# Investigations of *Ophiognomonia clavigignenti-juglandacearum*: Inhibition by Butternut Bark Extracts and Viability of Conidia

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ברוך אתה יהוה אלחינו מלך העולם שהחינו וקימנו והגיענו לזמן הזה

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# Chapter 1. General Introduction and Literature Review

## 1.1. A Cultural History of Butternut

The butternut tree (*Juglans cinerea*) has a long and noteworthy past. Long before any Europeans reached the shores, the indigenous peoples of eastern North America valued the butternut tree in many ways. It was among the many species that they would cultivate and harvest for food, and like many nuts it was especially suitable for winter storage. However, it was the tree's numerous other properties that brought the most recognition in cultural records (55). The oil from the seeds was used as a hair dressing and as fuel for lamps. The hulls and bark were used for a brown or black dye. The hulls or bark, when placed in a small pond or stream could stun fish for an easy catch. The list of native medicinal uses for butternut bark was long, including a cathartic, toothache remedy, de-wormer, pain reliever, wound treatment and general tonic (9).

The very earliest European explorers of North America also found the butternut useful, and it thus became an important archaeological clue in the analysis of a historical site. The oldest and the only widely accepted known site of Norse presence in North America is L'Anse aux Meadows, on the northern tip of modern Newfoundland (4). There Norse explorers built a small settlement in the 11$^{th}$ century, around the time of the famed Leif Ericson. In the course of excavations of this site, archeologists found the remains of butternut shells and carved butternut wood. There is no evidence of butternuts ever growing in Newfoundland, so researchers have surmised that these Norse explorers used L'Anse aux Meadows as a winter camp or base camp and brought these nuts back from excursions further south into New Brunswick or beyond as a winter food supply. Clearly they found in the butternut tree something worth keeping: a source of both food and wood.

When European colonists arrived permanently in the New World, they found a vast country filled with a rich abundance of unique plants and trees. Nut trees were especially common, such as walnuts, hickories, chestnuts, and oaks. Not only did these trees produce wood for building and tools, but also provided food, medicines, dyes, and chemical products. The vast unknown botanical treasures of North America intrigued

Pehr Kalm, a student of Carl Linnaeus, and he was sent to the Colonies in 1748 to study, sample and describe plants useful for agriculture and industry (49). He lived and travelled in North America for three years and is credited for cataloging sixty new species. One of the nut trees that intrigued him was the butternut. He first named it *Juglans alba*, white walnut, so called because its wood was much lighter than black walnut. Kalm's description was that the "white walnut" was quite common in North America and Canada, and it preferred to grow on gradually sloping banks of rivers and brooks and hillsides, often where oaks were common. He noted the abundant large, oily nuts that were used for food and oil, and the many uses of its wood and bark. The butternut tree was later named *Juglans cinerea* because of its ashy grey bark (3).

Through the years the butternut kept an enduring history of usefulness in eastern North America. Pioneering Americans used its sap to make syrup much like that of the maple tree (30) and probably learned from the Native Americans how to use it for dye and medicines. The brown-colored dye was so common that rural Confederate soldiers (who could not obtain the official Richmond grey uniform) used it for their uniforms, giving them the moniker "butternuts" (45). It was so prized that wagon trains carried seed westward and homesteaders planted butternuts in new locations such as Oregon and Washington. One of the first settlers of the Olympic Peninsula, George Bush, planted a butternut tree on his homestead in the 1840's which still survives to this day (2). In fact, one of its progeny has been planted on the lawn of the State Capital of Washington as a memorial tree.

Continuing into more recent times, the wood of butternut has been highly prized for woodcarving, paneling and furniture making, having a beautiful grain that resembles black walnut when stained but it is softer to work with. For example, the library of Grey Towers National Historical Site, home of the first chief of the Forest Service Gifford Pinchot, is paneled entirely with butternut (99). The nuts are noted for their sweet, rich nutmeats, and a traditional confection in the Northeast is a candy made of maple sugar and butternuts. Unfortunately, the nut crops are inconsistent and cultivars with high quality nuts are difficult to find (31). The trees are difficult to propagate and train into a

plantation-like setting that would be necessary for commercial production. Still, it is highly valued as a yard tree or in small woodlots.

Interest in butternut has also come from ethnopharmacology, the study of medicinal products used by indigenous communities. Native peoples' use of butternut as a medicine has been validated by recent studies showing high levels of antifungal and antibacterial properties in butternut bark (62, 26). The enduring informal use of butternut bark for medicinal purposes is illustrated by the point that butternut bark is available for sale on herbal websites. Thus the ancient uses are coming full circle to the present.

### 1.2. Ecological History and Silvics

Fossil nuts very similar to *J. cinerea* have been found in the Beaufort Formation in Arctic Canada (37). They have been classified as *Juglans eocinerea* and are a probable ancestor of our current species. Evidence suggests this *Juglans* species developed in the North during the Miocene and then moved south during the Ice Ages. Analysis of pollen in sediment cores corresponding to the Late Quaternary period in Tennessee shows that butternut was among the earliest of the deciduous trees to increase in importance after the glaciers receded, along with ash, ironwood, hickory, birch, willow and elm (23).

In early land surveys, butternut was occasionally recorded in bearing-tree data. Since butternut is generally not a large tree, it would not frequently be chosen for these records. Still, its presence in these records helps in a small part to piece together the ecological history of the tree. For example, butternut was a component of bottomland hardwoods in southern Indiana bearing trees (82). Butternut was also a component of the "Big Woods" forest type in south-central Minnesota, as opposed to the fire-tolerant oaks and aspens that bordered the prairie (32).

The known native range of butternut in North America is from southeastern New Brunswick south to northwestern South Carolina, west to northeastern Arkansas, with a western limit of central Missouri and Iowa. Its northern limit reaches from central Minnesota through southern Ontario and southern Quebec (Figure 1.1)(85). It is the most cold-hardy of the *Juglans* species. It is a minor component of common mixed hardwood types and is a short-lived tree, rarely living more than 75 years. In the past, West

Virginia, Wisconsin, Indiana and Tennessee have been the leading producers of butternut timber.

The butternut has an important ecological niche. Its rapid growth in sunny, open locations favors its establishment in old fields, road cuts and fence rows (99). It is often found in sloping riparian areas and may stabilize the soil in erosion-prone sites. The nuts provide food for a number of animals, and are especially favored by squirrels. In the early autumn during nut fall, the squirrels often compete with humans to gather the crop first, and can develop large caches of the nuts. In fact, it is probably squirrels that deserve the most credit for moving seed and regenerating butternut in forest openings and old fields. Deer find the tree especially useful for an antler rub (9). Apparently the bark texture and/or hemostatic compounds in the bark are helpful during antler rubbing and shedding seasons. In locations where deer are abundant, antler rub can be a serious threat to the health of young trees, creating large wounds on the bark and broken branches (Moore, personal observations).

Butternut is monoecious. Flowers of each sex usually do not mature at the same time, providing for cross-pollination in most cases. Fruit is oblong to oval, averaging about 5 cm long, in clusters of two to five, maturing in September and October. Nut crops are irregular, with good crops of nuts generally every two to three years, and must have a period of cold stratification in order to germinate (85).

Young butternuts are limited by weed and brush competition, and must have direct sunlight (71). Butternut is very shade-intolerant, and regeneration in a mature forest is rare. Most young trees are found near forest edges, disturbed areas and old pastures. Recent studies of regeneration conditions have shown quite limited success in both direct planting and creating openings with reserve trees (76). Lack of natural regeneration is becoming an increasing concern, and more study is needed to develop consistent regeneration methods.

### 1.3. Butternut Canker and Other Threats

Although Pehr Kalm listed the “white walnut” as “quite common”, it is now becoming increasingly rare. It is susceptible to stresses and diebacks due to the fungus

*Melanconis juglandis* and other opportunistic fungi. It is also susceptible to the bunch disease phytoplasma or "witch's broom" (87). The butternut curculio (*Conotrachelus juglandis*) and eriophyid mites (43) can severely weaken infested trees. Adding these problems to the aforementioned regeneration issues reveals some of the reasons why the tree generally is not very abundant.

By far the most dramatic reason for the decline in butternut tree numbers is the presence of a lethal disease, butternut canker, caused by the fungus *Ophiognomonia clavigignenti-juglandacearum* (*Oc-j*), formerly known as *Sirococcus clavigignenti-juglandacearum* (*Sc-j*). It was first noticed in 1967 in Wisconsin, where unusual bark cankers were observed on butternut trees (84). Further investigation revealed that this was a new disease caused by a previously undescribed fungus (58). In 1977 cankered butternuts were found in fourteen out of sixteen states surveyed (1). At that time, North and South Carolina reported that the disease had virtually eliminated butternut trees in the areas surveyed, and Wisconsin reported widespread mortality.

Recent USDA Forest Service Forest Inventory and Analysis data reveals the alarming trend in the decline of butternut. In 2008, data revealed a decrease in the number of butternuts averaging 23% in seven Midwest states across all size classes from the previous survey period (usually five years)(96). In 2012, the trend continued with another average 28% decrease over five years for the same seven states. The greatest decline listed was in Iowa with 570,000 live butternut trees in 2007 to 46,000 in 2012, a decrease of 92%. Other states with large changes over the same five years included Michigan and Missouri, with decreases of 73% and 52%, respectively.

Even though the butternut has not been a large percentage of the forest mix, it nonetheless has an important role. Now that butternut canker is decimating the few that are there, there is concern for loss of a species. The canker fungus has been found nearly everywhere butternuts grow in their native range, with the exception of isolated yard trees and Western states where occasional butternuts are planted outside their natural range. From the standpoint of biodiversity, the loss of this species in the landscape could have serious ramifications. In response to a call for more information on butternut in general,

an extensive study has been done on butternut literature and a bibliography published (74).

### 1.4. Disease Etiology and Epidemiology

Butternut canker development usually begins on twigs and small branches in the crown, when the fungus invades the bark tissue through wounds and leaf scars (94). Evidence of new cankers is seen as sunken, darkened, elliptical areas on the bark, often with a black center and a sooty gray margin. Peeling the bark reveals dark brown to black areas of killed cambium and wood with distinct margins. Older cankers often have callus tissue at the margins, shredded or fissured bark, and hyphal pegs of the fungus. Abundant conidia are produced in stroma, which spread throughout the crown via rain splash. Multiple cankers, both annual and perennial, develop and coalesce on large branches, main stems and root flares, eventually girdling the tree (72).

The spores are disseminated over short distances to other trees via rain splash (92), but the nature of its long-term dispersal is still unclear. Airborne conidia can be dispersed up to about 40 meters during rain events (92). Viability of airborne conidia has been studied. While such conidia can survive over 32 hours under ideal laboratory conditions, their viability is much reduced (over 8 hours) in field experiments (93).

The possibility that an insect or other animal transmits the pathogen over longer distances has been explored (34, 46). A number of insects have been found capable of disseminating *Oc-j* conidia, and three beetle species were found capable of carrying viable conidia for up to 16 days (90). These potential vectors may explain some of the observed long-distance dispersal, but more studies are needed to explore the conditions and timing in which such an event happens.

Butternut canker predominantly affects butternut, but naturally-produced cankers have been found on other *Juglans* species, namely black walnut (*Juglans nigra)*(73) and Japanese walnut (*Juglans ailantifolia*)(64). Artificial inoculations have found that *Oc-j* is able to colonize and survive in the wood of several genera, including other *Juglans* spp., *Carya, Corylus, Prunus,* and *Castenea* (69). This is evidence that the fungus may be

surviving on other plant material in a non-pathogenic state as a reservoir when butternuts are not present.

### 1.5. Fungal Taxonomy and Genetics

The butternut canker fungus was first described and characterized in 1979 as *Sirococcus clavigignenti-juglandacearum* (*Sc-j*) (58). It is an Ascomycete of the Diaporthales, family Gnomoniaceae. No teleomorph stage has been found for the fungus, making it difficult to classify on morphological characteristics alone. In culture and on its host it produces numerous pycnidia, from which extrude large masses of spindle-shaped two-celled hyaline conidia in pink-to-tan matrix. It was originally classified as *Sirococcus* because of the structural similarity of its pycnidia, conidiophores, and conidia to *Sirococcus strobilinus*. *S. strobilinus* Preuss, now named *Sirococcus conigenus* (DC.) Cannon and Minter, is a shoot blight pathogen of conifers. Phylogenetic analysis of *Sirococcus* species via DNA sequences showed only a limited relationship between *Sc-j* and *S. conigenus* (48). More detailed studies have shown that the butternut canker fungus aligns more closely with *Ophiognomonia* (88). An extensive study was done with a number of isolates of *Sc-j* and related genera using gene regions coding for β-tubulin, actin, calmodulin, ITS 1 and 2, and tef1-α (13). The researchers found *Sc-j* clearly grouping in a clade with other *Ophiognomonia* species and proposed a name change. The butternut canker fungus is now referred to as *Ophiognomonia clavigignenti-juglandacearum* (*Oc-j*).

This new classification places *Oc-j* genetically close to another *Juglans* pathogen, *Ophiognomonia leptostyla*, the walnut anthracnose fungus, and other common anthracnose and leaf-inhabiting pathogens. Although the majority of damage done by *Oc-j* is from bark cankers, the pathogen has frequently been found on leaves and creates lesions on butternut leaves nearly indistinguishable visually from those created by *O. leptostyla* (Moore, personal observations). The pathogen has also been isolated from overwintered butternut leaves (Moore, unpublished data). This opens up new questions as to inoculum reservoirs and transmission routes of the *Oc-j* pathogen. It could easily survive and be carried some distance on wind-blown fallen leaves.

Like chestnut blight and Dutch elm disease, this fungus shows a high probability of being an introduced pathogen, probably from Asia (14). First, it was never noted until the 1960's, despite decades of forest disease surveys in many areas where butternuts grow. Secondly, it has spread quickly, with little sign of innate or co-evolved resistance from the native butternut population. Thirdly, walnuts native to Asia, namely *Juglans ailantifolia*, are relatively resistant to the disease and they were frequently imported and planted in the US in the late 1800's. They could easily have been a reservoir for latent infections, and spread the pathogen to native butternuts growing in close proximity. In an early genetic study of the fungus (28), randomly amplified polymorphic DNA (RAPD) markers were used to examine *Oc-j* isolates from throughout the range of butternut, with the fragments showing no variation between isolates. This evidence points to most likely clonal (asexual) reproduction and recent introduction. A more recent and detailed study using single nucleotide polymorphisms (SNP's) revealed at least three different genetic groupings of the fungus, pointing to probable multiple introduction events (14). One other piece of evidence pointing to an Asian origin of the fungus is in a recent report that cultured *Oc-j* growing as an endophyte on maple leaves in China (91). This is the only time this fungus has been reported from outside North America.

### 1.6. Regulatory Status and Current Listings

Increasing mortality of butternut trees has caused governmental agencies to take notice. In the US it is listed as a species of special concern in the National Forest system. In Canada it is considered an endangered species. Various states in the US have taken measures to protect the tree. For example, the Minnesota Department of Natural Resources has placed a moratorium on the harvest of healthy butternuts on state land, and has recently moved the species' status from "special concern" to "endangered" (5).

In the early days of the disease epidemic, some forest mangers started cutting all marketable butternut in order to preserve some value from the wood. In order to give some guidance into the matter, a retention guide was published for forest managers and decision makers covering when to cut down a butternut tree and when to retain it (71).

Results of inoculation tests have shown that commercial Persian walnuts (*Juglans regia*) are highly susceptible (69). Persian walnuts are the common walnuts found in grocery stores, and the majority of the crop is grown in California, where it is of high economic value. Concern over the spread of both bunch disease and butternut canker has led to regulatory action including quarantine measures, and no *Juglans* material can be moved from the Eastern US into California (16). If the *Oc-j* fungus were to spread to the commercial groves, there could be serious economic consequences.

### 1.7. Resistance, Selection and Restoration

On a more hopeful track, beginning in the 1990's, healthy butternut trees were being found despite growing in close vicinity to diseased trees. Numerous surveys by forest agencies as well as contacts from private individuals recorded a number of putatively resistant trees throughout the range. There appeared to be hope of finding resistance to butternut canker within the natural population (65). Researchers at the US Forest Service developed a plan to preserve these trees. One major problem was the manner of preservation. Seed crops of butternut are unpredictable, and in forest settings, difficult to collect before scavenging rodents get them. They do not survive long-term storage, with a maximum time reported as four years (12). Also open-pollinated progeny are variable and never genetically identical to the mother tree. Because outcrossing occurs naturally with butternut, it is nearly impossible to control the male parent. Clearly vegetative propagation was the necessary method for propagation of promising material, namely grafting, since there were no reports of successfully rooted cuttings from a mature tree.

Starting in 1990, research scientists with the NCRS, US Forest Service, began collecting scion wood from healthy trees located throughout the northeastern US. They were then grafted onto black walnut seedlings via techniques commonly used for *Juglans* grafting. This was done in a greenhouse and the grafted trees were outplanted for preservation and study. The first outplantings were made at the University of Minnesota experimental station (UMore Park) at Rosemount, MN by US Forest Service researchers (NCRS and now Northern Research Station). A number of other locations and agencies

that have had active past or present grafting and planting programs include the Oconto River Seed Orchard on the Chequamegon-Nicolet National Forest in WI; the Hardwood Tree Improvement and Regeneration Center (HTIRC) at Purdue University, IN; and several Ministry of Natural Resources locations in Ontario, Canada. At some locations researchers have also planted and evaluated seed sources. These clonal archives preserve genetic material, but also provide a location for inoculation and disease resistance studies, and provide scion wood and seeds for propagation and breeding purposes.

A number of these trees have been used for artificial inoculation studies, and some selected lines have been shown to be more resistant than unselected lines, both in number and size of cankers that developed (70). Continuing studies using half-sib progeny from these and other trees done at the HTIRC have yielded variable results, but are still showing some differentiation between resistant and susceptible lines. More time is needed to get a clearer evaluation of disease progression over several years (54). Trees in the original planting at Rosemount are currently developing natural cankers, and over a number of years it will be clear if the selected trees survive the disease.

During the course of surveys looking for resistant trees, an unusual phenotype was discovered among many of the healthier trees. A large proportion of the healthier, putative resistant butternuts had a distinctive bark, darker than the typical light grey, with deeper furrows. At first glance, it was easy to mistake them for black walnut trees. A survey of a woodlot where this phenomenon was common revealed a correlation between tree health and bark characteristics (75). Preliminary molecular studies have been done with leaf samples of this population and studies are in progress to discover if there is a genetic basis between bark type and disease resistance (K. Woeste, personal comm.). If a clear phenotype can be correlated with disease resistance, the process of selecting resistant trees will be much more efficient.

Once resistance has been clearly established, propagation of selected lines will be important in order to increase numbers for outplantings. The grafting process, although successful, is labor intensive and also results in a butternut tree growing on black walnut rootstock (56). Rooted cuttings are a possible alternative. A study has shown that it is possible to produce roots from cuttings from young butternut trees that have been

severely pruned (three years' growth removed), cuttings taken from the re-growth, dipped in rooting hormone, and placed in the mist chamber (81). These rooted cuttings were outplanted and showed good survival and growth (80).

Another helpful tool would be the long-term storage of selected germplasm, similar to what is being done for seed banks of crop plants. A cryopreservation method was developed for long-term storage of scion wood, similar to what is used for long-term preservation of apple cultivars (24). Butternut scion cuttings survived desiccation and liquid nitrogen treatments and were able to be grafted successfully, though at low percentages. This allows the potential of preserving elite germplasm long-term for future grafting needs.

### 1.8. The Hybrid Dilemma

*Juglans cinerea* is a member of the walnut family (Juglandaceae), which includes hickories and pecans (*Carya* spp.), eastern black walnut (*Juglans nigra*), Persian walnut (*J. regia*), and Japanese walnut (*J. ailantifolia*), among others. Butternut can hybridize with Persian and Japanese walnuts but not with black walnuts (83). In the late 1800's a form of Japanese walnut called the heartnut (*J. ailantifolia* var. *cordiformis*) was imported to the US and became a popular yard and plantation tree because its hardiness and unique easy-to-crack shell. As time went on there were "heartnut" trees that produced butternut-shaped nuts instead of heart-shaped nuts. It was discovered that these heartnuts had hybridized with the native butternuts (11). The resulting trees were named "buarts", and were often so similar to butternuts that it was difficult to distinguish the parentage of a given tree. Recent studies have matched phenotypic traits to genetic markers and have found large groups of trees formerly believed to be natural butternuts but with hybrid parentage (39).

Both heartnuts and buarts have been observed to be relatively resistant to the butternut canker disease. This has been confirmed by inoculation tests, where both heartnut and buart were found moderately resistant (63). Histological studies showed that heartnut has a thicker periderm and greater phenolic production than butternut (57), which may explain its greater resistance. In the forest, hybrids tend to be faster growing

and larger than native butternuts, which alone can be an advantage against disease. They tend to have larger and more frequent crops of nuts, and can sometimes self-pollinate (Moore, personal observations), which also may help to explain their prevalence in certain areas. Often butternut seedlings sold in nurseries are actually hybrids, since the seeds are nearly indistinguishable from pure butternut. In the search for potential resistant material for propagation, hybrids are often selected for their vigor and apparent resistance, and are difficult to distinguish with the untrained eye from pure butternuts. Studies have now delimited specific phenotypic differences (25) and molecular differences (101) so now there are definitive means by which hybrids can be identified. These tools have been used in field studies to map the distribution of pure butternut versus hybrids (79, 100).

