposium; 2004 July 25-28; Lafayette, IN. Gen. Tech. Rep. NC-243. St. Paul, MN: U.S. Department of Agriculture, Forest Service, North Central Research Station. 188 p.

were made of trees, these reports may have mistakenly attributed tree decline to *M. juglandis* and butternut canker may have been present much earlier than reported.

In Canada, butternut canker was first detected and confirmed from Ontario and Quebec in 1991 (Davis and others 1992) and in New Brunswick in 1997 where it was thought to have been present for at least 7 years (Harrison and others 1998).

The most recent U.S. Department of Agriculture, Forest Service, Forest Inventory and Analysis survey data examined for butternut (NCRS, FIA Web site, October 2003) revealed that overall in seven Midwestern states the number of butternut trees in all size classes decreased by 23%, however, the number of trees recorded increased in three of the states. The states with a decrease in the number of trees and the inventory interval from which the data were collected are as follows: Michigan, 89% (1993-2001); Illinois, 87% (1998-2002); Wisconsin, 44% (1996-2001); and Iowa, 40% (1990-2001). An increase in the number of butternut trees was recorded in Minnesota, 55% (1990-2002); Indiana, 41% (1998-2002); and Missouri, 25% (1989-2002). This increase in number of trees was predominantly in the smallest size class (1.0-2.9 inch); the number of trees in all other size classes revealed decreases ranging from 13% (11.0-12.9 inch) to 100% (21.0+).

Butternut and eastern black walnut (*J. nigra* L) seed are known to harbor *S. clavigignenti-juglandacearum* (Innes 1997). There is no evidence that it can be spread on Japanese walnut (*J. ailanthifolia* Carr.) seed or seedlings but this walnut species has been widely planted throughout the eastern United States (Bixby 1919). One can only speculate whether the fungus could have been inadvertently introduced into the United States from Asia on seed. There also is evidence that several insect species are closely associated with healthy and diseased butternut and because some of these insects were shown to be contaminated with the fungus they may act as vectors of the pathogen although the exact mode of spread is unknown (Katovich and Ostry 1998, Halik and Bergdahl 2002). Birds also may come into contact with the sticky spores and spread them from diseased to healthy trees within and between forest stands.

NATURAL AND EXPERIMENTAL HOST RANGE

Butternut is the only species that is killed by this canker disease. However, other *Juglans* species and hybrids are diseased to varying degrees. Orchard and others (1982) inoculated 10- to 20-year-old trees of several *Juglans* species and found that Japanese walnut, heartnut (*J. ailanthifolia* var. *cordiformis* (Maxim.) Rehd.) and hybrids between them and butternut expressed greater resistance than either eastern black walnut or Persian walnut (*J. regia* L.) with the latter developing the most severe disease symptoms.

In Canada and the United States tree species other than butternut have been affected by butternut canker over the past several years. It was reported eastern black walnut and butternut seedlings were naturally infected by *S. clavigignenti-juglandacearum* in a nursery in Quebec (Innes 1997). Stem cankers were confirmed on a 48 cm diameter eastern black walnut in North Carolina, and branch cankers were detected on 20-year-old eastern black walnut trees in Minnesota (Ostry and others 1997). Branch cankers were also found on a 25-year-old heartnut in Iowa (Ostry 1997a). These reports indicate that this fungus may be a potential threat to walnut plantations.

Nearly the entire U.S. Persian walnut crop is produced in California (Beede and Hasey 1998). *S. clavigignenti-juglandacearum* is not known to be present in California and a quarantine on importing *Juglans* species from the eastern U.S. was put in place, but it is unknown what impact, if any, this pathogen may have on walnut cultivation should it become established there. Grafted plants of several *Juglans* species and hybrids have been artificially inoculated in the greenhouse, including several accessions from the National Clonal Germplasm Repository in Davis, California. Three of the leading cultivars grown, 'Hartley', 'Chandler', and 'Payne' (Beede and Hasey 1998) were among the most susceptible Persian walnut selections tested (Ostry and Moore, unpublished data) indicating caution should be exercised to avoid the movement of the pathogen into California. Interestingly, a "Paradox' hybrid (*J. hindsii* x *J. regia*), a hybrid commonly used as rootstocks for Persian walnut (Beede and Hasey 1998), was highly resistant.