The existence of these hybrids is a mixed blessing. On one hand, hybrids are surviving in areas where most pure butternuts have been eliminated by the canker disease. They could be used in the future for breeding purposes much like what has been done for the American chestnut, by crossing it with a chestnut blight-resistant Chinese chestnut. If canker disease resistance is not clearly found in butternut germplasm, this may be one means of keeping the tree viable in some form. On the other hand, hybridization represents dilution and loss of a distinctive species, with unknown ecological consequences (99). Therefore, any plan that includes use of hybrids for future restoration should be approached with caution.

### 1.9. Thesis Objective One: Inhibition of *Ophiognomonia clavigignenti-juglandacearum* by *Juglans* species bark extracts

An important question that needs to be explored is the nature of butternut canker resistance, if and when found. Disease resistance is a complex phenomenon, especially in trees, and one rarely has the genetic resources available to target resistance genes as in traditional agricultural crops. For long-lived plants like trees, breeding cycles are extremely long and conventional crossing and backcrossing usually takes too long for most practical research. In all practicality one must observe phenotypic traits that may

confer an increased level of partial resistance and look for incremental improvements, such as increased callusing or reduction in size or number of cankers.

Up to the present, butternut canker resistance work has involved a long and laborious process of finding putative resistant trees, grafting, waiting till they grow to sufficient size, then challenging them with artificial inoculations of the fungus or exposing them to natural disease pressure (70). One whole research cycle could take ten years or more. It would be useful to have a quicker screening method by which one could tell if a candidate tree was likely to be resistant. Preliminary studies of this thesis research have shown that there may be a chemical basis for resistance to butternut canker (67). Chapter Two of this document reports investigations of butternut bark extracts that may lead to a rapid screening method. Bark from selected young butternut branches and other *Juglans* species was peeled, dried, and crude filtrates extracted with ethanol. The hypothesis was that butternut bark contains significant levels of naphthoquinones, and that these and/or other substances have an inhibitory effect on the germination of *Oc-j* conidia that is distinguishable by selection, thereby explaining observed differences in canker resistance. The overall goal was to develop a rapid screening method to aid in the process of selection for disease resistant butternut trees.

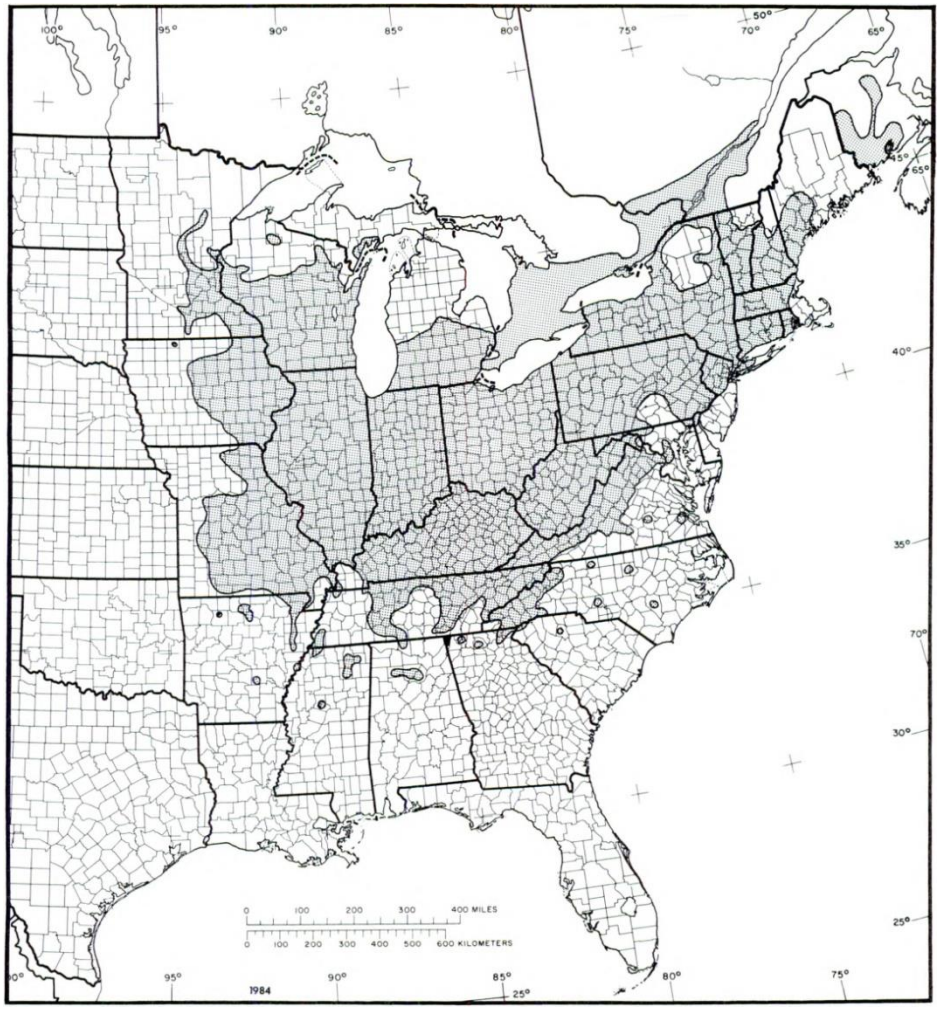
### 1.10. Thesis Objective Two: Influence of temperature and humidity on the viability of *Ophiognomonia clavigignenti-juglandacearum* conidia

Parts of the life cycle of the *Oc-j* fungus have been studied by various researchers, but a number of questions remain. One question is the means of *Oc-j* survival during long distance dissemination. The fungus has no known long-lived stage that is resistant to desiccation or temperature extremes, and no known sexual state that could overwinter on a plant part. The only known infective agents are conidia produced in pycnidia within stroma under the bark. Butternuts often grow in isolated pockets, with miles between them, yet the fungus is present wherever they grow. Rain-splash and air movement is an obvious and proven means of short-range dispersal (95), but it does not explain dispersal in terms of longer distances. Inoculum can be carried on infected seed (41), but that

would only explain spread via human or rodent dispersal. An insect vector would be the most obvious dispersal agent, and several butternut-inhabiting beetles have been found to carry *Oc-j* spores, implicating them as potential vectors (46, 34). One study has shown *Oc-j* spores to survive on a beetle exoskeleton for up to 16 days (90).

Still, the conditions needed for long-term survival of *Oc-j* conidia have not been explored, and Chapter Three presents studies done to explore the effect of temperature and humidity on the viability of *Oc-j* conidia. *Oc-j* conidia from two-to four-week-old cultures were first used to find the germination at a range of temperatures. Then conidia were placed on nylon membranes, allowed to dry, and held at a range of temperatures and humidities to test for viability. The hypothesis was that *Oc-j* conidia could survive for a considerable time dried on a surface, and the length of time would be affected by temperature and humidity. The overall goal was to gather information useful in ascertaining probable means and conditions of long distance conidial dispersal on insects or plant material.

**Figure 1.1.** Native range of butternut (*Juglans cinerea*) in North America. Source: Rink, 1990.



## Chapter 2. Inhibition of *Ophiognomonia clavigignenti-juglandacearum* by *Juglans* species bark extracts

A rapid and reliable disease resistance screening technique is needed for detecting *Juglans cinerea* (butternut) with resistance to butternut canker. We investigated the potential of a bark extract bioassay to detect levels of resistance to *Ophiognomonia clavigignenti-juglandacearum* (*Oc-j*), the causal agent of butternut canker. Both reagent grade naphthoquinones and crude bark extracts of *Juglans* species inhibited germination of *Oc-j* conidia. The *in vitro* bioassay, in which 2 mg of each extract was placed on a filter disc on a petri dish seeded with *Oc-j* conidia, revealed that the level of germination inhibition varied between extracts depending on the selections of butternut or species of *Juglans* tested. Over a three year period, groups of butternut accessions selected for disease resistance could be distinguished from unselected trees, depending on the month of bark collection. The levels of inhibition of conidia germination roughly correlated to the level of resistance observed in field inoculations of the trees. The naphthoquinone compounds juglone and plumbagin were found in butternut bark using ultra-performance liquid chromatography. Concentrations of these two compounds varied by month and by accession, and juglone levels correlated with the bark extract bioassay in some months. Juglone may account in part for the observed range of inhibition levels in the assay and variation in canker resistance among selections of butternut exhibited in the field. This assay may have potential use for selecting butternut with resistance for conservation and restoration purposes.

### 2.1. Introduction

There is concern over the rapid loss of the butternut tree (*Juglans cinerea*) throughout North America to butternut canker caused by *Ophiognomonia clavigignenti-juglandacearum* (*Oc-j*) (13) since the disease was first reported in 1967. Investigators in the United States and Canada have examined the potential conservation of individual trees that may have resistance to the disease (86). Occasionally one to several healthy butternut trees have been found growing among groups of similarly aged diseased and

dying butternut. These trees may have resistance to the disease, and a number of these selections have been grafted and grown in research plantings and could have value for breeding and restoration of the species. The hope is that these surviving trees, assumed to be under the same disease pressure as their neighbors, have some genetic factor that provides resistance. Likely there were some in those selections that were only "escapes", in other words, not genetically different from their neighbors but not as fully exposed to the pathogen, or lacking conditions suitable for infection. However, one would expect that within the grafted selections many would have a greater degree of resistance than the general population.

Differences in susceptibility to *Oc-j* among *Juglans* species and selected butternut have been demonstrated using artificial wound inoculation tests in a plantation (63). Heartnut (*Juglans ailantifolia* var. *cordiformis*) and black walnut (*J. nigra*) were among the least susceptible, and Persian walnut (*Juglans regia*) was the most susceptible among the species tested. Inoculations of putative resistant butternuts revealed significant differences between accession, month of inoculation and fungal isolate (70). Resistance mechanisms among different *Juglans* species have been only minimally explored. It has widely been observed that butternut x heartnut hybrids, often referred to as "buarts", are more resistant to the canker disease than pure butternuts (39). It is hypothesized that the thicker periderm of heartnut provides it resistance against the fungus, and the high phenolic content of black walnut bark confers disease resistance to that species (57).

The capability of plants to produce chemical substances involved in resistance to pathogens has been extensively studied. Phenolics such as salicylic acid are well known as signal molecules for both the hypersensitive response and systemic acquired resistance (47). Disease resistance may be correlated with a higher level of these and other substances, and chemical assays for detecting disease resistance have been developed. Baiocchi and others (6) found varying levels of phenolics among poplars displaying different levels of resistance to *Discosporium populeum*. Bucciarelli and others (15) found that aspen phenotypes resistant to *Entoleuca mammata* produced wound callus rich in phenolics that was absent in the susceptible phenotypes. Reservatrol production has been investigated as a possible indicator of resistance in grapevines to *Plasmopara*

*viticola* and *Botrytis cinerea* (7, 42). Gao and Shain (29) found that different levels of a polygalacturonase inhibitor in American and Chinese chestnut explained the difference in levels of resistance of these species to *Cryphonectria parasitica*, the cause of chestnut blight.

There is evidence that substances in butternut bark have substantial fungicidal and antimicrobial properties. Butternut bark extracts were the most antagonistic and had the broadest spectrum of activity of the tree species tested against several human pathogenic bacteria and several fungi (62, 26). It is probable that these substances are a part of the tree's own defense mechanisms against disease.

It is generally established that *Juglans* species contain a number of structurally related, double-ring compounds called naphthoquinones. Many naphthoquinones have been found to inhibit the growth of plant pathogens. Several naphthoquinones known to be present in *Juglans regia* husks including 1,4-naphthoquinone, juglone, menadione, and plumbagin were found effective at inhibiting *Aspergillis flavis* in culture and reducing aflatoxin production (52). Naphthoquinones also inhibited the growth of several human pathogenic bacteria (59, 77, 78).

The most predominant and most thoroughly studied naphthoquinone is juglone. It has long been observed that walnut trees are detrimental to the growth of plants such as alfalfa, apples, and tomatoes grown in close proximity. Root exudates were implicated in this allelopathic effect and the substance was found to be juglone (53, 22). Juglone is present in black walnut and butternut in their roots, leaves, fruit hulls, and bark (36). Pure juglone and crude extracts from green walnut hulls have been found inhibitory against a wide range of microorganisms including bacteria, filamentous bacteria, algae and dermaphytes (50). Juglone was an effective inhibitor of *Botrytis cinerea*, *Cladosporium herbarum*, and *Fusarium avenaceum* growth (33). Inhibition by juglone of the growth of the wood-rotting fungus *Pleurotis sajor-caju* (21) and the pecan scab fungus, *Fusicladium effusum* (98) has also been demonstrated. It has been suggested that the high levels of juglone in black walnut may be responsible for its greater resistance to scab than pecan (35).

In a study comparing both leaf pathogens and non-pathogens of black walnut, juglone was more effective in inhibiting growth of non-pathogens (*Gnomonia quercina*, *G. platani*, and *Sclerotinia sclerotiorum*) and one pathogen (*Cristulariella moricola*) than against two other pathogens, *Cylindrosporium juglandis* and *Gnomonia leptostyla* (18) . This may indicate a tolerance to juglone among some *Juglans* pathogens, therefore higher concentrations of juglone may be required to inhibit their growth. In a related study the juglone concentration in leaves was dependent on leaf age, with young leaves having a higher juglone concentration and being more resistant to anthracnose fungi than older leaves (19).

We tested reagent grade naphthoquinones and crude bark extracts of *Juglans* species and a *Juglans* hybrid for their effects against Oc-j conidia using a disc diffusion bioassay. Bark extracts were then analyzed chemically to determine naphthoquinone content. The objectives of this research were to determine a possible resistance mechanism in butternut to infection by *Oc-j* and to develop a technique to select for resistance among trees. The hypothesis was two-fold; first, that a disc diffusion bioassay using bark extracts could distinguish differences in levels of *Oc-j* conidial germination inhibition among butternut selections; second, that butternut bark contained significant levels of naphthoquinones, and that these substances correlated with the level of inhibition found in the bioassay.

## 2.2. Materials and Methods

### 2.2.1. Fungal Cultures

Cultures of *Oc-j* isolated from butternut cankers collected in Wisconsin and Minnesota (Table 2.1) were grown on 3% malt agar in Petri plates at 20$^{o}$ C in the dark until sporulation occurred (usually 15-30 days). Sporulating cultures were flooded with sterile deionized water and rubbed lightly with a sterile, bent plastic rod to dislodge conidia. Suspensions were vortexed, then heavier debris containing agar and mycelia were allowed to settle out. The remaining conidial suspension was adjusted to the desired concentration with sterile deionized water using a hemacytometer. A mixture of conidia

obtained from four isolates was used in the naphthoquinone assay and in the bark extract assays in 2010 and 2011. Conidia from two separate isolates were used in the bark extract assays in 2006.

### 2.2.2. Disc Diffusion Bioassay with Reagent Grade Naphthoquinones

The disc diffusion bioassay procedure used was similar to standard antibiotic sensitivity tests (8). It was based on the principal that inhibiting substances diffuse out into a medium at increasingly lower concentrations by distance from the source (the chemical-infused disc), and that fungal growth inhibition is measurable and predictable for a given set of conditions. A bioassay was conducted using several related naphthoquinones to determine their activity against *Oc-j*. The naphthoquinones tested included juglone, 1, 4-naphthoquinone, plumbagin, menadione, and lawsone; all were obtained from Sigma-Aldrich (St. Louis, MO) (Table 2.2). The naphthoquinones were dissolved in 95 percent ethanol and applied to sterile 6.5 mm diameter cellulose discs at a rate of 5, 10, 20, 50, and 100 μg per disc. A control of ethanol alone was also prepared. To each 100 mm petri plate containing malt agar, a suspension of $4 \times 10^5$ *Oc-j* conidia per plate (mixed isolates, Table 2.1) was added and spread over the surface evenly using a sterile, bent plastic rod. One disc of naphthoquinone was applied to the center of each plate and eight replicate discs of each naphthoquinone were tested for each treatment level. Plates were placed in the dark and incubated at $20^oC$.

After incubation for 72 hours, the fungal growth was visible on the plates as a solid lawn, except for a clear inhibition zone around the discs. The diameter of each of these inhibition zones (including the disc) was measured to the nearest 0.5 millimeter using a circular template, and samples with no inhibition were recorded as 6.5 mm, the diameter of the disc. The experiment was conducted twice.

Level of inhibition or "inhibition zone", measured in millimeters, will be the term used henceforth to determine the degree of inhibition of the *Oc-j* conidial germination. Inhibition of mycelial growth, though it occurred, was not an objective of the study, and was more difficult to measure because of the presence of aerial mycelia within the inhibition zone.

### 2.2.3. Plant Material

All bark samples were collected from a plantation near Rosemount, MN, consisting of trees planted from 1994-1996. Species included *J. cinerea*, *J. nigra*, *J. ailantifolia* var. *cordiformis* and the hybrid *J. cinerea* x *J. ailantifolia*. These trees included both unselected, seedling butternut of unknown origin and grafted trees selected for possible disease resistance (Table 2.3). The selected trees were propagated from disease-free trees growing among those exhibiting active cankers.

Bark samples were collected monthly April through October from ten trees in 2006 and fifteen (the same ones as 2006 plus five additional selected trees) in 2010. In 2011 bark samples were collected from 69 trees, including the fifteen trees used previously, in May and August. The trees included ten unselected seedling butternuts, two black walnut, three hybrids, one heartnut and 53 grafted butternuts. The grafted trees included those selected for possible disease resistance (42 trees) and a collection of named varieties selected in the past for good nut characteristics but not for disease resistance (11 trees). Observations in other studies have found the majority of these named varieties were susceptible to butternut canker (69) . A minimum of three 30 cm lengths (0.5 to 2.5 cm diameter) of 4- to 6-year-old branches per tree were collected each month. Branches were collected from different sides of each tree and kept cool ($4^{o}$C) until they were peeled, within one to two weeks after collection.

### 2.2.4. Bark Extraction

In 2006 branches were collected in mid-month from April through October (Table 2.4). Bark tissue was divided by age: current year (greenwood), 1- to 2-year-old, and 3- to 4-year-old. Based on preliminary studies, outer (green layer) bark was discarded and only the inner, fibrous bark was used. Current-year bark was collected starting in June, with no removal of the outer bark. The following extraction procedure was according to Omar and others (62). Bark was air-dried and ground in a Wiley mill to a fineness of a 20 mesh screen (0.8 mm). For the extraction, the bark powder was soaked in 95 percent ethanol at a rate of 3 g per 15 ml for 48 hours with occasional agitation and the resultant extractives were filtered with Whatman #1 paper and air dried. A total of 190 extract

samples from 10 trees (Table 2.3) were prepared and stored at -20$^{o}$ C. Extracts from each collection were prepared once and two bioassay experiments were carried out on each sample of the extract.

In 2010 and 2011 the inner bark of 1- to 6-year-old branches was used. The extraction procedure was modified somewhat to reduce heating and oxidative processes during grinding and to increase yield of extractives. Bark was ground with dry ice and stored at -70$^{o}$ C. The extraction process was started by mixing 1 g of bark powder in 10 ml of cold (-20$^{o}$ C) 95 percent ethanol and soaking the mixture overnight at -20$^{o}$C. Mixtures were then agitated at room temperature for 24 hours, centrifuged, and the supernatant removed. Two successive extractions of 10 ml of 95 percent ethanol each were performed on the same bark powder and added to the original aliquot for a total of 30 ml of combined extract. Extracts were evaporated under vacuum to near dryness, and then air-dried to a tarry consistency.

Bark samples were generally collected the third week of each month in 2010 (Table 2.4). A total of 105 samples from 15 trees (Table 2.3) were prepared, stored at -70$^{o}$ C, and three bioassay experiments were carried out on each sample. The chemical analysis was performed on the same extracts. In 2011 bark samples were collected on May 25 and August 16 from 68 and 69 trees, respectively. The same extraction procedures were used as in 2010 and three bioassay experiments and the same chemical analysis carried out on each sample.

### 2.2.5. Disc Diffusion Bioassay with Bark Extracts

Bark extracts were re-suspended in 95 percent ethanol and applied to sterile 6.5 mm cellulose discs at a rate of 2 mg per disc, then air dried. Ethanol controls were also prepared. To each malt agar petri plate, a conidial suspension of 4 x 10$^{5}$ *Oc-j* spores per plate was added and spread over the surface evenly using a sterile, bent plastic rod. Four discs of each extract were placed equidistant on each malt agar plate, with two replicate plates for each combination of month, accession and in 2006, isolate and bark age. Plates were incubated at 20$^{o}$ C in the dark. After 72 hours the inhibition zone was marked and

the diameter was measured to the nearest 0.5 millimeter. The bioassay was carried out twice in 2006 and three times in 2010 and 2011.

### 2.2.6. Chemical Analysis

The same extracts used for the bioassay were used for the chemical analysis. For each extract, 20 mg was dissolved in 200 µl of 20% acetonitrile in water with 0.1% formic acid. The mixture was sonicated and filtered using Nanosep MF 0.2 micron spin filters (Pall Life Sciences, Ann Arbor, MI) to remove fine particles. Solutions of known naphthoquinones used for the first bioassay were used for comparison for component identification in 2006. Later analyses used a standard curve of known concentrations of only juglone and plumbagin. Samples were analyzed by reversed-phase ultra-high performance liquid chromatography-mass spectrometry (UPLC-MS) using a Waters $C_{18}$ BEH column, UPLC and SQD MS detector in negative electrospray ionization mode (Waters Corp., Milford, MA). The samples were separated on a water-acetonitrile gradient from 20 to 98% acetonitrile over 11 minutes.

Standard naphthoquinones were used to identify the compounds by matching elution time and mass to confirm their presence or absence in each extract. Standard curves for juglone and plumbagin were used to determine their concentration by integrated peak area for extracts collected in 2010 and 2011. A linear regression equation was produced for the best fit in the range of areas found for the extracts (Figure 2.1).

Each extract was analyzed three times, and peak areas were determined for juglone and plumbagin peaks by integrating over the same time period for each peak using MassLynx software (Waters). This area was transformed into a concentration quantity by the regression equations, in millimolar quantities, and "concentration" was used as the quantification or dependent variable for all related statistics.

### 2.2.7. Statistical Analysis

All bioassay experiments were a randomized complete block design, with blocking where necessary for tissue age and isolate. In 2006, because extracts from the current year's growth had significantly less inhibitory effect, that data was disregarded

and data from one through four year's growth combined in the final analysis. Inhibition zone data points were based on the mean of eight measurements per accession per month. Mean inhibition zones and juglone and plumbagin concentrations were the dependent variables and were subjected to analysis by means and standard errors, one-way and mixed model ANOVA (Enterprise Guide 4.2, SAS Institute, Cary, NC). Tree accession, source, and month of collection were included as fixed effects and tissue age and isolate were included as random effects where applicable; least square means separation tests were conducted using a Fisher's Least Squares Difference procedure with a significance level of 0.05.

The two different methods of analysis, namely the bioassay resulting in a mean inhibition zone, and the chemical analysis resulting in a mean juglone or plumbagin concentration, were plotted for each individual extract as a scatterplot, and subjected to linear regression, revealing the degree of linear correlation between the two datasets.

## 2.3. Results

### 2.3.1. Inhibition of *Oc-j* by Reagent Grade Naphthoquinones

The bioassay revealed that the mean inhibition zone varied by type of naphthoquinone (Figure 2.2). Menadione, 1,4-naphthoquinone, and plumbagin were highly effective against *Oc-j* conidial germination, showing mean inhibition zones of 62, 47, and 45 mm, respectively, at a concentration of 100 μg per disc. Juglone was also inhibitory, but to a lesser extent, of 23 mm at 100 μg per disc. Lawsone was minimally inhibitory, 11 mm at 100 μg, and the ethanol controls exhibited no inhibition of spore germination.

### 2.3.2. Inhibition of *Oc-j* by Bark Extracts

#### 2.3.2.1. Comparisons of Bark Age and Isolate

Bark extracts from the current year's growth in 2006 had a significantly ($p<0.0001$) weaker inhibitory effect than extracts from older bark (Figure 2.3). The

inhibitory effect of extracts from 1-to 2-year-old bark were similar to extracts of the 3- to 4- year-old bark ($p=0.99$). The level of inhibition in the first replication was consistently greater than in the second replication ($p<0.0001$). However, the difference had no effect on the ranking of the accessions in the experiments (data not shown). The level of inhibition varied by *Oc-j* isolate with isolate 1347 being inhibited less than isolate 1344 ($p<0.0001$) (Figure 2.3). Isolate however, had no effect on the ranking of the accessions (data not shown).

#### 2.3.2.2. Comparison of Collection Month

In 2006 and 2010 the inhibition zone varied significantly ($p<0.0001$) by month of bark collection (Figure 2.4). In 2006 inhibition peaked in May and reached a secondary peak in August and September. The level of inhibition of extracts from bark collections in August and September were not significantly different from each other ($p=0.99$). In 2010 there was no May peak, with a late summer peak relatively larger than spring. As in 2006, extracts collected in August and September of 2010 were not significantly different from each other. Although there was a difference ($p<0.0001$) between the three experimental replications in 2010 (data not shown), the ranking of the accessions in terms of the size of the inhibition zone in each experiment was similar (Figure 2.5). In 2011, the mean inhibition zone was significantly larger ($p<0.0001$) in August (19.0 mm) than in May (17.4 mm).

#### 2.3.2.3. Comparison of Tree Source and Species

Extracts from unselected butternuts had significantly smaller inhibition zones ($p<0.0001$) than the selected butternuts in every month but July in 2006 (Figure 2.6). The greatest mean differences between the groups occurred in September, April and August with mean differences of 3.0, 2.9 and 2.8 mm, respectively. The comparison extracts from black walnut, heartnut, and butternut x heartnut hybrid varied from month to month, each species having its own pattern of inhibition (data not shown). Each of these species was represented by only one tree, while the unselected and selected trees were a mean of five and two trees, respectively.

In 2010, the differences in mean inhibition zones among sources were most clearly evident using the extracts from the August bark collection (Figure 2.7). While bark extracts from all accessions yielded peak inhibition in late summer or early fall, the inhibition effect of extracts from the selected butternuts peaked earlier and higher (August) than the unselected butternuts (September). When analyzed by ANOVA, the mean separation was confirmed for unselected versus selected for every month but September and October (Figure 2.8). The August mean separation was 4.6 mm, followed by July and April, at 2.6 and 2.1 mm, respectively. Inhibition by extracts from walnut, heartnut, and hybrid were still variable, but with fewer wide swings than in 2006 (data not shown).

Another source group, named variety, was added in 2011. When analyzed by ANOVA, extracts from unselected butternuts (10 trees) yielded significantly smaller inhibition zones than selected butternuts (43 trees), only in August (Figure 2.9). The named varieties were indistinguishable from either the selected or unselected group. When individual accessions were analyzed by ANOVA, significant differences were found in both May and August and the accessions ranked in broadly overlapping groups (Figure 2.10).

#### 2.3.2.4. Comparison of Accession in August

The most consistent mean separation over the three years between extracts of selected and unselected butternut was with the August collection. When extracts from the same individual tree accessions were compared over all three years, inhibition zones of extracts of some accessions were similarly ranked (Table 2.5). For example, inhibition zones by extracts of B16 in August consistently ranked as the smallest in all three years, and B03 ranked moderately small. Inhibition zones of bark extracts from S22 were larger than all other accessions in 2006 and continued to rank among the largest in 2010 and 2011. Inhibition zones of extracts of S67 were large for both of the years it was used (2010, 2011). Extracts from the hybrid (Y128) varied little, from moderate to relatively large inhibition zones. Other accessions had more variable results, such as B04 extracts which had moderately small inhibition zones in 2006 and 2010 but large in 2011. Both

the walnut and the heartnut extracts yielded inhibition zones that were also somewhat variable, ranking moderate to small depending on year.

### 2.3.3. Chemical Analysis

#### 2.3.3.1. Identification and Quantification of Naphthoquinones

Peaks that were observed via UPLC matched both juglone and plumbagin standard compounds in time of elution and observed mass. Juglone peaks had a mass of 175 and a time of elution approximately 5.5 minutes, and plumbagin peaks had a mass of 189 and a time of elution approximately 4.5 minutes (Figure 2.11). Trace amounts of 1,4-naphthoquinone may also have been present, but were too low to confirm. Consequently, the remainder of the analyses will focus only on juglone and plumbagin.

Juglone concentrations in 2010 varied from a low of 0.14 mM (W01, August) to a high of 2.32 mM (S48, April). Juglone concentrations in 2011 varied from 0.67 mM (S39, August) to 2.2 mM (Y165, August). Plumbagin concentrations were generally tenfold lower; in 2010 they varied from a low of 0.018 mM (Y28, August) to a high of 0.27 mM (S54, July). In 2011 they varied from 0.13 mM (S67, August) to 0.82 mM (S19, August).

#### 2.3.3.2. Comparison of Juglone and Plumbagin Concentrations in 2010

Juglone levels in the 2010 bark extracts varied by month and accession. Overall juglone levels were highest in April (mean of 1.60 mM) and decreased until August (mean of 0.31 mM), then increased again in the fall (Figure 2.12). By accession, the lowest juglone levels were found in B16 (mean 0.65 mM, months combined) and highest in S20 (mean 0.97 mM, months combined). When analyzed by ANOVA, the concentration of juglone varied significantly by both month and accession ($p<0.0001$). Concentrations in both April and August were significantly different from all other months using LSD pairwise comparisons ($p<0.0001$). There were significant differences in juglone concentrations between accessions in every month but May and September ($p<0.0001$). For example, analysis by ANOVA found that in July there was significant

mean separation between accessions (Table 2.6). When analyzed by source group, unselected butternuts were significantly lower in juglone concentration than selected butternuts in April, July, and August (Figure 2.13).

Plumbagin concentrations also varied by month and accession. They were highest in 2010 in May, June and July (mean 0.17 mM), lowest in August (mean 0.033 mM), then recovered slightly in the fall (Figure 2.12). ANOVA analysis revealed significant differences between months, with LSD pairwise comparisons showing May-July mean separations from August-October. Variation by accession ranged from an overall mean of 0.086 mM in black walnut to 0.13 mM in heartnut, with ANOVA showing significant differences between accessions for each month (data not shown). When analyzed by source, no clear pattern emerged (Figure 2.14).

#### 2.3.3.3. Comparison of Juglone and Plumbagin Concentrations in 2011

Extracts from May were significantly higher in juglone and plumbagin than extracts collected in August, similar to the 2010 data and the reverse of the results of the bioassay. Juglone levels showed variation by tree source, with unselected butternut significantly lower in juglone than selected butternut in both months (Figure 2.9). Named varieties were not significantly different from selected butternuts in either month. Ranking and separation patterns were very similar between the bioassay and the juglone data in regards to tree source in 2011. When juglone concentrations were compared by accession, ranking of accessions and mean separations were relatively similar to the bioassay data (Figures 2.10 and 2.15). Plumbagin concentrations did not vary significantly by tree source groupings (selected versus unselected), nor did they follow a pattern similar to either juglone concentrations or bioassay data.

#### 2.3.3.4. Correlation of Concentrations with Bioassay Results

The results of the bioassay and the chemical analysis of the bark extracts were subjected to linear regression, revealing if there was a linear correlation between the two datasets. Extracts collected in several months had a significance level of .05 or less, showing a strong linear correlation between conidial germination inhibition levels and

juglone concentration (Figure 2.16). In July, June and April of 2010, the p-values were <0.0001, 0.001, and 0.002, respectively. In 2011 p-values were both <0.0001 for May and August.

When the bioassay data was correlated with plumbagin, the correlations were not as strong. The only significant linear correlation was in April 2010, with a p-value of 0.036 (Figure 2.17). No significant correlation was found between the bioassay and plumbagin in 2011.

## 2.4. Discussion

Restoration of butternut will require a reliable procedure to select trees that have resistance to butternut canker. Some success has been reported by investigators challenging trees directly with the pathogen in common garden orchards. However, propagating candidate trees, establishing orchards and testing trees in this manner is time and cost prohibitive in most cases. A rapid, repeatable test that distinguishes highly disease resistant trees from susceptible trees is needed.

The hypothesis of this study was that there would be discernable differences between the selected and unselected butternut trees. A statistically significant difference between inhibition of *Oc-j* conidia germination by bark extracts from unselected butternuts and selected butternuts was detected in this study (Figures 2.8, 2.9). The greatest distinction between selected and unselected was found with extracts from bark collected in August in the 2010 study, with similar results in both 2006 and 2011, providing evidence that the procedure is repeatable. In addition, individual accessions ranked similarly in the same month over several years (Table 2.5),

The small group of trees used in 2006 and 2010 may have magnified individual tree-to-tree differences and limited the ability to see source group differences. In 2011 the purpose was to compare results of both the bioassay and chemical analysis using a greater number of selected and unselected trees, as well as repeating the procedure on the previously analyzed trees for two key months. The overall results were comparatively similar to the previous data, with the broader range of values that would be expected with a larger group of trees. ANOVA tests did not reveal any significant mean separation

between unselected butternut, named varieties and selected butternut in May (Figure 2.9). There was mean separation in August, however, with a significant difference between unselected and selected butternuts, but not for named varieties. This confirms the findings found in August 2010, but with a less distinct separation between selected and unselected. The bioassay was not able to separate out the named varieties, which have been shown to be a generally susceptible group.

The chemical analysis confirmed the presence of both juglone and plumbagin in the bark extracts. While juglone has long been confirmed present in *Juglans* species, including *J. cinerea*, plumbagin is less studied and appears to be present in smaller amounts (52, 10). Plumbagin levels were generally 10-fold less in concentration than juglone, and did not follow the same monthly pattern (Figure 2.12).

When juglone concentration was analyzed by tree source, ANOVA did not separate groups as clearly as it did with the inhibition zones of the bioassay in 2010. The mean separation in the bioassay between unselected and selected was observed in five out of seven months (Figure 2.8). For juglone concentration, only in three months was there any separation between the unselected and selected groups (Figure 2.13). In 2011 the bioassay ranking by tree source was very similar to the ranking by juglone concentration in both months tested (Figure 2.9), showing evidence of a connection between juglone and the bioassay.

The juglone levels correlated with the bioassay (p-value $< 0.05$) for five months out of nine (Figure 2.16). This high correlation shows strong evidence that the bioassay effect on spore germination inhibition is due at least in part to the action of juglone. Correlation is not proof of causation, however, and there clearly could be other substances involved that have yet to be analyzed. The monthly patterns of juglone concentration and of bioassay inhibition were different, at least in 2010 (Figure 2.18). The strong inhibition effect by extracts collected in August of 2010 despite low levels of juglone and plumbagin points to other substances involved in inhibiting *Oc-j* conidia germination. Juglone concentration did not correlate well with inhibition in four months. Although plumbagin performed strongly in the original naphthoquinone test (Figure 2.2),

in the bark extract bioassay it correlated poorly or not at all in all but one month (Figure 2.17).

Chemicals constituents in plant extracts have often been found to fluctuate throughout the growing season. A variety of phenolics, flavonoids and quinones in walnut (*J. regia*) leaves were shown to vary by time of collection (89). In that study, juglone levels were relatively low at the end of May and increased to a peak in mid-July. Results from this study also shows distinct differences in both the bioassay data and the juglone and plumbagin levels depending on month of bark collection. It would be instructive to track seasonal differences not only by date but by phenological changes from year to year. A distinctly higher peak in the inhibition zone was found in bark collected May 2006 than the same month in 2010 (Figure 2.4). This could be due to phenological or climatic variation in different years.

May and August were selected as collection months for the 2011 assays since they were peak times of larger inhibition zones in the 2006 and 2010 assays. The bark collection date that had the greatest mean separation of inhibition zone between source groupings was August 19, 2010. The collection on August 16, 2011 did not have as great a separation. That may be due to the greater variability inherent in the larger number of trees tested, but it may have been due to a yearly difference in phenology or physiology of the trees. Timing of bark collection would need to be carefully considered if an extract screening test were to be implemented. Both juglone and plumbagin concentrations changed dramatically from July to August of 2010 (Figure 2.12), therefore bark samples collected during this late summer period could be quite variable in chemical constituents and collection timing would need to be fine-tuned to develop a reliable assay.

The greatest consistent level of inhibition in the bioassay was obtained using extracts from bark collected in August or September. Early fall was also the period of greatest separation of susceptible and resistant butternut selections based on artificial inoculations of trees with the fungus (70). This suggests that this may be a key period for butternut trees to produce active defense compounds. Further study would be needed to identify these active compounds that are likely present as well as the known

naphthoquinones, since the juglone and plumbagin levels were shown to be relatively low during this period.

This study included representative samples from black walnut, heartnut, and buart (hybrid) trees for comparison purposes. In past studies, these species have been observed to be more resistant to *Oc-j* than butternut (63, 38). It could be expected that bark extracts of these species would result in larger inhibition zones and/or higher concentrations of juglone than butternut in the studies described here. The hybrid bark extract often was moderately high in inhibition and in juglone concentration compared to the butternuts, but black walnut extracts produced moderate to low levels of inhibition in most months of 2010 (Figure 2.7) and had among the lowest levels of inhibition and juglone in 2011 (Figure 2.9). Since black walnut is clearly resistant to *Oc-j* in the field, but performed poorly in these tests, its resistance mechanism is likely different than that of butternut. For example, it may have a different chemical profile and/or physical structures that provide a barrier to the fungus. Also, data for these studies was taken from only one black walnut tree in 2006 and 2010, and two trees in 2011, so that it would be difficult to make inferences based on a small dataset. The heartnut also frequently performed moderately or poorly in these tests, pointing to other mechanisms of resistance or a small (one tree) dataset.

Another topic that is beyond the scope of the present study is the question of constitutive versus induced defenses. The assumption of this research was that juglone and/or other defense compounds, although fluctuating in time, were constitutive and represent a consistent baseline for comparison between other factors. Research in other pathosystems has revealed defensive substances that are induced when the tree is wounded or under attack by a pathogen or herbivore. Classic examples of these are the stilbenes that are synthesized when Scots pine is infected by *Heterobasidion*. These stilbenes are correlated with higher decay resistance (49). If production of juglone or other substances in the bark extract were influenced by wounding or the presence of active *Oc-j* cankers, the interpretation of the bioassay and the chemical analysis could be very different. Although all the trees used were free of obvious cankers, several collections revealed staining and/or small incipient cankers during the bark peeling

process. The presence of trees with undetected cankers in the bark collections could be influencing the outcomes of both the bioassay and the chemical analysis. A possible next step in this research would involve comparing bark samples from healthy versus cankered trees to see if the bioassay and/or the chemical analyses are different, or to track chemical changes after inoculation and/or wounding.

Results of the bioassay produced results similar to screening 7- to 11-year-old trees in the field by introducing the fungus into wounds, where a statistically significant separation was found between selected (putative *Oc-j*-resistant) and unselected trees (70). This bioassay screen is considerably faster and less dependent on variable field conditions, and may be useable as a rapid screening test.

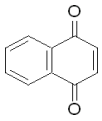
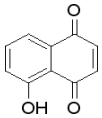
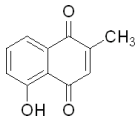
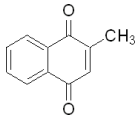
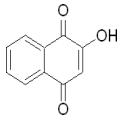
The results of this research have demonstrated several principles that would be needed in a disease screening test. First, the bioassay was relatively easy to perform without overly complex equipment or expensive chemicals. Second, it was repeatable with similar results over three years. The amount of variation was reasonable due to what would be expected by changes in phenology and lab conditions from year to year. Third, the bioassay test resulted in quantifiable data that was able to be analyzed by typical ANOVA methods, showing statistically significant differences between individual accessions and between selected and unselected groups. Fourth, juglone is correlated to the inhibition zone measurement at least in some months. The strong correlation between juglone levels and bioassay data in five out of nine months tested gives evidence that the bioassay inhibition of *Oc-j* conidia germination is due at least in part to the juglone level in the extract. With refinement and more experimentation, especially comparing healthy and cankered trees, a bark extract bioassay could be a reasonable screening technique to shorten the time needed to evaluate butternut trees for resistance to butternut canker.