Using artificial inoculations of greenhouse seedlings, several other hardwood species have been shown to be susceptible and may be able to harbor the fungus (Ostry 1997b). Species in *Carya*, a genus in the walnut family (*Juglandaceae*) that were demonstrated to be susceptible include pecan (*C. illinoensis* [Wangenh.] K. Koch) and shagbark hickory (*C. ovata* [Mill.] K. Koch). Although not causing large cankers, the fungus was recovered beyond the inoculation point from northern red (*Quercus rubra* L.), black (*Q. velutin*a Lam.), and white oak (*Q. alba* L.) and black cherry (*Prunus serotina* Ehrh.). Bitternut hickory (*C. cordiformis* (Wangenh.) K. Koch) also has been shown to support the growth of the pathogen in greenhouse tests (Ostry and Moore, unpublished data). These preliminary results indicate that species of genera

other than *Juglans* may serve as a reservoir of the pathogen within forest stands.

EVIDENCE OF RESISTANCE

In many areas throughout its range, healthy butternut have been found growing adjacent to trees infected and killed by the disease. Some of the trees we have monitored have remained healthy for over 12 years despite the severe disease on neighboring trees, minimizing the likelihood that disease escape is responsible for trees being symptom-free. Although these relatively rare trees may be disease resistant, we do not have experimental data as yet to demonstrate the existence of effective resistance.

Our current evidence of resistance mechanisms is circumstantial based on examining butternut over the years in search of trees that may have disease resistance. During our examinations we have detected two bark phenotypes on trees of the same size and relative age. One is a dark colored bark with deep bark fissures resembling the bark of eastern black walnut. The other is a light gray bark color with shallow bark fissures. These bark types and various intermediate types have been found on adjacent trees in many woodlots in Minnesota and Wisconsin.

Often the dark/deep bark phenotype is associated with healthy trees and the light/shallow bark with diseased trees (Ostry and others 2003). Part of our research is directed at determining if bark phenotype and disease severity are genetically based traits that may help elucidate the mechanism of host resistance and potentially be used in conservation and genetic improvement of the species.

Disease resistance screening was initiated in one of the five grafted butternut clonal archives in 2003 (Ostry and others 2003). Three trees 7-11 years old from each of 22 accessions propagated from diseased and healthy source trees and unselected 9-year-old butternut trees were wound inoculated each month from April through October with two isolates of *S. clavigignenti-juglandacearum*. The objective was to mimic natural infection in the field to compare time of inoculation and host responses of selected grafted lines of butternut with putative disease resistance to grafted clones of butternut that are known to be highly susceptible.

Although it is too early for reporting definitive results from this screening trial, indications are that infection resulted from all inoculation dates and several selected butternut lines have limited canker development compared to unselected or diseased source trees (Ostry and Moore, unpublished data). As with inoculations of plants in the greenhouse, screening trees in the field this way may allow us to separate groups of highly resistant selections from those that are highly susceptible.

The potential for plant pathogens to overcome host resistance can be high, especially with pathogens associated with long-lived trees. Agriculture is in a constant race with plant pathogens to develop and incorporate new genetic resistance into important crops as pathogens evolve and overcome them. Pathogens with a high evolutionary potential are more likely to overcome resistance compared to pathogens with a low evolutionary potential and knowledge of the genetic structure of a pathogen may be useful in predicting its future evolutionary potential (McDonald and Linde 2002). Pathogens having both a sexual and asexual reproduction system, high genotype flow, large effective population size and a high mutation rate will have the greatest potential to overcome host resistance.