**Table 2.1.** Sources of *Ophiognomonia clavigignenti-juglandacearum* (*Oc-j*) isolates used in assays.

| | | Assay used | |
|---|---|---|---|
| **Isolate** | **Origin** | **2006 bark extracts**[a] | **Reagent grade naphthoquinone assay, 2010 & 2011 bark extracts**[b] |
| 1344 | Forest Co., WI | X | |
| 1347 | Kanabec Co., MN | X | |
| 1384 | Goodhue Co., MN | | X |
| 1385 | Ramsey Co., MN | | X |
| 1387 | Langlade Co., WI | | X |
| 1388 | Forest Co., WI | | X |

[a]Isolates were used separately
[b]Multiple isolates were mixed

**Table 2.2.** Description of the naphthoquinones[a] used in bioassay and as chemical standards.

| Naphthoquinone | Structure | Molecular weight | Species derivation | Common uses and/or biological effects |
|---|---|---|---|---|
| **1,4-Naphthoquinone** |  | 158.15 | Multiple | Precursor to other naphthoquinones |
| **Juglone** |  | 174.15 | *Juglans* spp. | Allelopathic<br>Dye<br>Anthelmintic (Expelling worms) |
| **Plumbagin** |  | 188.18 | *Plumbago* spp.<br>*Drosera* spp.<br>*Nepenthes* spp.<br>*Juglans* spp. | Dye<br>Antimicrobial<br>Anti-inflammatory<br>Anti-carcinogenic |
| **Menadione** |  | 172.18 | Multiple (mainly leafy green vegetables) | Vitamin K precursor<br>Blood coagulation |
| **Lawsone** |  | 174.15 | *Lawsonia inermis*<br>*Impatiens balsamina* | Skin and hair dye (henna) |

[a]Naphthoquinones were reagent grade and obtained from Sigma-Aldrich

**Table 2.3.** Trees used for bark extraction in 2006, 2010, and 2011. Unless otherwise noted, trees were *J. cinerea* scion grafted onto *J. nigra rootstock.*

| Accession[a] | 2006 | 2010 | 2011 | Source |
|---|---|---|---|---|
| B03 | X | X | X | Butternut seedling of unknown origin |
| B04 | X | X | X | Butternut seedling of unknown origin |
| B05 | | | X | Butternut seedling of unknown origin |
| B06 | | | X | Butternut seedling of unknown origin |
| B07 | | | X | Butternut seedling of unknown origin |
| B08 | | | X | Butternut seedling of unknown origin |
| B09 | | | X | Butternut seedling of unknown origin |
| B10 | X | X | X | Butternut seedling of unknown origin |
| B11 | X | X | X | Butternut seedling of unknown origin |
| B16 | X | X | X | Butternut seedling of unknown origin |
| S01 | | X | X | Selected, Dunn Co., WI |
| S06 | | | X | Selected, Caledonia, MN |
| S10 | | | X | Selected, Arlington, WI |
| S19 | | | X | Selected, Olmstead Co., MN |
| S20 | | X | X | Selected, Whitewater, WI |
| S22 | X | X | X | Selected, Whitewater, WI |
| S23 | | | X | Selected, Whitewater, WI |
| S28 | | | X | Selected, Red Wing, MN |
| S36 | | | X | Selected, Trade Lake, WI |
| S37 | | | X | Selected, Trade Lake, WI |
| S38 | | | X | Selected, Trade Lake, WI |
| S39 | | | X[b] | Selected, Trade Lake, WI |
| S54 | | X | X | Selected, Nicolet NF, WI |
| S55 | | | X | Selected, Nicolet NF, WI |
| S60 | | X | X | Selected, Whitewater, WI |
| S61 | | | X | Selected, Whitewater, WI |
| S67 | | X | X | Selected, Mazaska Lake, MN |
| S69 | | | X | Selected, Plymouth, MN |
| S71 | | | X | Selected, Trade Lake, WI |
| S78 | | | X | Selected, Rochester, MN |
| S83 | | | X | Selected, Mark Twain NF, MO |
| S86 | | | X | Selected, Mark Twain NF, MO |
| S87 | | | X | Selected, Mark Twain NF, MO |
| S95 | | | X | Selected, Perch River, NY |
| S96 | | | X | Selected, Perch River, NY |
| S97 | | | X | Selected, Perch River, NY |
| S109 | | | X | Selected, Charlotte, MI |
| S132 | | | X | Selected, Berlin, VT |
| S134 | | | X | Selected, Berlin, VT |
| S135 | | | X | Selected, Berlin, VT |
| S136 | | | X | Selected, Williston, VT |
| S140 | | | X | Selected, Red Wing, MN |

| Accession | 2006 | 2010 | 2011 | Source |
|---|---|---|---|---|
| S140 | | | X | Selected, Red Wing, MN |
| S141 | | | X | Selected, Trade Lake, WI |
| S143 | | | X | Selected, Raddison, WI |
| S144 | | | X | Selected, Nicolet NF, WI |
| S146 | | | X | Selected, Whitewater, WI |
| S147 | | | X | Selected, Whitewater, WI |
| S148 | X | X | X | Selected, Whitewater, WI |
| S153 | | | X | Selected, Daniel Boone NF, KY |
| S173 | | | X | Selected, Isanti Co., MN |
| S180 | | | X | Selected, Chalk Hills, MI |
| S188 | | | X | Selected, Polk City, IA |
| N154 | | | X | Named variety "Montauk" |
| N155 | | | X | Named variety "Kinneyglen" |
| N156 | | | X | Named variety "Weschcke" |
| N157 | | | X | Named variety "Creighton" |
| N158 | | | X | Named variety "Painter" |
| N160 | | | X | Named variety "Ayres" |
| N161 | | | X | Named variety "George Elmer" |
| N162 | | | X | Named variety "Ft. Wood A" |
| N164 | | | X | Named variety "Bear Creek" |
| N167 | | | X | Named variety "New Discovery" |
| N169 | | | X | Named variety "Booth" |
| H133 | X | X | X | *J. ailantifolia*, Berlin, VT |
| Y92 | | | X | Probable *J. cinerea* x *J. ailantifolia*, Loudon, NH |
| Y128 | X | X | X | *J. cinerea* x *J. ailantifolia*, Sanford, ME |
| Y165 | | | X | *J. cinerea* x *J. ailantifolia*, Mitchell Hybrid |
| W01 | X | X | X | *J. nigra*, Seedling of unknown origin |
| W02 | | | X | *J. nigra*, Seedling of unknown origin |

[a]Source group designators are as follows: B, unselected butternut; S, selected (putative resistant) butternut; N, named butternut variety; H, heartnut; Y, hybrid.
[b]S39 was used only in August 2011.

**Table 2.4.** Bark collection dates

| | 2006 | 2010 | 2011 |
|---|---|---|---|
| **Month** | **Date** | **Date** | **Date** |
| April | 18 | 23 | |
| May | 16 | 20 | 25 |
| June | 14 | 24 | |
| July | 12 | 19 | |
| August | 16 | 19 | 16 |
| September | 13 | 24 | |
| October | 19 | 21 | |

**Table 2.5.** Inhibition of germination of conidia of *Oc-j* at 72 hours using a bark extract disc assay with bark collected in August in each of three years.

| 2006[a] | | | | 2010[b] | | | | 2011[b] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Inhibition zone, mm | | | | Inhibition zone, mm | | | | Inhibition zone, mm | | |
| Accession | Mean diameter[c] | Range | SE | Accession | Mean diameter[c] | Range | SE | Accession | Mean diameter[c] | Range | SE |
| B16 | 14.0 a | 11-18 | 0.74 | B16 | 14.9 a | 13-17 | 0.79 | B16 | 15.2 a | 12.5-17 | 0.59 |
| W01 | 18.0 b | 14.5-22 | 0.67 | B10 | 15.7 a | 13-19 | 0.89 | H133 | 15.7 ab | 14.5-17 | 0.27 |
| B03 | 18.2 b | 14-23 | 0.84 | B04 | 16.0 ab | 13-19 | 1.25 | S54 | 15.9 ab | 14.5-17.5 | 0.40 |
| B04 | 19.9 c | 15-25 | 0.87 | B03 | 16.1 ab | 14-20 | 1.26 | S60 | 16.7 abc | 15-18 | 0.25 |
| Y128 | 19.9 c | 17-23 | 0.54 | B11 | 16.8 abc | 14.5-19 | 0.98 | B03 | 16.9 abc | 15.5-18.5 | 0.08 |
| H133 | 20.3 c | 18-24 | 0.57 | H133 | 17.8 abcd | 15-21 | 1.07 | W01 | 17.1 bc | 15.5-20 | 0.72 |
| B11 | 20.3 c | 16.5-24 | 0.73 | S148 | 19.1 bcde | 17-22 | 0.95 | S148 | 17.9 cd | 16.5-20 | 0.64 |
| S148 | 20.9 c | 17-25 | 0.70 | W01 | 19.2 bcde | 16.5-22 | 1.21 | Y128 | 18.0 cd | 16.5-20 | 0.57 |
| B10 | 22.7 d | 18-28 | 0.83 | S54 | 19.5 cde | 17-22 | 0.97 | B10 | 18.4 cd | 16-20 | 0.62 |
| S22 | 22.8 d | 18-28 | 0.93 | S01 | 19.6 cde | 17-23 | 1.21 | S20 | 19.4 de | 17.5-21.5 | 0.69 |
| | | | | Y128 | 20.0 de | 17-23 | 1.17 | S22 | 19.4 de | 17.5-21 | 0.36 |
| | | | | S22 | 20.3 de | 18-23 | 0.93 | S01 | 20.7 e | 18.5-23 | 0.90 |
| | | | | S20 | 20.7 de | 17-24 | 1.26 | B11 | 20.9 e | 18-23 | 0.97 |
| | | | | S60 | 22.0 e | 19-25 | 1.46 | S67 | 23.8 f | 22-25.5 | 0.58 |
| | | | | S67 | 22.3 e | 19-26 | 1.23 | B04 | 23.8 f | 22.5-25 | 0.40 |

[a]Data for August collection, combined 1-4 yr old bark, 2 isolates and 2 replications.
[b]Data from August collection, 3 replications.
[c]Values with the same letter do not differ significantly according to Fisher's LSD test ($p<0.05$)

**Table 2.6.** Mean juglone concentrations of bark extracts collected July 2010, by ranked accession. Accessions with the same letter do not differ significantly according to Fisher's LSD test ($P<0.05$), n=3.

| Accession | Mean juglone concentration, mM | Mean separation | Range | Standard error |
|---|---|---|---|---|
| B16 | .39 | a | .31-.43 | .038 |
| B03 | .45 | a | .40-.48 | .025 |
| S01 | .55 | b | .48-.59 | .034 |
| Y28 | .55 | b | .44-.63 | .059 |
| B11 | .56 | b | .49-.62 | .038 |
| H33 | .61 | bc | .56-.64 | .023 |
| S54 | .62 | bcd | .58-.65 | .019 |
| B04 | .63 | bcd | .53-.68 | .047 |
| W01 | .66 | cd | .62-.71 | .027 |
| S22 | .67 | cde | .63-.71 | .023 |
| B10 | .68 | cde | .62-.72 | .031 |
| S20 | .69 | cdef | .65-.72 | .023 |
| S48 | .70 | def | .67-.74 | .020 |
| S67 | .76 | ef | .72-.78 | .017 |
| S60 | .77 | f | .71-.81 | .030 |

**Figure 2.1.** Standard curve plots of peak area versus concentration for juglone and plumbagin, 2010 and 2011.

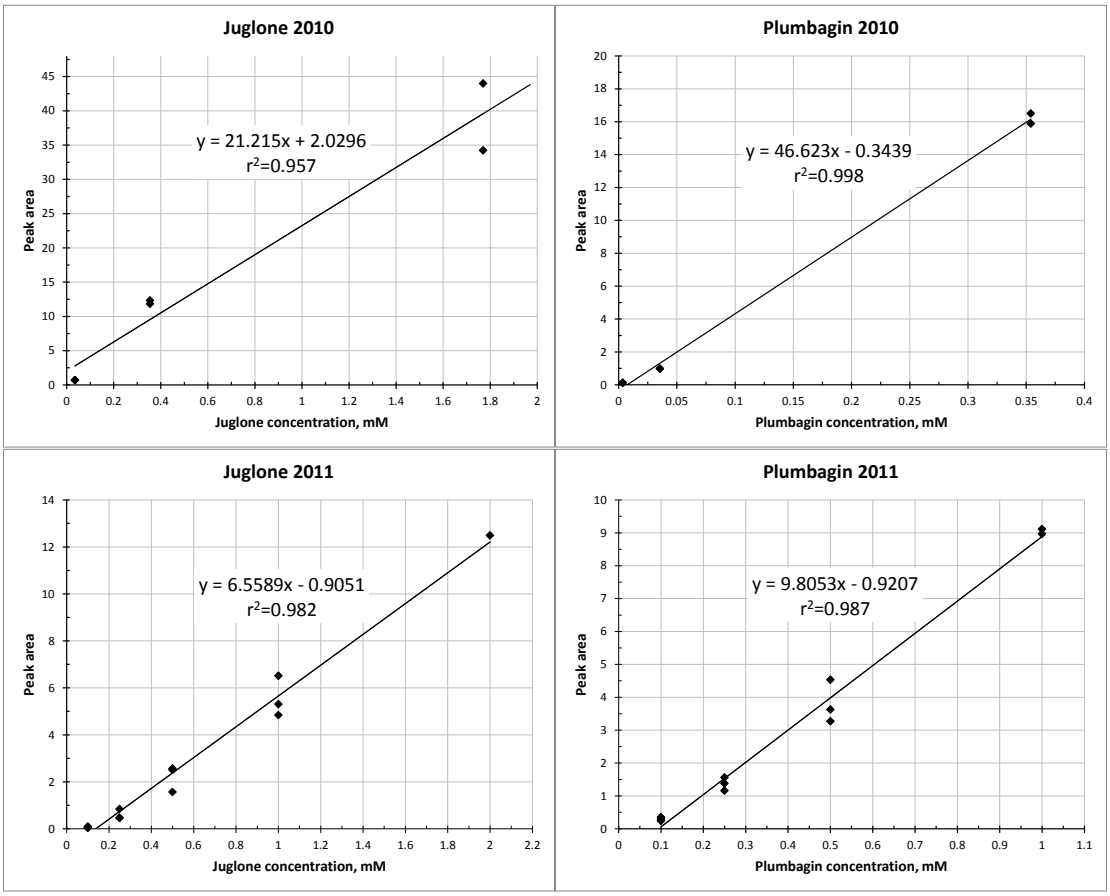


**Figure 2.2.** Inhibition of *Oc-j* conidia germination by reagent grade naphthoquinones using a disc assay after 72 hours. Data points are mean diameters of inhibition zones of two replications. Bars are standard error.

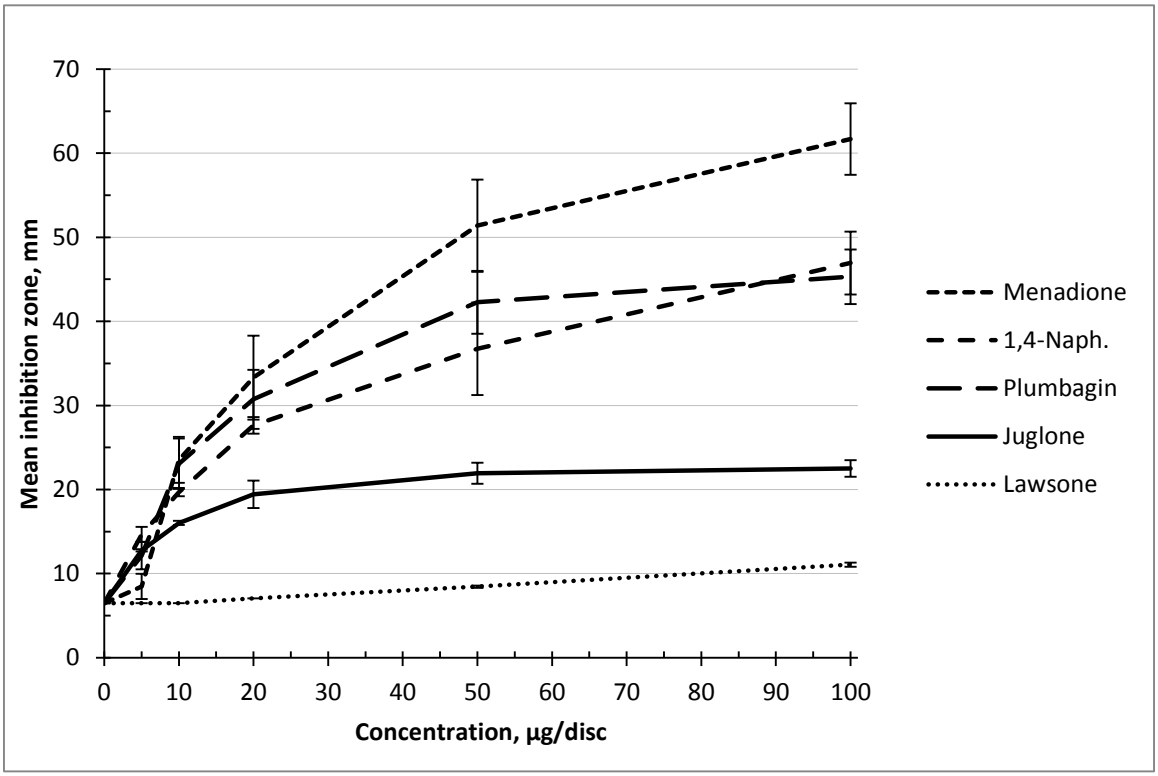


**Figure 2.3.** Inhibition of *Oc-j* conidia germination at 72 hours using a bark extract disc assay, comparing bark age, isolate (1344 or 1347) and replication, April-October, 2006. Data points are mean diameters of inhibition zones for all collection months and accessions combined. Bars represent standard error.

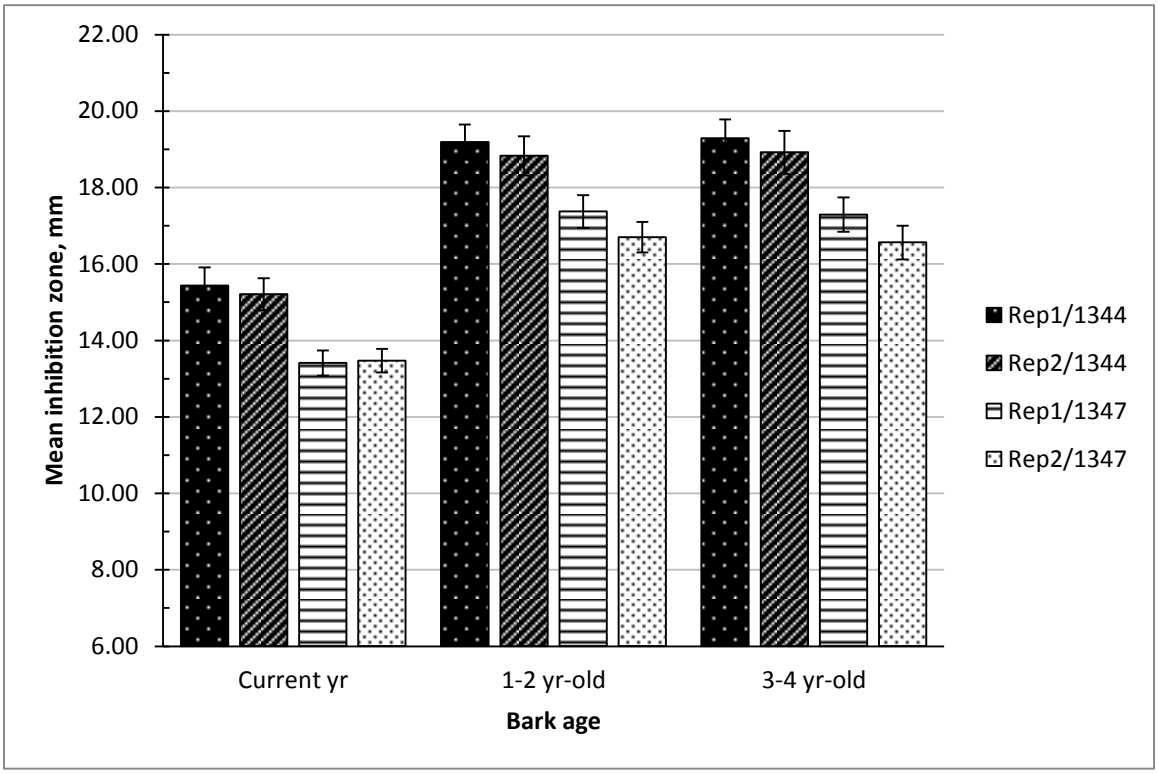


**Figure 2.4.** Inhibition of *Oc-j* conidia germination at 72 hours using a bark extract disc assay, comparing experimental year and month of bark collection. Data points are mean diameters of inhibition zones for all accessions combined. The experiment had 2 replications in 2006 and three replications in 2010. Bars represent standard error.

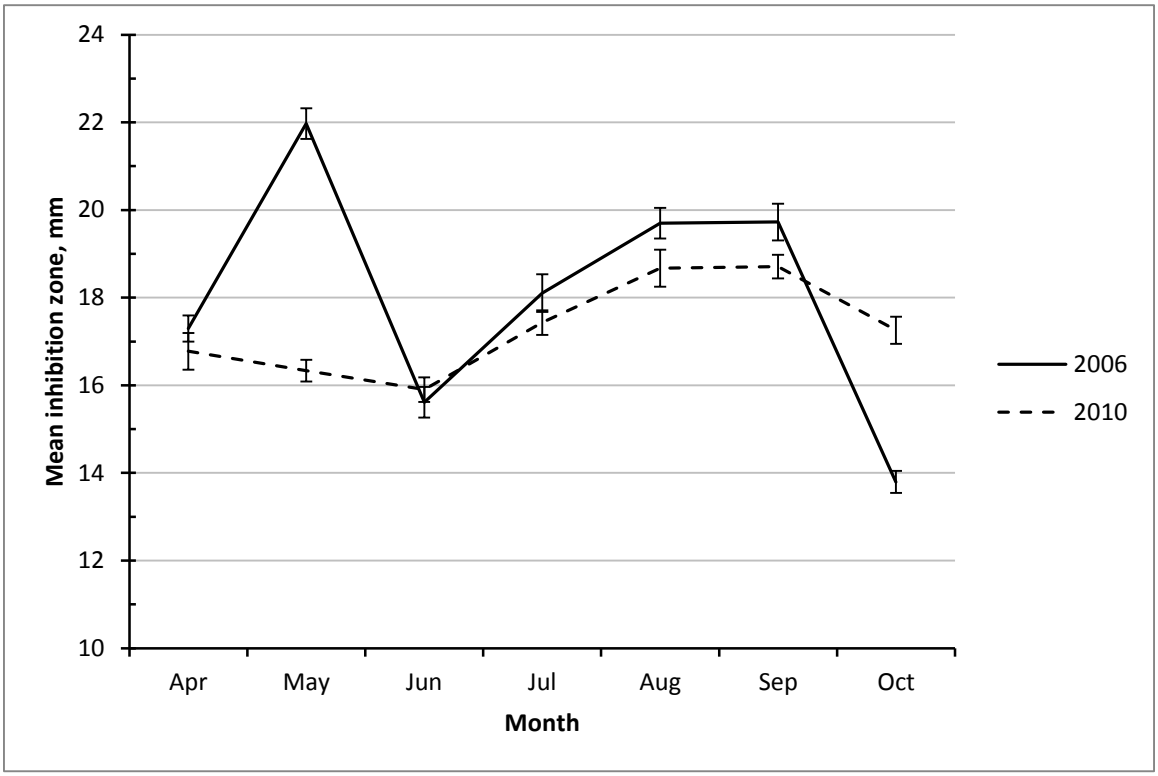


**Figure 2.5.** Inhibition of *Oc-j* conidia germination at 72 hours using a bark extract disc assay in 2010, ranking tree accessions and replications. Data points are mean diameters of inhibition zones, months combined. Bars represent standard error.

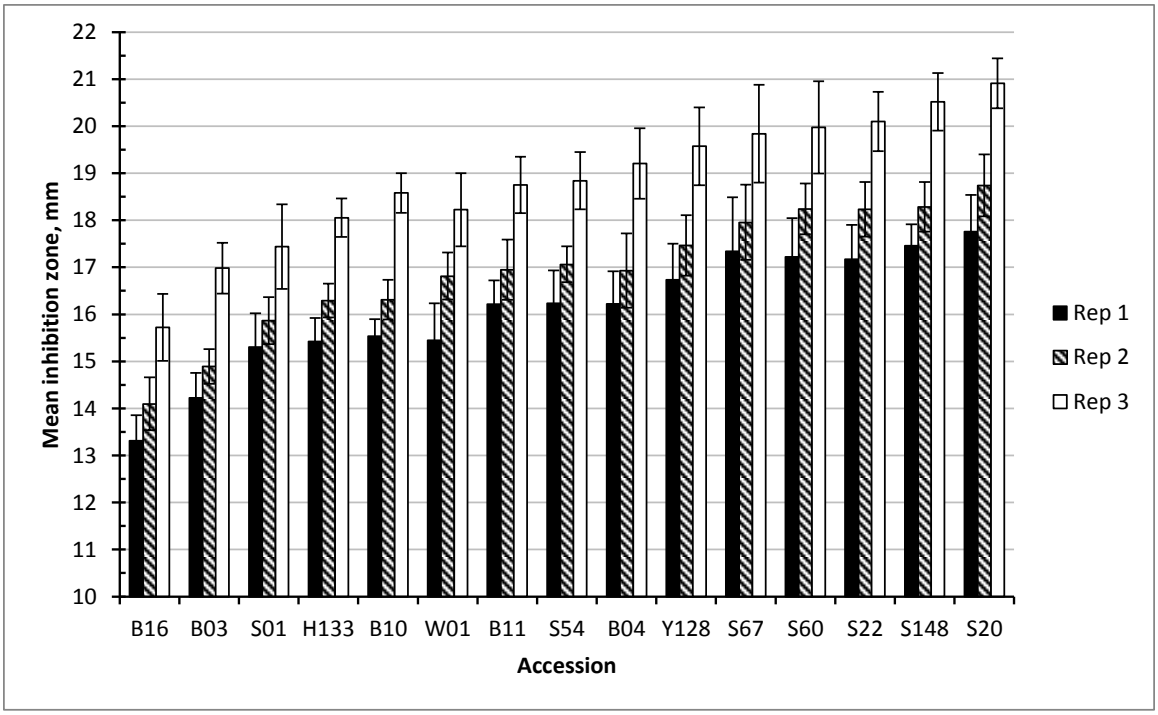


**Figure 2.6.** Inhibition of *Oc-j* conidia germination at 72 hours using a bark extract disc assay in 2006, by tree source and month. Data points are mean diameters of inhibition zones, combining isolate and bark age. B = unselected butternut (n=5), S = selected butternut (n=2). Values with the same letter do not differ significantly according to ANOVA ($P<0.05$).

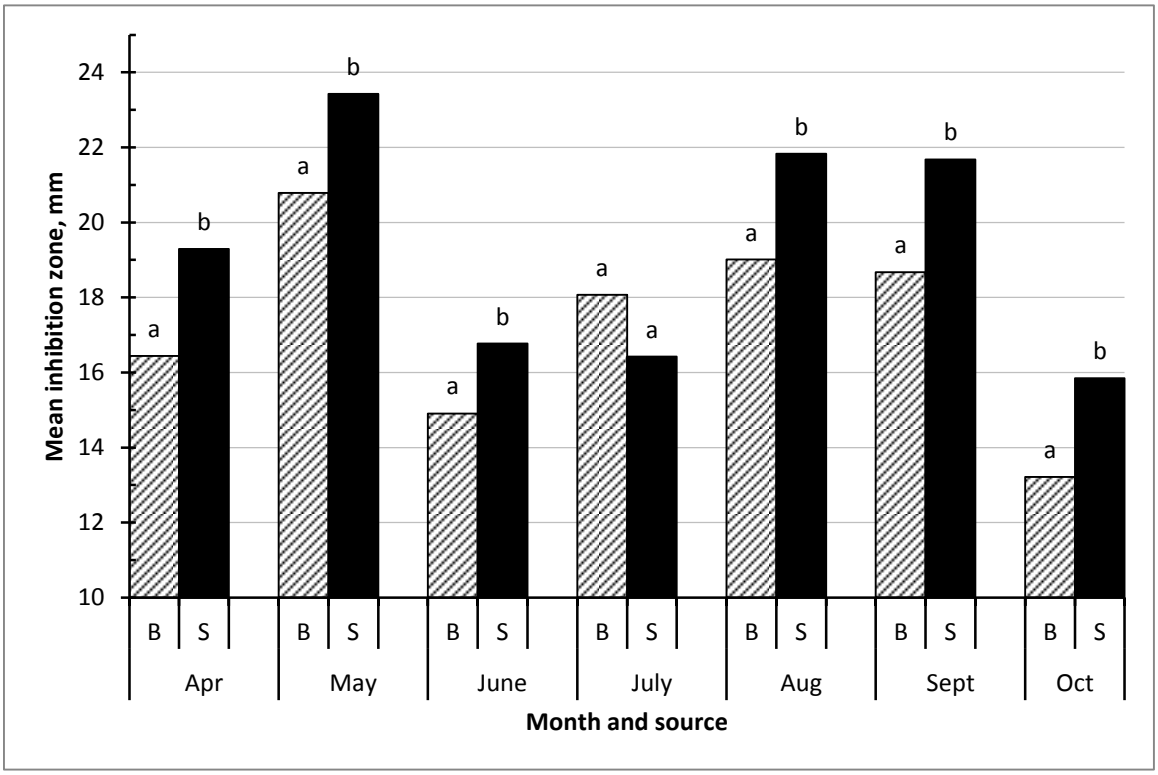


**Figure 2.7.** Inhibition of *Oc-j* conidia germination at 72 hours using a bark extract disc assay, comparing month of bark collection and source, 2010. Data points are mean diameters of inhibition zones. B = unselected butternut, S = selected butternut, H = heartnut, W = black walnut, Y = hybrid. Bars represent standard error.

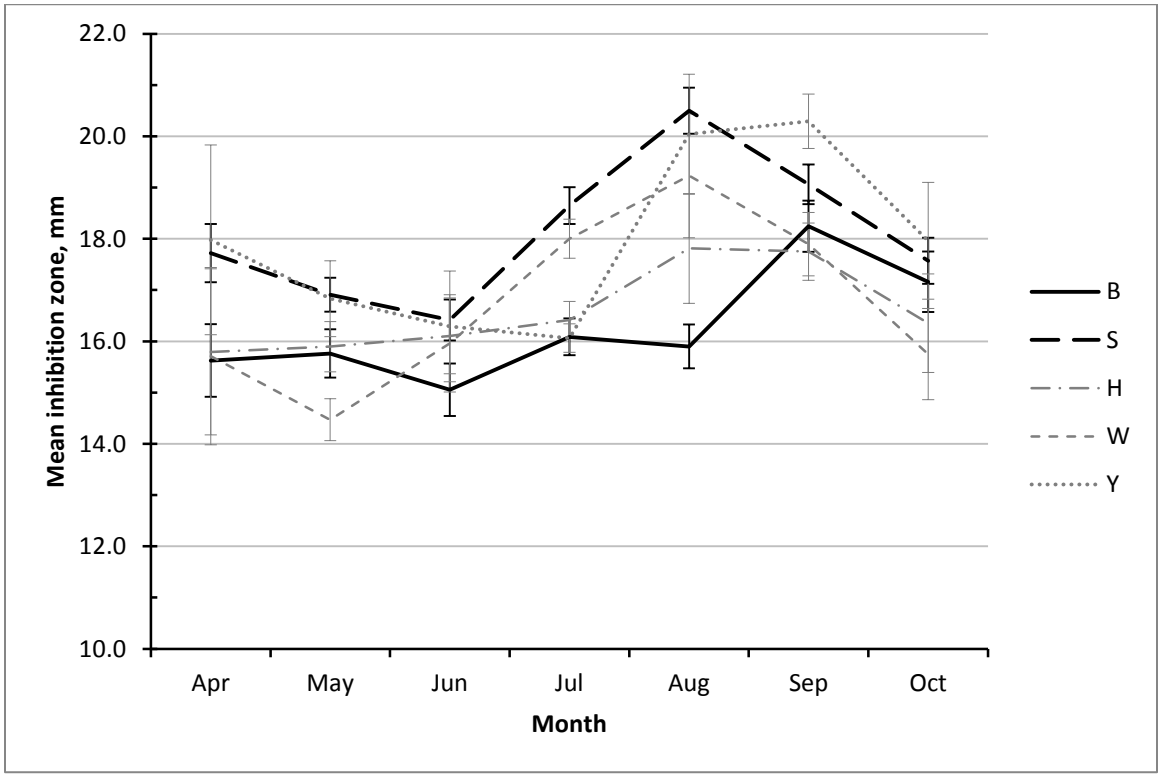


**Figure 2.8.** Inhibition of *Oc-j* conidia germination at 72 hours using a bark extract disc assay in 2010, by tree source and month. Data points are mean diameters of inhibition zones. B = unselected butternut (n=5), S = selected butternut (n=7), three replications. Values with the same letter do not differ significantly according ANOVA (P<0.05).

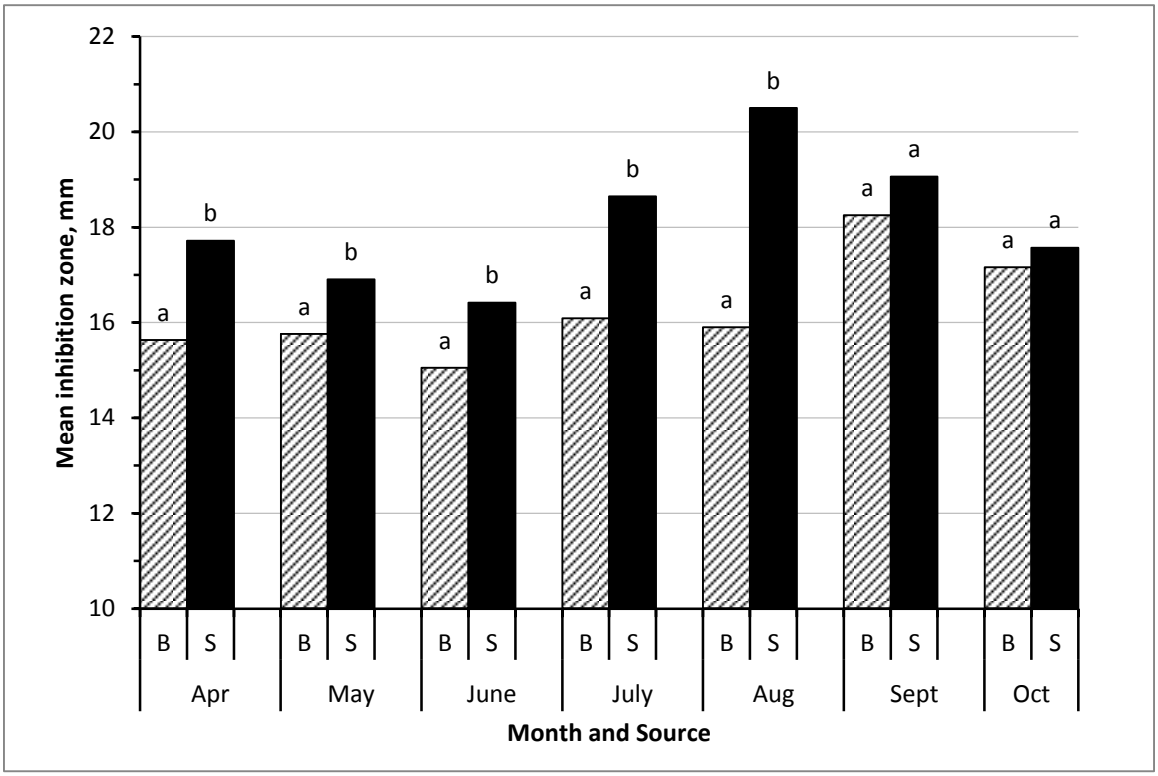


**Figure 2.9.** Inhibition of *Oc-j* conidia germination at 72 hours using a bark extract disc assay in 2011 (A), compared to juglone concentration of the same extracts (B), by tree source and month. Values are means of three replications. B = unselected butternut (n=10), N = named variety (n=11), S = selected butternut (n=43). Values with the same letter do not differ significantly according to Fisher's LSD ($P<0.05$). Separate analyses were performed for each response variable. Included for comparison, but not in statistical analysis: H = heartnut, W = black walnut, Y = hybrid (heartnut x butternut).

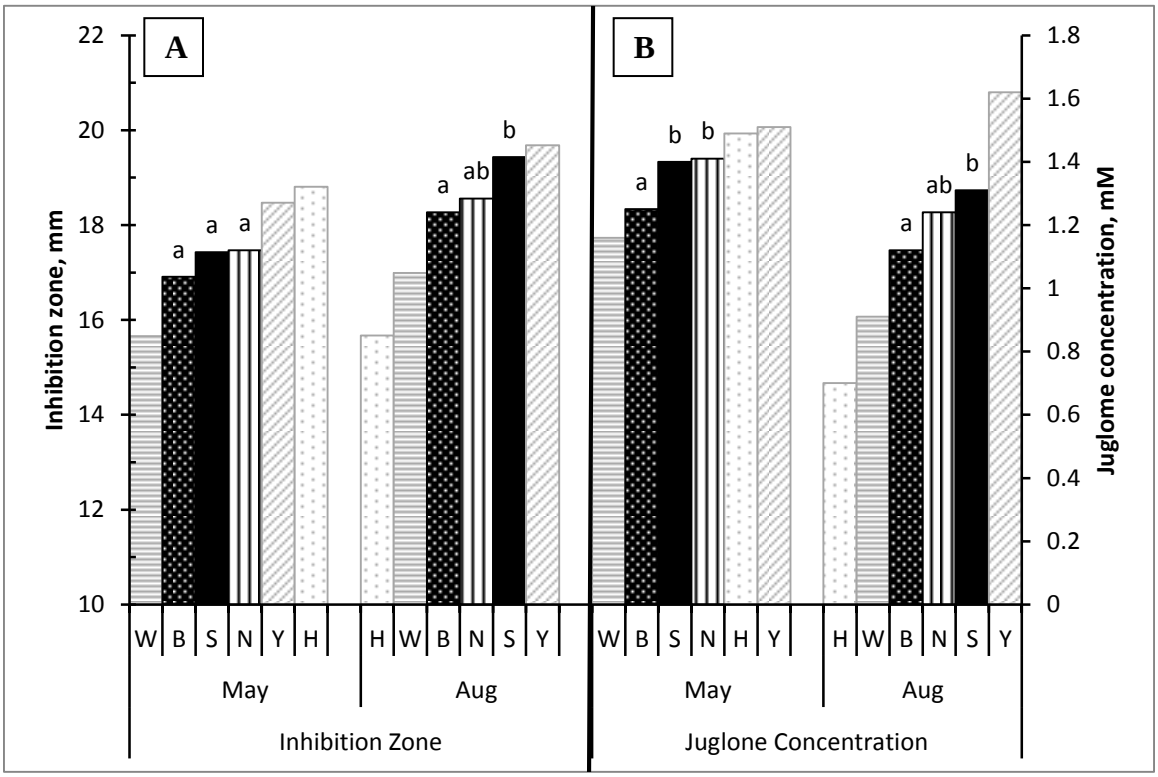
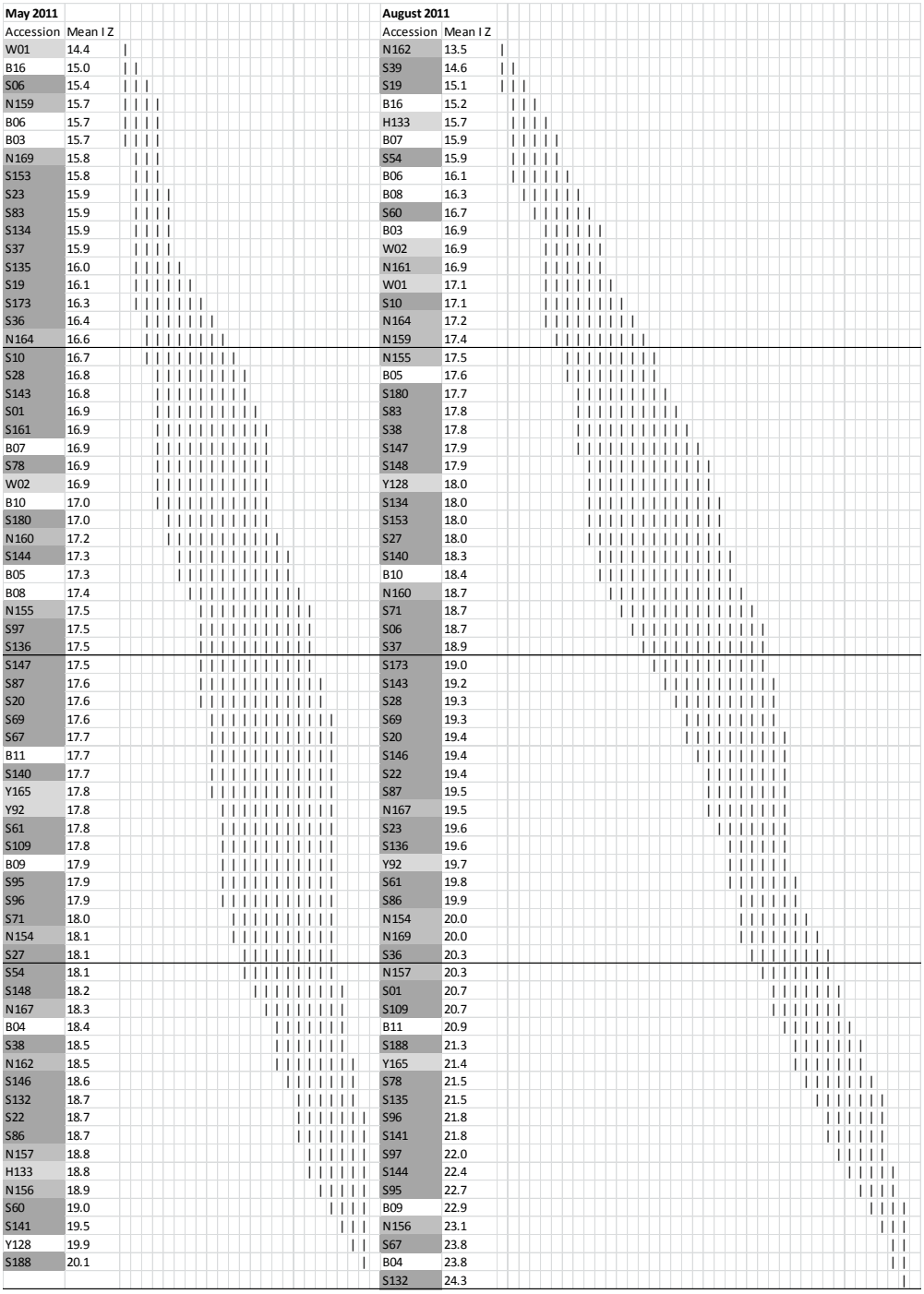