Evaluating the potential for *S. clavigignenti-juglandacearum* to overcome resistance in butternut within the framework outlined above results in guarded optimism that resistance may be long-lasting. First, a sexual state of *S. clavigignenti-juglandacearum* is not known to be present, therefore recombination via outcrossing resulting in new gene combinations that could overcome resistance is not likely. DNA fingerprinting (Furnier and others 1999) revealed limited genotype diversity supporting this theory.

Second, gene flow, exchange of either alleles (genes) or individual clones (genotypes) among populations is more limited with *S. clavigignenti-juglandacearum* than many other tree pathogens because it lacks an efficient long-range airborne spore stage. However, the sticky spores may be moved considerable distances by insects or birds countering this. Another mode enabling pathogens to move beyond their natural dispersal range is through human transport of infected plants or plant parts and *S. clavigignenti-juglandacearum* can be seedborne and also moved on logs and scionwood. Thus, although lacking an efficient airborne state, *S. clavigignenti-juglandacearum* can still be dispersed long distances.

Another source of genetic variation in pathogens is mutation resulting in new strains that could overcome host resistance genes. However, these mutations are more likely to occur and be selected for in pathogens that exist in large populations in individual plants, such as with bacteria and viruses. Butternut canker is not systemic and small populations of the fungus exist within relatively

few diseased trees in any given area. Thus, the potential for a mutant strain of *S. clavigignenti-juglandacearum* to multiply, spread to a susceptible host, infect, successfully colonize and then reproduce on that host is probably not very high.

In summary, considering what we know about the genetics of *S. clavigignenti-juglandacearum*, there is realistically a low to moderate risk that it will evolve strains capable of overcoming disease resistance in butternut populations that may exist today or will be developed in the future.

BUTTERNUT CONSERVATION GENETICS

The decline in butternut populations at local, regional and national levels raises questions about whether the long-term genetic viability of the species has been compromised. Stated another way, how much genetic variability did butternut have historically, how much remains, and is there enough for butternut to fulfill its ecological functions, resist disease and adapt to environmental change (Yang and Yeh 1992)? At present, there are few answers.

Available data indicate that butternut has considerably lower genetic diversity (as measured by percent polymorphic loci and number of alleles per locus) than similar species based on allozyme and RFLP marker systems (Morin and others 2000, Fjellstrom and Parfitt 1994). Morin and others (2000) reported that less than 20% of the loci they evaluated in butternut were polymorphic, with 1.3 alleles per allozyme locus or fewer. By comparison, eastern black walnut had 42 – 88% polymorphic loci and about 2.9 alleles per locus. The preliminary findings of Morin and others (2000) indicate that butternut may be slightly more genetically diverse in the US than in Canada, but they were unable to determine the cause or causes of the lower-than expected genetic diversity of butternut.

Microsatellite DNA polymorphisms (SSRs) are rapidly becoming the marker system of choice for population genetic studies, and several of the SSRs originally identified in black walnut (Woeste and others 2002) are also polymorphic in butternut (Woeste, unpublished data). Nuclear SSRs used in tandem with chloroplast markers can potentially be used to confirm if butternut has been through a genetic bottleneck and to predict whether the bottleneck was recent or ancient. SSRs are also an excellent tool for evaluating regional and local genetic diversity of butternut. Comparative studies of allele sizes of SSRs that can be amplified in butternut, eastern black walnut, and Japanese walnut might also be useful for identifying hybrids.