**Figure 2.10.** Mean inhibition (IZ) of *Oc-j* conidia germination of conidia at 72 hours using a bark extract disc assay in 2011, by month and ranked accession. Accessions connected by vertical lines are not significantly different according to Fisher's LSD test (P=0.05)



| May 2011 | |
|---|---|
| Accession | Mean I Z |
| W01 | 14.4 |
| B16 | 15.0 |
| S06 | 15.4 |
| N159 | 15.7 |
| B06 | 15.7 |
| B03 | 15.7 |
| N169 | 15.8 |
| S153 | 15.8 |
| S23 | 15.9 |
| S83 | 15.9 |
| S134 | 15.9 |
| S37 | 15.9 |
| S135 | 16.0 |
| S19 | 16.1 |
| S173 | 16.3 |
| S36 | 16.4 |
| N164 | 16.6 |
| S10 | 16.7 |
| S28 | 16.8 |
| S143 | 16.8 |
| S01 | 16.9 |
| S161 | 16.9 |
| B07 | 16.9 |
| S78 | 16.9 |
| W02 | 16.9 |
| B10 | 17.0 |
| S180 | 17.0 |
| N160 | 17.2 |
| S144 | 17.3 |
| B05 | 17.3 |
| B08 | 17.4 |
| N155 | 17.5 |
| S97 | 17.5 |
| S136 | 17.5 |
| S147 | 17.5 |
| S87 | 17.6 |
| S20 | 17.6 |
| S69 | 17.6 |
| S67 | 17.7 |
| B11 | 17.7 |
| S140 | 17.7 |
| Y165 | 17.8 |
| Y92 | 17.8 |
| S61 | 17.8 |
| S109 | 17.8 |
| B09 | 17.9 |
| S95 | 17.9 |
| S96 | 17.9 |
| S71 | 18.0 |
| N154 | 18.1 |
| S27 | 18.1 |
| S54 | 18.1 |
| S148 | 18.2 |
| N167 | 18.3 |
| B04 | 18.4 |
| S38 | 18.5 |
| N162 | 18.5 |
| S146 | 18.6 |
| S132 | 18.7 |
| S22 | 18.7 |
| S86 | 18.7 |
| N157 | 18.8 |
| H133 | 18.8 |
| N156 | 18.9 |
| S60 | 19.0 |
| S141 | 19.5 |
| Y128 | 19.9 |
| S188 | 20.1 |

| August 2011 | |
|---|---|
| Accession | Mean I Z |
| N162 | 13.5 |
| S39 | 14.6 |
| S19 | 15.1 |
| B16 | 15.2 |
| H133 | 15.7 |
| B07 | 15.9 |
| S54 | 15.9 |
| B06 | 16.1 |
| B08 | 16.3 |
| S60 | 16.7 |
| B03 | 16.9 |
| W02 | 16.9 |
| N161 | 16.9 |
| W01 | 17.1 |
| S10 | 17.1 |
| N164 | 17.2 |
| N159 | 17.4 |
| N155 | 17.5 |
| B05 | 17.6 |
| S180 | 17.7 |
| S83 | 17.8 |
| S38 | 17.8 |
| S147 | 17.9 |
| S148 | 17.9 |
| Y128 | 18.0 |
| S134 | 18.0 |
| S153 | 18.0 |
| S27 | 18.0 |
| S140 | 18.3 |
| B10 | 18.4 |
| N160 | 18.7 |
| S71 | 18.7 |
| S06 | 18.7 |
| S37 | 18.9 |
| S173 | 19.0 |
| S143 | 19.2 |
| S28 | 19.3 |
| S69 | 19.3 |
| S20 | 19.4 |
| S146 | 19.4 |
| S22 | 19.4 |
| S87 | 19.5 |
| N167 | 19.5 |
| S23 | 19.6 |
| S136 | 19.6 |
| Y92 | 19.7 |
| S61 | 19.8 |
| S86 | 19.9 |
| N154 | 20.0 |
| N169 | 20.0 |
| S36 | 20.3 |
| N157 | 20.3 |
| S01 | 20.7 |
| S109 | 20.7 |
| B11 | 20.9 |
| S188 | 21.3 |
| Y165 | 21.4 |
| S78 | 21.5 |
| S135 | 21.5 |
| S96 | 21.8 |
| S141 | 21.8 |
| S97 | 22.0 |
| S144 | 22.4 |
| S95 | 22.7 |
| B09 | 22.9 |
| N156 | 23.1 |
| S67 | 23.8 |
| B04 | 23.8 |
| S132 | 24.3 |

**Figure 2.11.** Sample chromatograms for 2011 bark extract S169, May. Top: plumbagin peak; Middle: juglone peak; Bottom: Total ion chromatogram. Horizontal scale is time of elution in minutes, vertical scale is relative peak size.

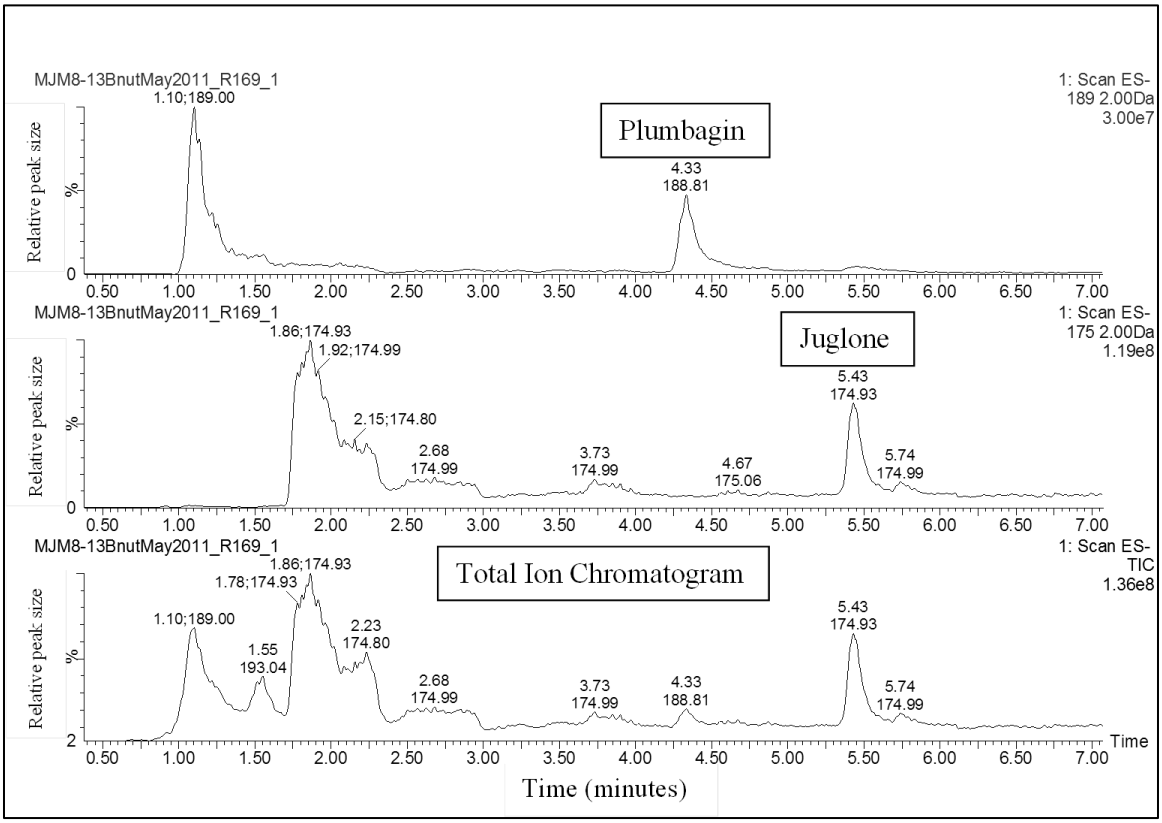


**Figure 2.12.** Juglone and plumbagin concentrations in bark extracts by month, 2010. Vertical axis is log scale (base 10). Vertical bars represent stand error, n=3.

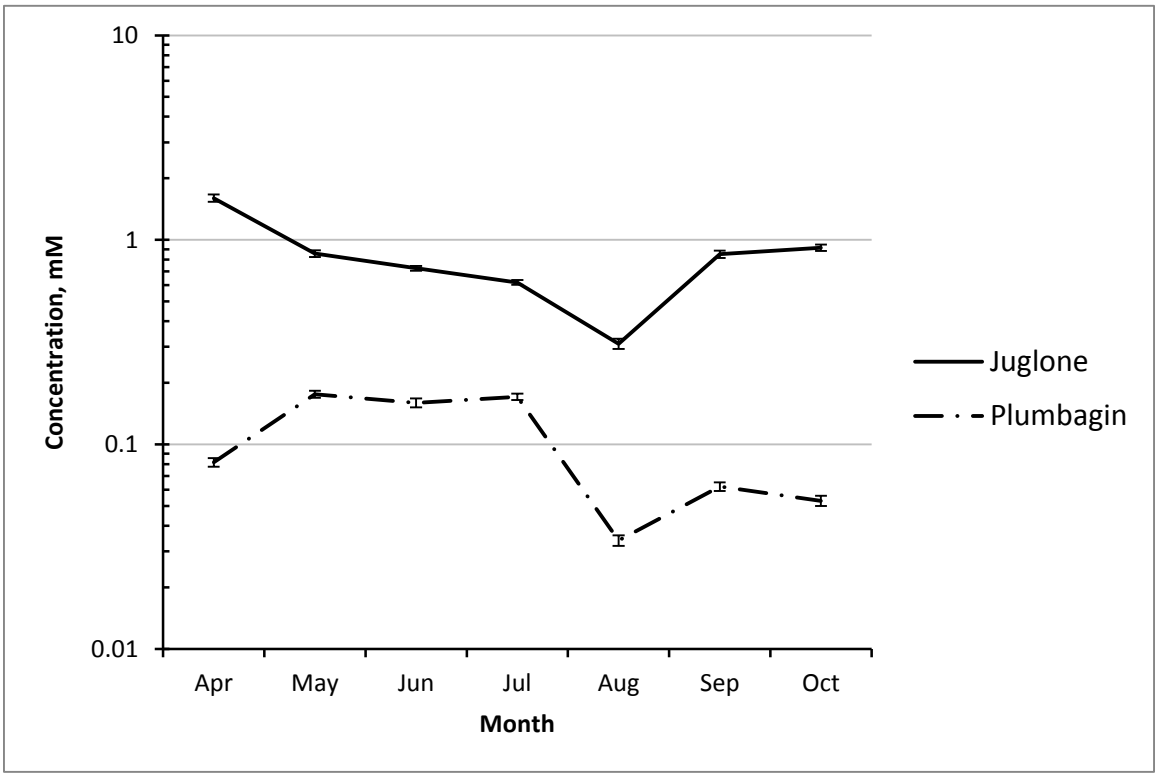


**Figure 2.13.** Juglone concentration of bark extracts in 2010, by tree source and month. B = unselected butternut (n=5), S = selected butternut (n=7), mean of three replications. Values with the same letter do not differ significantly according to ANOVA (P<0.05).

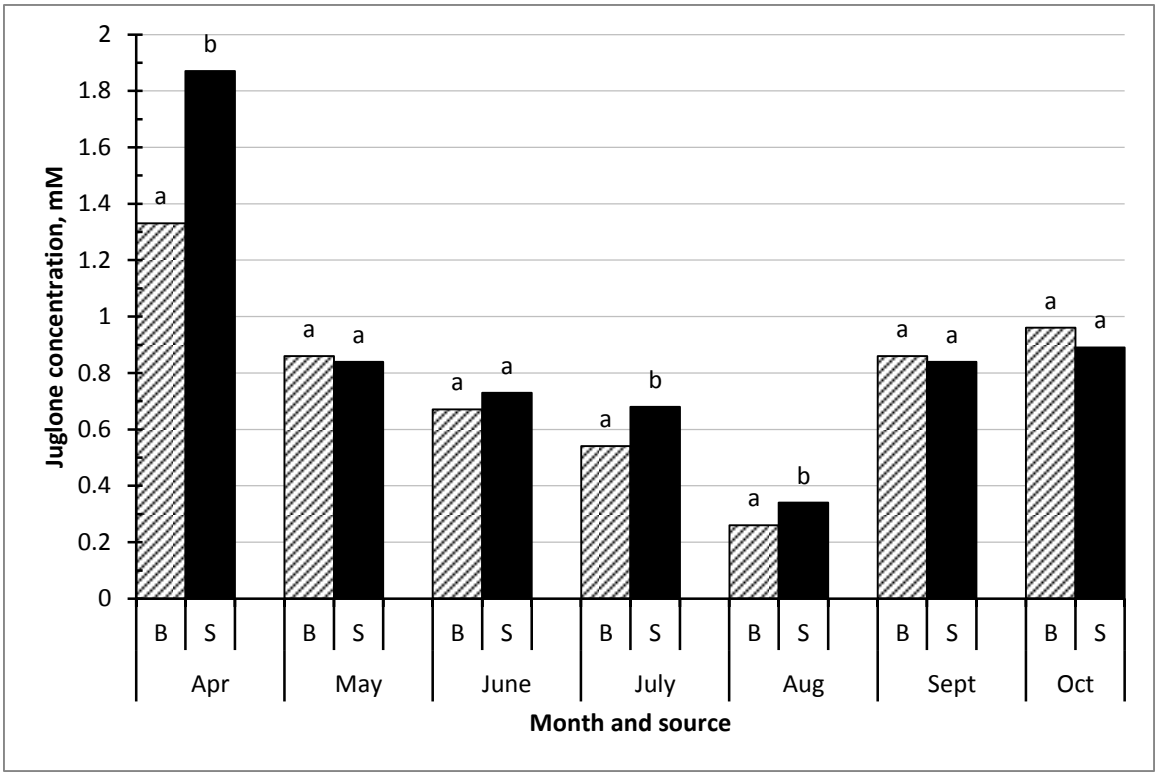


**Figure 2.14.** Plumbagin concentration of bark extracts in 2010 by month and tree source. W = black walnut (n=1), B = unselected butternut (n=5), S = selected butternut (n=7), Y = hybrid (n=1), H = heartnut (n=1). Bars represent standard error, three replications.

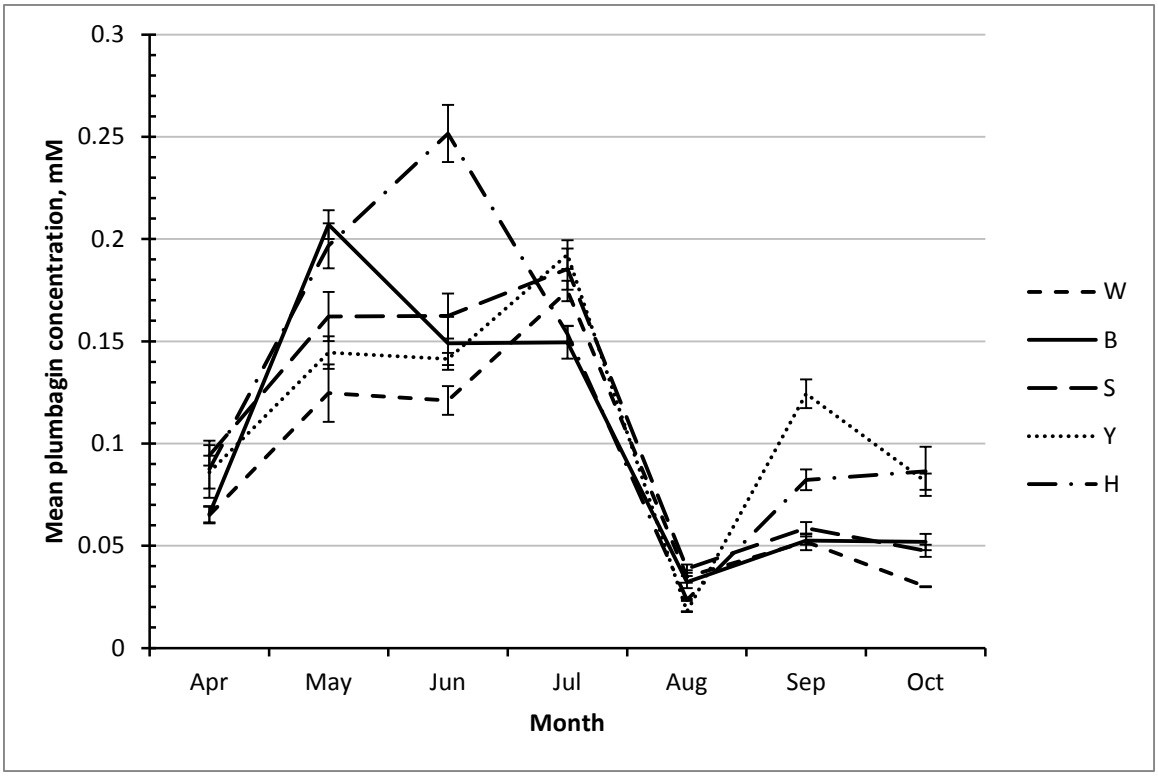
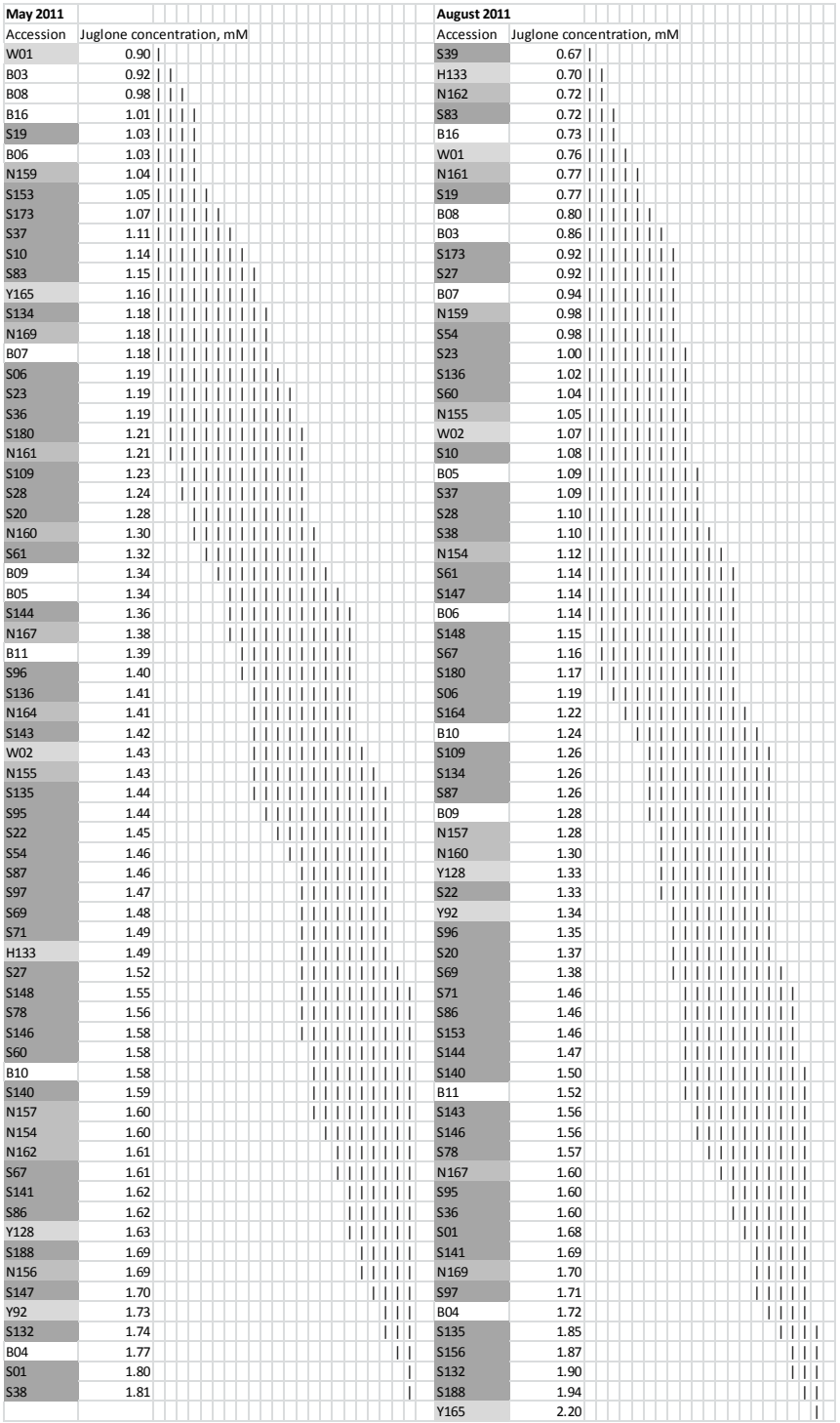


**Figure 2.15.** Mean juglone concentration of bark extracts in 2011 by month and ranked accession. Accessions connected by vertical lines are not significantly different according to Fisher's LSD test ($P<0.05$).



| May 2011 | |
|---|---|
| Accession | Juglone concentration, mM |
| W01 | 0.90 |
| B03 | 0.92 |
| B08 | 0.98 |
| B16 | 1.01 |
| S19 | 1.03 |
| B06 | 1.03 |
| N159 | 1.04 |
| S153 | 1.05 |
| S173 | 1.07 |
| S37 | 1.11 |
| S10 | 1.14 |
| S83 | 1.15 |
| Y165 | 1.16 |
| S134 | 1.18 |
| N169 | 1.18 |
| B07 | 1.18 |
| S06 | 1.19 |
| S23 | 1.19 |
| S36 | 1.19 |
| S180 | 1.21 |
| N161 | 1.21 |
| S109 | 1.23 |
| S28 | 1.24 |
| S20 | 1.28 |
| N160 | 1.30 |
| S61 | 1.32 |
| B09 | 1.34 |
| B05 | 1.34 |
| S144 | 1.36 |
| N167 | 1.38 |
| B11 | 1.39 |
| S96 | 1.40 |
| S136 | 1.41 |
| N164 | 1.41 |
| S143 | 1.42 |
| W02 | 1.43 |
| N155 | 1.43 |
| S135 | 1.44 |
| S95 | 1.44 |
| S22 | 1.45 |
| S54 | 1.46 |
| S87 | 1.46 |
| S97 | 1.47 |
| S69 | 1.48 |
| S71 | 1.49 |
| H133 | 1.49 |
| S27 | 1.52 |
| S148 | 1.55 |
| S78 | 1.56 |
| S146 | 1.58 |
| S60 | 1.58 |
| B10 | 1.58 |
| S140 | 1.59 |
| N157 | 1.60 |
| N154 | 1.60 |
| N162 | 1.61 |
| S67 | 1.61 |
| S141 | 1.62 |
| S86 | 1.62 |
| Y128 | 1.63 |
| S188 | 1.69 |
| N156 | 1.69 |
| S147 | 1.70 |
| Y92 | 1.73 |
| S132 | 1.74 |
| B04 | 1.77 |
| S01 | 1.80 |
| S38 | 1.81 |

| August 2011 | |
|---|---|
| Accession | Juglone concentration, mM |
| S39 | 0.67 |
| H133 | 0.70 |
| N162 | 0.72 |
| S83 | 0.72 |
| B16 | 0.73 |
| W01 | 0.76 |
| N161 | 0.77 |
| S19 | 0.77 |
| B08 | 0.80 |
| B03 | 0.86 |
| S173 | 0.92 |
| S27 | 0.92 |
| B07 | 0.94 |
| N159 | 0.98 |
| S54 | 0.98 |
| S23 | 1.00 |
| S136 | 1.02 |
| S60 | 1.04 |
| N155 | 1.05 |
| W02 | 1.07 |
| S10 | 1.08 |
| B05 | 1.09 |
| S37 | 1.09 |
| S28 | 1.10 |
| S38 | 1.10 |
| N154 | 1.12 |
| S61 | 1.14 |
| S147 | 1.14 |
| B06 | 1.14 |
| S148 | 1.15 |
| S67 | 1.16 |
| S180 | 1.17 |
| S06 | 1.19 |
| S164 | 1.22 |
| B10 | 1.24 |
| S109 | 1.26 |
| S134 | 1.26 |
| S87 | 1.26 |
| B09 | 1.28 |
| N157 | 1.28 |
| N160 | 1.30 |
| Y128 | 1.33 |
| S22 | 1.33 |
| Y92 | 1.34 |
| S96 | 1.35 |
| S20 | 1.37 |
| S69 | 1.38 |
| S71 | 1.46 |
| S86 | 1.46 |
| S153 | 1.46 |
| S144 | 1.47 |
| S140 | 1.50 |
| B11 | 1.52 |
| S143 | 1.56 |
| S146 | 1.56 |
| S78 | 1.57 |
| N167 | 1.60 |
| S95 | 1.60 |
| S36 | 1.60 |
| S01 | 1.68 |
| S141 | 1.69 |
| N169 | 1.70 |
| S97 | 1.71 |
| B04 | 1.72 |
| S135 | 1.85 |
| S156 | 1.87 |
| S132 | 1.90 |
| S188 | 1.94 |
| Y165 | 2.20 |

**Figure 2.16.** Scatterplots and linear regression between bioassay data (mean diameter of inhibition zone) and juglone concentration for each extract, for 2010 and 2011, listed by month. $r^2$ = coefficient of determination.

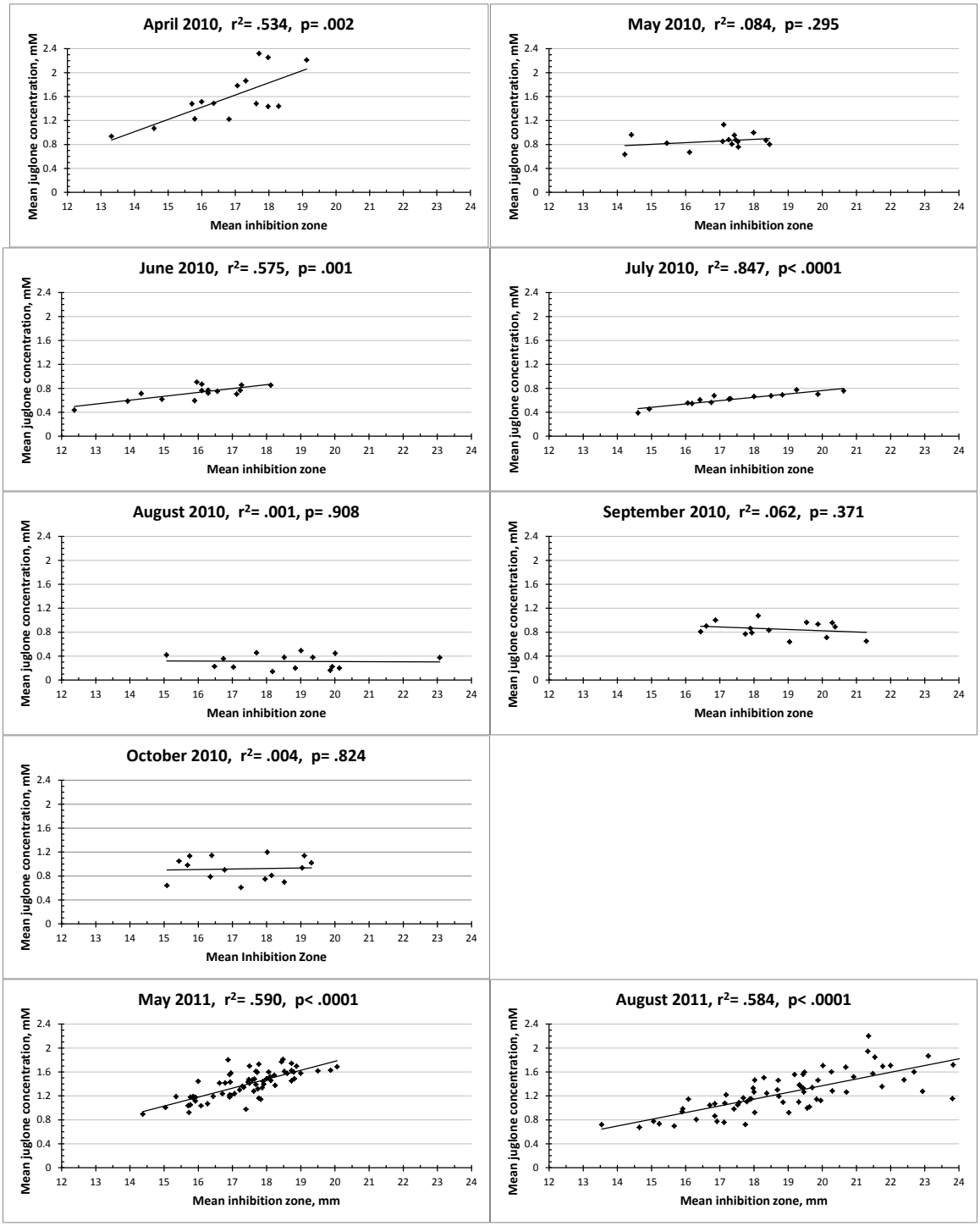


**Figure 2.17.** Scatterplots and linear regression between bioassay data (mean diameter of inhibition zone) and plumbagin concentration for each extract, for 2010 and 2011, listed by month. $r^2$ = coefficient of determination.

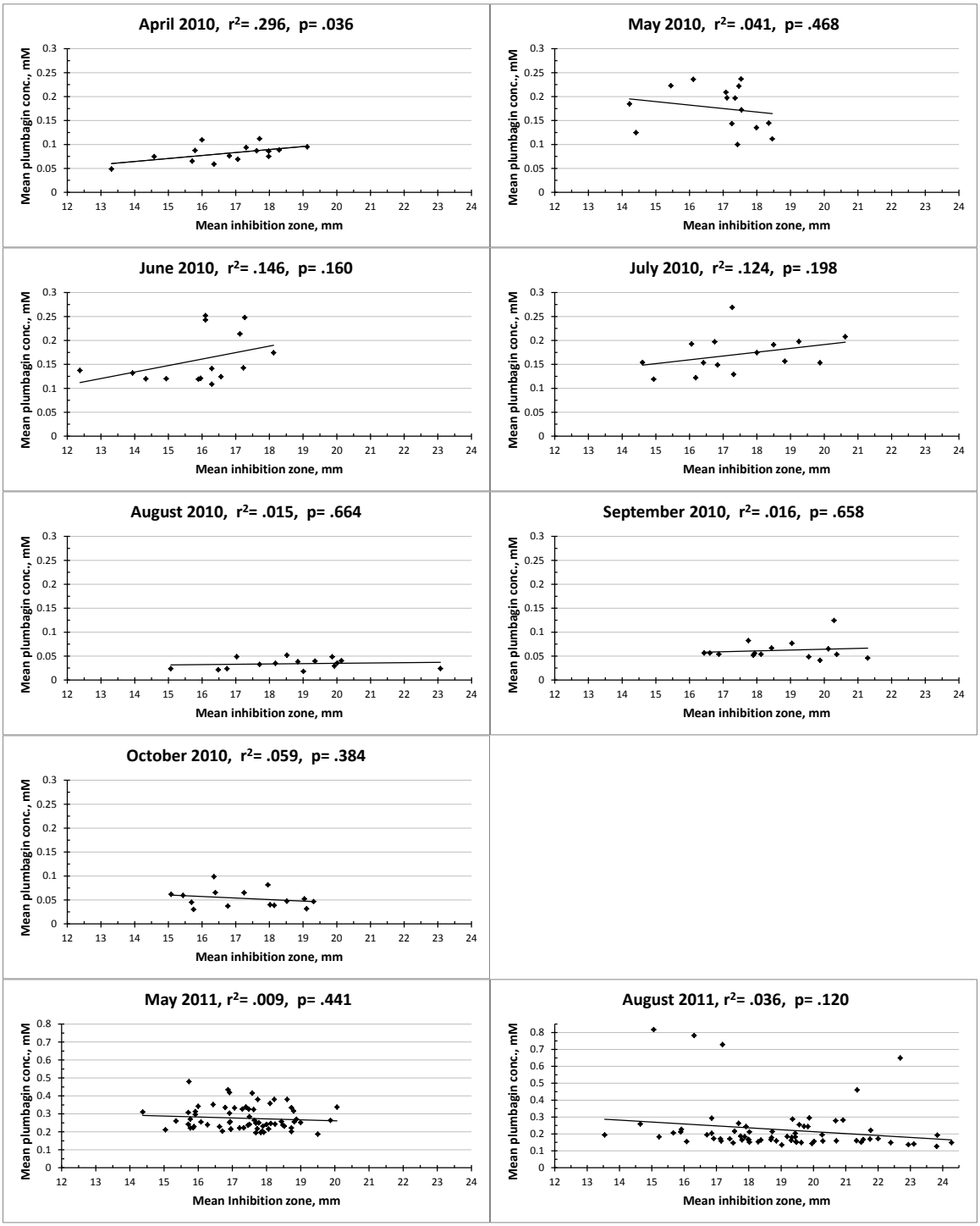
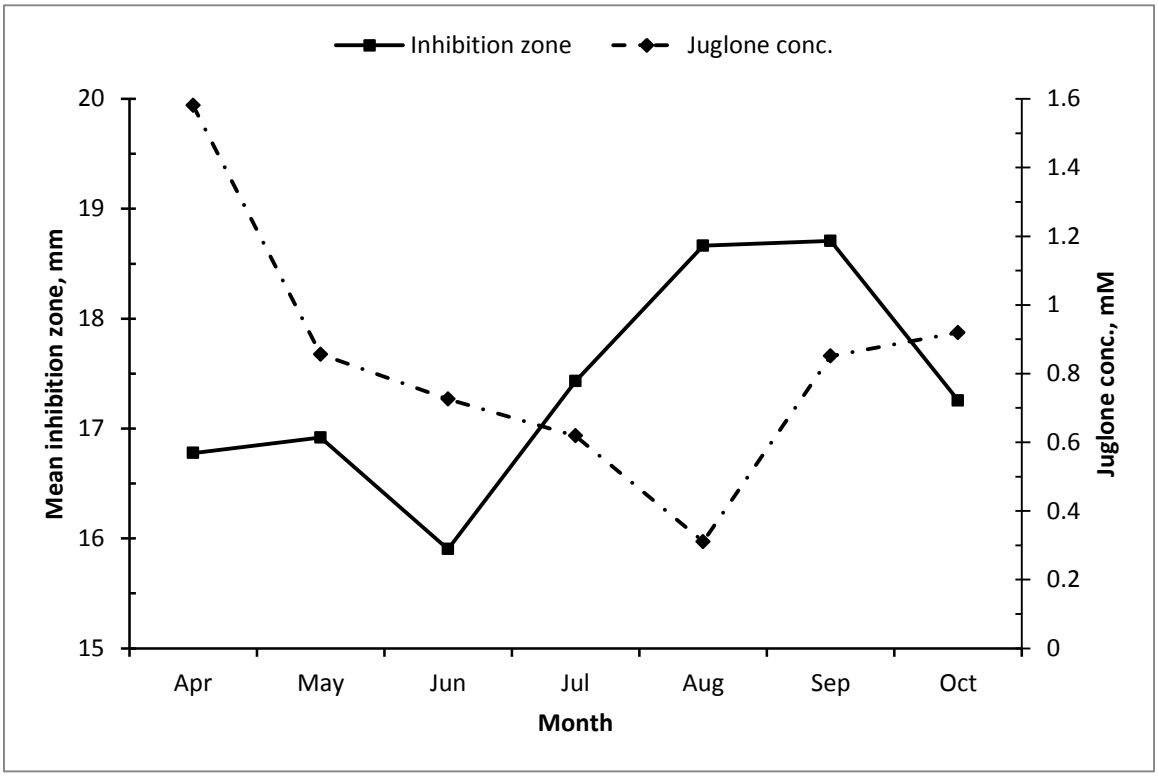


**Figure 2.18.** Inhibition of *Oc-j* conidia germination at 72 hours using a bark extract disc assay (mean diameter of inhibition zone) compared to mean juglone concentration of the same bark extracts, 2010.



## Chapter 3. Influence of temperature and humidity on the viability of *Ophiognomonia clavigignenti-juglandacearum* conidia

Butternut canker, caused by the fungus *Ophiognomonia clavigignenti-juglandacearum* (*Oc-j*) primarily kills butternut (*Juglans cinerea*). Rain splash and local air currents are the primary means of conidia dispersal, but that does not explain its long-distance spread and infection of isolated trees. Dispersal by insect or animal vectors or plant material likely necessitates the ability for conidia to tolerate drying for a period of time over variable temperature and humidity conditions. The objective of this study was to determine the influence of temperature and humidity on conidial germination and survival of air dried conidia. Conidia collected from one-month-old cultures germinated on water agar over a wide range of temperatures (4- 32$^{o}$C) and were viable after brief periods at 36$^{o}$C when returned to lower temperatures. Viability of air dried conidia held on nylon membranes at various temperatures and humidities varied from less than a day at 28$^{o}$C and 90% relative humidity (RH) to a mean of 16 days at 20$^{o}$C and 80% RH. Relative humidity had the least effect on longevity at 12$^{o}$C, with conidia remaining viable for 7 days at most humidity levels tested. Conidia held at 100% RH remained viable for 168 days at all temperatures tested. Conidia in a water suspension also remained viable for 168 days, at all temperatures tested. These results suggest that *Oc-j* conidia may remain viable on the surface of a vector or plant material and seed for over two weeks, given the proper conditions, or for much longer if in water or in an environment of saturated humidity. This potential may in part explain the frequent presence of the disease on isolated trees.

### 3.1. Introduction

Butternut canker, caused by the fungus *Ophiognomonia clavigignenti-juglandacearum* (*Oc-j*) (13) is responsible for killing butternut (*Juglans cinerea*) throughout eastern North America. The disease was first reported in the U. S. in Wisconsin in 1967 (84) and is threatening the survival of the species throughout its native range (66). Butternut, although never commercially important for timber or nut

production, is an ecologically important species in eastern hardwood forests. Scarcity of large trees resulting from tree mortality predominately caused by butternut canker has increased the value of the wood, highly prized for woodworking, and has reduced the availability of an important mast species for wildlife (68). Butternut bark extracts may have value for their high level of antibiotics (62) including some that inhibit human pathogenic fungi (26).

Butternut is the primary species that is killed by the disease, but cankers have been found on black walnut (*J. nigra*) (73) and heartnut (*J. ailantifolia* var. *cordiformis*) (64). Artificial inoculations have produced cankers on Persian walnut (*J. regia*) in a plantation (63) and grafted plants in a greenhouse were highly susceptible (69). If the disease was to become established in California, where most of the U.S. commercial Persian walnut crop is grown, it could be devastating.

The fungus may be an exotic species, possibly brought to North America on Asian species of walnut seed and/or walnut planting stock (14). Recently DNA evidence of *Oc-j* as an endophyte in twigs of *Acer truncatum* was reported from northern China (91). Stromatal columns (hyphal pegs) produced by the fungus lift and split the outer bark, exposing the pycnidia that exude a sticky matrix of conidia during wet conditions. The fungus is able to infect leaves of butternut (13) and butternut hybrids (Ostry and Moore, unpublished data). Transmission of the fungus has been attributed to dissemination of conidia by rain splash and aerosols up to 40 meters from the source (92). Conidia have been collected on spore traps over 45 meters from a source only during periods of rainfall (60). The fungus can sporulate on dead trees for nearly two years (94). *Oc-j* conidia in a simulated airborne state were viable for 8 hours in the field and at least 32 hours in an environmentally controlled chamber (93). Duration of conidia viability increased with reduced temperature and relative humidity. Conidia germinated after 48 hours on either water agar or potato dextrose agar at temperatures from 8-32$^{o}$C, with the optimum being 24-28$^{o}$C (95). The germination of conidia beyond that period has not been studied.

Dissemination of conidia in rain splash and air currents account for localized spread of the fungus, yet the disease is found nearly everywhere butternuts grow, even in

isolated locations. The fungus is seed borne on butternut and black walnut (*J. nigra*) (41) but the large heavy seeds are only moved locally by squirrels and other rodents, and long distance spread by seed would be dependent on human dispersal. Human movement of plant materials is also a potential pathway for the spread of *Oc-j*. The potential of *Oc-j* survival on the surface of seeds, seedlings and scion wood during transport and storage is unknown. Butternut trees are often found in riparian environments. Water runoff could move conidia to exposed buttress roots of butternut trees; however the viability of conidia after extended periods in water is unknown.

The importance of insects in vectoring tree pathogens such as Dutch elm disease and oak wilt is well known (97, 44). Insects can carry large numbers of conidia on their bodies and thus move a fungus from a diseased tree to a healthy one, especially during feeding activity, but reports of the time period in which the conidia remain viable are sparse. The convergent lady beetle *Hippodamia convergens* was found to be able to carry viable conidia of dogwood anthracnose (*Discula destructiva*) for up to 16 days in a laboratory study (20).

Several potential insect vectors of *Oc-j* have been identified (46). In Vermont, 17 species of beetles were found to carry *Oc-j* conidia (34). Stewart and others (90) found that conidia remained viable on the bodies of three beetle species for up to 16 days. In order to be an effective vector, an insect would need to visit both healthy and diseased trees regularly with viable inoculum during a time when infection is probable (51).

The conditions needed for maximum duration of *Oc-j* conidia viability have not been studied. Viability of fungal conidia has been more frequently studied in regards to the practical application of biocontrol agents against pathogens and insects. Conidia of *Beauveria bassiana*, an entomopathogenic fungus, could be stored for the longest at cool temperatures and low humidity (17). Viability decreased with added light, increased humidity, and increased temperature. Researchers have produced mathematical models of conidia longevity, but the models have been species dependent and even strain dependent (40). Knowledge of conidia survival requirements is also used in the study of disease epidemiology and pathogen spread.

The objectives of this study were to (1) determine the temperature range in which conidia of *Oc-j* can germinate, (2) determine the viability of *Oc-j* conidia after air drying and storage under several temperature and relative humidity (RH) regimes and (3) determine the viability of *Oc-j* conidia after an extended time in water.