As previously mentioned, many of the apparently canker resistant butternut trees we have examined are characterized by deeply fissured, darkly colored bark. This phenotype, not typically associated with butternut, is similar to the bark of black walnut. The origins of this dark-barked phenotype are unknown. It is possible that dark-barked trees are an ecotype of butternut that was previously unnoticed, either because it was rare or because dark-barked butternuts were mistaken for walnuts by casual observers. Because of the phenotypic similarity between dark-barked butternuts and black walnut, we investigated whether the ITS region of some of the dark-barked trees indicated hybrid origins. Published literature was clear that the (*J. nigra* x *J. cinerea*) hybrid was not possible (Funk 1970), but unsubstantiated claims of the existence of such hybrids infrequently arise. For example, trees catalogued as *J. nigra* x *J. cinerea* hybrids were maintained at the Tree Improvement Center (TIC) of the Carbondale work unit of the North Central Research Station, and seeds of putative *J. nigra* x *J. cinerea* have been sold by nurseries. The trees in the TIC were grown from seeds provided by Michigan State University researchers in the late 1950s. We have concluded from preliminary analyses of the dark-barked butternuts and putative *J. nigra* x *J. cinerea* hybrids that some of the dark-barked butternuts are true butternuts (non-hybrids) and that at least one of the many putative *J. nigra* x *J. cinerea* hybrids at the TIC may in fact be such a hybrid.

Several unknowns confound these results, but first among them is that we do not know if we are able to accurately differentiate among *J. cinerea* x *J. ailanthifolia*, *J. nigra* x *J. cinerea* and all possible three-species hybrids such as (*J. nigr*a x *J. ailanthifolia*) x *J. cinerea*, in part because there are not as yet any positive controls for the experiment. Furthermore, it is not known if hybridization that may have occurred two or three generations ago can be detected accurately using internal transcribed spacer (ITS) sequences, although the phenotypic impact of such hybridization may still be present. In other words, dark-barked butternuts with greater canker resistance may be butternuts; they may be the product of a rare, natural hybridization between eastern black walnut and butternut that occurred a few generations ago; or they may be something else. Simple analysis of ITS regions only may not be able to demonstrate or rule out any of the possibilities. Butternut may have hybridized with eastern black walnut in the recent past, the more distant past, or both. Detailed analysis of the chloroplast sequences of both species and the putative hybrids may provide some insight into this question. Since chloroplasts are strictly maternally inherited in *Juglans*, the presence in dark-barked trees of eastern black walnut chloroplast sequences would

indicate a hybrid between eastern black walnut and butternut in which butternut was the male.

It should be noted that hybridization between butternut and eastern black walnut seems ecologically unlikely since interspecific hybridization usually happens in the contact zones of spatially separated species, and the ranges of eastern black walnut and butternut overlap across almost the entire northeast quarter of the US (Funk 1970).

Butternut hybridizes with Persian walnut to produce *J.* x *quadrangualata* (Carr.) Rehd., with Japanese walnut to produce *J.* x *bixbyi* Rehd and with heartnut to produce "buartnut". The striking vigor of the F1 hybrid between butternut and Japanese walnut is one phenotype that can be used to distinguish these hybrids, also called buarts or butterjaps, from butternuts. Field observations indicate that buarts are more common in old, abandoned farmyards, on pasture edges, and in the yards of houses in small, rural towns. The leaves of buarts may be greener and more persistent than those of butternut, not abscising until well into October; whereas butternut leaves typically turn yellow and abscise in early to mid-September. There are reports that butternut also hybridizes with little walnut (*J. microcarpa* Berland) and Manchurian walnut (*J. mandshurica* Maxim.).

To identify and characterize hybrids it will be essential to develop markers that clearly differentiate among *Juglans* species, specifically eastern black walnut, butternut, and Japanese walnut. A full study of the variability of the nuclear, chloroplast and mitochondrial genomes of all three species will probably be necessary before any marker system can be used to detect and identify the parents of hybrids with a high level of confidence. One method for finding species specific DNA sequence signatures is the analysis of DNA sequences from highly conserved genes. It may be possible to identify species-specific polymorphisms in the introns or nearby non-coding regions from six or seven genes, and these could be used to detect backcrosses or even three-species hybrids. Unfortunately, there is almost no DNA sequence data available in public databases for butternut and Japanese walnut, and very little for eastern black walnut. All methods for evaluating species diversity and hybridity have drawbacks and blind spots, and as such a combination of morphological and molecular techniques seems likely to produce the most reliable results.

At present, the best tool for discriminating *Juglans* species and their hybrids is the sequence polymorphism found within the ITS regions of the nuclear ribosomal DNA. The ITS region is present in most genomes as thousands of copies of tandem repeats at one or many loci (Baldwin and others 1995). Because the sequence undergoes rapid, concerted evolution leading to a high level of intergenic uniformity, the ITS region has proven generally useful in studies of angiosperm taxonomy (Baldwin and others 1995) and it has been used specifically to decipher the species identities and hybrid origins of *Juglans* rootstocks used in the California walnut nursery industry (Potter and others 2002). We have identified primers that amplify the ITS region of eastern black walnut, butternut, and Japanese walnut. By digesting the PCR products with restriction enzymes, DNA fragments of diagnostic sizes are produced for each species. This marker system is imperfectly co-dominant because there can be within genome length variation in the ITS (Sytsma and Schaal 1990 and our preliminary results from eastern black walnut), because the ITS regions of hybrids may homogenize to a single sequence at different rates, and because there may not be sufficient polymorphism within the ITS to determine which species among several possible are represented in a hybrid and at what percentage.

Taxonomists and dendrologists have traditionally used morphology to distinguish species and their hybrids. This approach is complicated in the genus *Juglans* because there are few readily discernable traits that distinguish the species. A careful evaluation of trichomes or other more subtle features may yet prove valuable in the identification of *Juglans* species and first generation hybrids. One potential problem with any approach to distinguishing hybrids is that Japanese walnut has been widely propagated in the US for over 150 years (Crane and Reed 1937). Some features that we now associate with butternut may have been introduced by gene flow from Japanese walnut to butternut a generation ago. Similarly, Persian walnut, a species that can hybridize with eastern black walnut, butternut, and Japanese walnut, has been propagated in the US since colonial times. In areas where butternut populations have undergone a severe decline, one may be justifiably skeptical concerning the identification of the remaining 'butternuts', especially if these trees express some morphological or genetic variability that is uncommon elsewhere. Do remnant trees represent the best opportunity to capture rare local diversity, or are they Trojan horses carrying genes, including perhaps genes for resistance to butternut canker, from other species? The study of herbarium sheets of butternut collected before 1860 may be one way to evaluate the morphological diversity of butternut before there was any potential impact by hybridization with other *Juglans* species.

CONCLUSIONS

Since the detection of butternut canker in 1967 researchers have clarified several aspects of the disease, including the description of the causal agent, its biology, a partial host range, and they have documented limited examples of potentially disease resistant trees. However, many gaps remain in our knowledge including the origin of the pathogen, the level of genetic diversity in butternut across its range, and silvicultural techniques to retain butternut in our forests and to restore the species where it has been eliminated by the disease.

Butternut canker and its spread raises fundamental issues with respect to the productivity and health of the central hardwoods landscape. Exotics invade native landscapes on several levels: physically, they occupy the space where endemics once grew, modifying the environment there and often making it less hospitable to native flora and fauna; they invade by introducing pests and they invade at the genetic level by hybridizing with endemic flora or other introduced species. The genetic invasion is often unseen and difficult to monitor unless (or until) the hybrids themselves become invasive weeds. More sensitive genetic and phenotypic marker systems are needed to monitor the genetic invasion of the exotics such as Japanese walnut into the central hardwoods region. Butternut has been the most affected by Japanese walnut. But over the long-term the possibility exists that black walnut could also be adversely affected by exotic invasion at the genetic level.

Butternut is rapidly being lost in our forests from a variety of causes in addition to butternut canker. Genetic diversity in species such as butternut is needed for its long-term survival, future adaptation and evolution. There is an urgent need to conserve genetic diversity among butternut populations before valuable populations are lost.

ACKNOWLEDGMENTS

We thank the many individuals in various state and federal agencies and the numerous private landowners who continue to support our research efforts. We especially thank M. Moore and Bo Li for technical support in the conduct of laboratory, greenhouse and field research.

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