## 3.2. Materials and Methods

### 3.2.1. Fungal Isolates and Preparation of Conidial Suspensions

*Oc-j* isolates were obtained from cankers on branches of affected butternut trees in Minnesota, Wisconsin and Indiana (Table 3.1). Outer bark was peeled from branches, and 5 mm chips of wood at the canker margin were excised. Samples were surface-sterilized in 10% commercial bleach for one minute, then rinsed twice in sterile deionized water. Cultures were grown on 3% malt agar in the dark at 20$^{o}$C. Conidia were harvested from sporulating one-month-old cultures by rubbing with a sterile bent plastic rod and placed in sterile deionized water. The suspension was allowed to sit for approximately ½ hour to allow any hyphal fragments, debris and agar to settle out. The conidial concentration was determined with a hemacytometer and diluted to 400-800 conidia per microliter (µl). Preliminary tests revealed that *Oc-j* conidial germination was inhibited at higher concentrations.

### 3.2.2. Temperature Effects on Germination of Conidia

The temperature range chosen for the conidia germination study was in 4-degree increments from 4$^{o}$C to 36$^{o}$C, temperatures typical of those experienced in the range of butternut during the growing season.

The studies were performed using 1.5% water agar on sterile Petri Slides (Millipore Corp, Billerica, MA). For each test, 50 µl of a conidial suspension (400-800 spores per µl) of one of four *Oc-j* isolates (Table 3.1) were placed on a slide and surface dried to remove free moisture in a laminar flow hood for approximately one half hour. They were then covered and incubated in the dark in controlled chambers at the

designated temperatures. Two replicate slides per treatment per isolate were prepared. Germination of 200 random conidia was determined daily for eight days at 200X using a light microscope until approximately 90% germination was obtained. After this, hyphae of germinated conidia made it difficult to distinguish the remaining ungerminated conidia. A conidium was considered germinated when a germ tube was seen to be at least half of the spore length. Conidia at low temperature levels were also examined at 9, 10 and 13 days. The experiment was carried out three times and results are reported as a mean of isolate and replication.

When it was clear that conidia did not germinate at 36$^{o}$C, an additional test was undertaken to determine if that temperature was lethal or merely inhibitory. Four plates (four different isolates) were incubated at 36$^{o}$C for 1-4 days and then incubated at 20$^{o}$C. Viability was assessed as described above after three days.

### 3.2.3. Temperature and Relative Humidity Effects on Viability of Air Dried Conidia

Glycerol solutions in a closed container control the relative humidity (RH) of the air in that container in direct proportion to the concentration of the glycerol. Solutions of water and glycerol were made to correspond to 40, 80, 90, and 100% RH according to published protocols (27). For each RH, 100 ml of solution was placed in the bottom of a square plastic food container (GladWare® 3 1/8 cup), and a 100 mm glass petri dish bottom was inverted over it to serve as a dry platform (Figure 3.1). Containers were sealed and allowed to equilibrate in temperature chambers of 12, 20, and 28$^{o}$C. RH was measured with a hygrometer, and glycerol solutions were adjusted if needed to +/- 3% of the target humidity. RH of 100% was achieved by using deionized water only.

Nylon membrane filters, 0.2 µm pore size, (MSI, Honeoye Falls, NY) were sterilized by dipping in 70% ethanol and air dried in a sterile laminar flow hood. Conidia suspensions, (50 µl of 400-800 conidia/µl) of two separate isolates were each placed on a membrane and allowed to air dry in open plastic 100 mm petri dishes in a laminar flow hood. When the membranes were dry they were covered (but not sealed) and placed in the prepared humidity chambers (Figure 3.1).

Daily for seven days, then at ten and fourteen days, one filter from each treatment of each isolate was removed. They were inverted onto Petri Slides containing water agar and after one half hour the membranes were peeled off, leaving the conidia behind. Petri Slides were covered, placed in a 20$^{o}$C dark incubator, and the percentage germination of 200 random conidia per plate was determined after three days at 200X with a light microscope. When germination was minimal at three days, the viability was confirmed qualitatively after seven days by visual presence of mycelial growth. After the initial fourteen day test, membranes that were still likely to contain viable conidia (had viable conidia within the previous two inspections) were monitored periodically for viability up to 24 weeks. The experiment was conducted three times. Results were reported as mean percentage germination of two isolates and three replications.

### 3.2.4. Viability of Conidia after Storage in Water at Various Temperatures

At the same time as the above experiment, 1 ml aliquots of conidial suspensions were removed and placed in sterile 1.5 ml microcentrifuge tubes. Tubes were placed in four temperatures, 4, 12, 20, and 28$^{o}$C, four tubes per isolate per treatment. At the same intervals as the above experiment but with additional inspections at 21 and 28 days, tubes were vortexed and 30 µl aliquots removed and placed on water agar on Petri Slides. They were surface dried to remove free moisture in a laminar flow hood for one half hour, covered and placed in the 20$^{o}$C incubator. For the most part, conidia did not germinate while in the water, but started germinating when surface-dried on the water agar. After three days on the Petri Slides, germination was determined as before. After the initial 28-day test, the storage suspensions were monitored every four weeks for 24 weeks as in the above experiment. A total of three replications were performed.

### 3.2.5. Statistical Analysis

Temperature effects on conidial germination were subjected to analysis by means and standard errors, one-way and mixed-model ANOVA. Survival effects of temperature and humidity on dried spores were subjected to logistic regression and survival analysis

(Enterprise Guide 4.2, SAS Institute, Cary, NC) and significance was tested at the 0.05 level.

## 3.3. Results

### 3.3.1. Temperature Effects on Germination of Conidia

Conidial germination occurred at a wide range of temperatures (Figure 3.2). Conidia at 24$^{o}$C and 28$^{o}$C reached 90% germination in 3 days. Germination of conidia at lower temperatures were progressively slowed, but all eventually reached 80% or greater except at 4 (45%), 32 (58%), and 36$^{o}$C (0%).

Germination rates varied by isolate, but were significant only for a few treatments. For example, with conidia held at 4$^{o}$C, isolate was only significant at day 9, when the mean germination ranged from a low of 18% to a high of 73% (Figure 3.3).

Ungerminated conidia held at 36$^{o}$C and then placed at 20$^{o}$C were found to be viable, with germination varying by the number of days held at 36$^{o}$C: after one day, 79%, 2 days, 26%, 3 days, 3%, and after 4 days less than 1%.

### 3.3.2. Temperature and Relative Humidity Effects on Viability of Air Dried Conidia

Significant ($p<0.0001$) effects were found for temperature and humidity using both logistic regression and survival analysis. There was no significant difference in viability between isolates (data not shown), so data from all isolates were combined.

Conidia at the highest temperature lost viability more quickly than at lower temperatures (Figure 3.4, Table 3.2). Conidia held at 28$^{o}$C had a maximum viability of one day at 40% and 90% RH and six days at 80% RH. Conidia held at 20$^{o}$C had a maximum viability of three days at 40% and 90% RH and 21 days at 80% RH. At both these temperatures, 80% RH increased conidia viability. Conidia held at 12$^{o}$C were less affected by RH. Maximum viability of conidia at 40% and 80% RH was seven days, and at 90% RH, ten days.

Some conidia held at 100% RH remained viable up to 168 days (24 weeks) at all temperature levels tested. However, at 4 to 24 weeks, some hyphal strands were seen

immediately after plating, evidence that some conidia had already germinated. Those that grew when in contact with the water agar were counted as viable. Over time there were also fewer intact conidia and increasing amounts of debris on the membrane, thus no percentages were possible and qualitative data was used beyond that point.

### 3.3.3. Viability of Conidia after Storage in Water at Various Temperatures

Conidial germination decreased steadily when held in water at all temperatures from an initial mean of 91%, to 43% and 26% at 14 days and 28 days, respectively. Differences in germination between temperatures were slight at first but increased with time (Figure 3.5). There was germination of at least some conidia over each temperature treatment for the full 24 weeks.

## 3.4. Discussion

The goal of this study was to better understand the potential long-range dispersal of *Oc-j* conidia by studying the effects of temperature and humidity on their long-term viability under laboratory conditions. Conidia of *Oc-j* germinated at a wide range of temperatures. Although rates of germination varied, 80% or greater of the conidia germinated in seven days or less at temperatures of 12 to 28$^{o}$C. This agrees with the results of an earlier study (95) which showed germination at a similar range of temperatures. However, in that study germination was monitored for only 48 hours, whereas in this study conidial germination was monitored up to 13 days for the lowest temperature. The earlier study did not detect conidial germination at 4$^{o}$C. In the current study, at 4$^{o}$C the conidia did germinate, though slowly. Germination of conidia was somewhat inhibited by 32$^{o}$C and completely inhibited by 36$^{o}$C, though it did not immediately kill them. When brought to 20$^{o}$C after an exposure at 36$^{o}$C for one or two days, germination occurred. This evidence suggests that *Oc-j* conidia, dispersed to a suitable host, could germinate over a wide range of temperatures that would be present throughout the growing season. Germination is not limited by cool periods, but possibly limited by excessive heat.

The key factor for long-distance dispersal of infective conidia is the survival of dried conidia on a surface, such as the exoskeleton of an insect on body parts of a mammal or bird. This was investigated using nylon membranes with air dried conidia stored at a wide range of temperature and humidity conditions. The results of this study demonstrated that conidial viability can be sustained for about 16 days at non-saturated humidity and for much longer periods in saturated humidity.

Tisserat and Kuntz (93) found a decrease in longevity of simulated airborne *Oc-j* conidia at their highest humidity (>95% RH). However, the lifespan of those conidia was in hours (32 hour maximum measured), not weeks as this study has demonstrated. Airborne conidia may be quite different physiologically than conidia dried on a surface.

Clerk and Madelin (17) generally found a decrease in viability of stored conidia as humidity increased, but did not test their conidia at saturated humidity. They also found that one fungus, *Metarrhizium ansiopliae*, had good viability at high and low humidities but very poor viability at 40-50% RH, demonstrating a unique viability curve. *Oc-j* conidia demonstrated relatively poor viability at 40 and 90% RH and better viability at 80% RH, suggesting a species-specific conidia viability pattern.

This study also demonstrated that *Oc-j* conidia may remain viable for extended periods of time in free water over a wide range of temperatures. This may have implications for dispersal in water in as well as on movement of plant material.

*Oc-j* conidia are produced by pycnidia which exude a sticky matrix, and in this study the matrix was diluted in order to produce a suspension of conidia of known concentration. Preliminary studies found very poor germination of conidia in suspensions of high concentrations, possibly because of inhibitors present in the matrix (unpublished data). In a natural setting the matrix includes substances, usually found to be polysaccharides and protein (61), which may increase longevity of conidia. *Colletotrichum graminicola* conidia survived up to four weeks when they remained embedded in the original matrix, versus washed conidia which survived 1-2 days. Further studies involving the role of the matrix in conidial viability of *Oc-j* and other factors that may influence the viability of conidia such as the effect of UV light, the presence of other microbes, and surface chemistry should be investigated.

**Table 3.1.** *Ophiognomonia clavigignenti-juglandacearum* (*Oc-j*) isolates from cankers on *J. cinerea* used in conidia viability studies. Isolates were kept separate in each study.

| | | **Temperature study** | | **Viability study** |
|---|---|---|---|---|
| **Isolate** | **Location** | Rep 1 & 2 | Rep 3 | All reps |
| 1343 | Whitewater, WI | X | | |
| 1352 | New Ulm, MN | X | | |
| 1363 | Rum River State Forest, MN | X | | |
| 1366 | LaCrosse, WI | X | X | |
| 1370 | Nicolet National Forest, WI | | X | |
| 1374 | Mille Lacs Wildlife Area, MN | | X | |
| 1378 | Hoosier National Forest, IN | | X | |
| 1391 | Roseville, MN | | | X |
| 1394 | Afton, MN | | | X |

**Table 3.2.** Longevity in days of air dried *Oc-j* conidia in humidity chambers and in water. Numbers represent the last day viable conidia were found. Conidia were not held past 168 days (24 weeks).

| | **Temperature** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Relative** | **12°C** | | | **20°C** | | | **28°C** | | |
| **Humidity** | Rep1 | Rep2 | Rep3 | Rep1 | Rep2 | Rep3 | Rep1 | Rep2 | Rep3 |
| 40% | 7 | 7 | 7 | 3 | 3 | 2 | 0 | 1 | 0 |
| 80% | 7 | 7 | 7 | 10 | 17 | 21 | 2 | 6 | 6 |
| 90% | 6 | 7 | 10 | 2 | 3 | 2 | 0 | 0 | 0 |
| 100% | 168 | 168 | 168 | 168 | 168 | 168 | 168 | 140[a] | 112[b] |
| In water | 168 | 168 | 168 | 168 | 168 | 168 | 168 | 168 | 168 |

[a] replication had insufficient membranes, endpoint unclear
[b] contamination occurred at later dates, endpoint unclear

**Figure 3.1.** Diagram of humidity chambers used to test viability of *Oc-j* conidia.

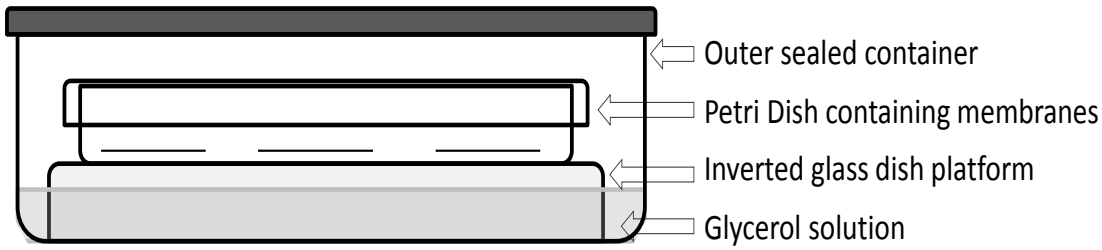
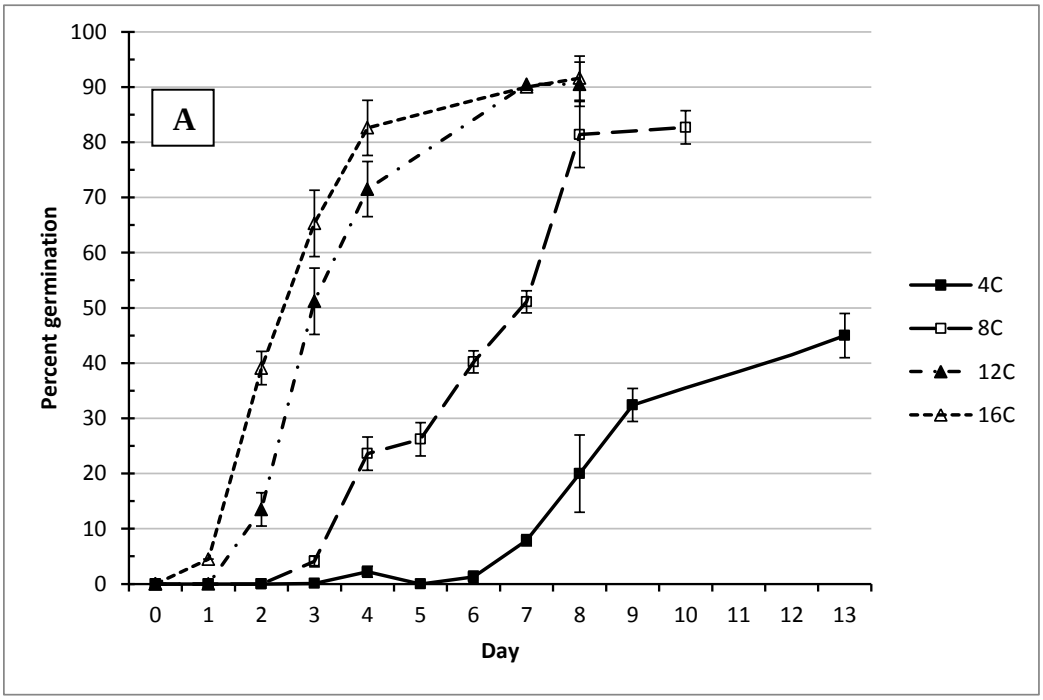


**Figure 3.2.** Germination of *Oc-j* by temperature and day, grown on water agar. Percentage rates are combined means of observations of 200 conidia on each of four single isolates, two plates each, and three replications. Bars represent standard error.



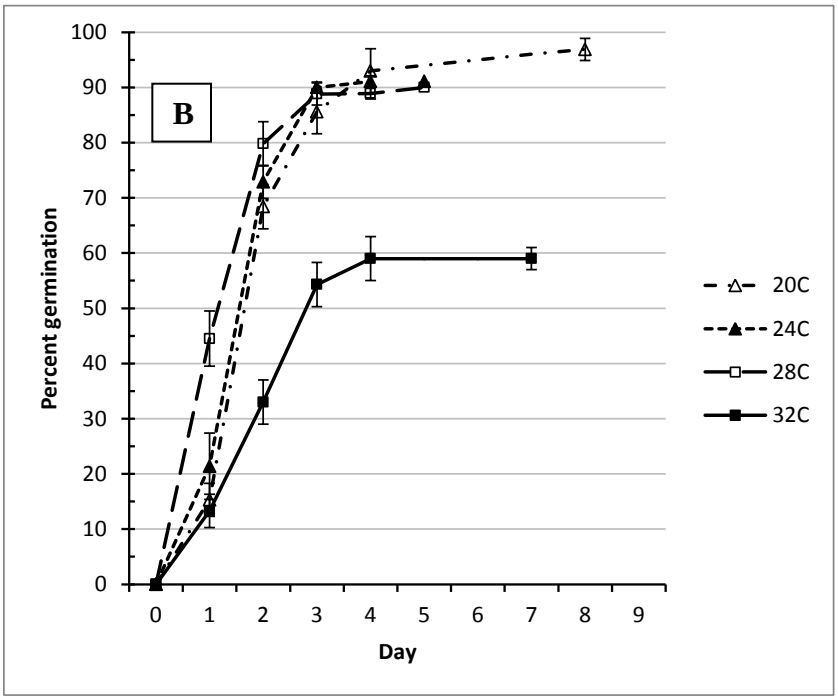



**Figure 3.3.** Germination of *Oc-j* conidia from several isolates at 4°C on water agar. Bars represent standard error of three replications. Plates not counted at 13 days were too overgrown by hyphae to distinguish remaining ungerminated conidia.

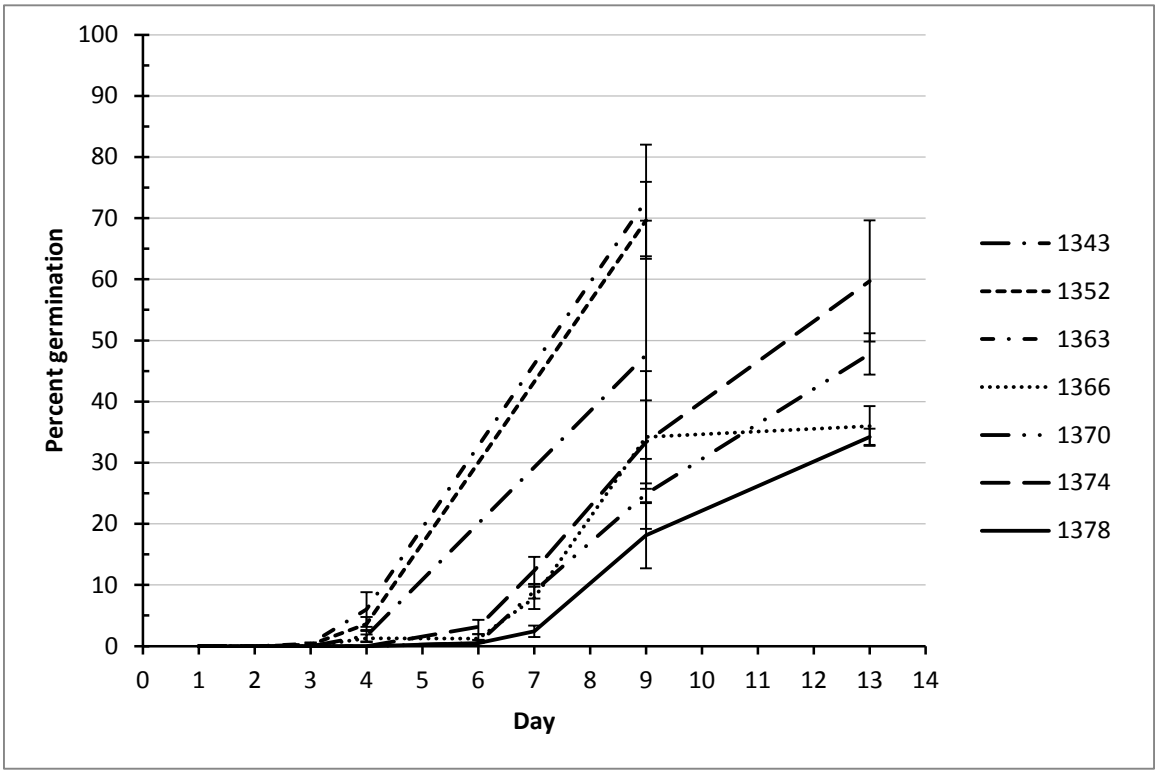


**Figure 3.4.** Germination of dried *Oc-j* conidia held on nylon membranes at 12, 20, and 28°C and 40, 80, 90 and 100% relative humidity over 14 days. Conidia were germinated on water agar at 20°C. Percentages are means of three replications and two isolates. Bars represent standard errors.

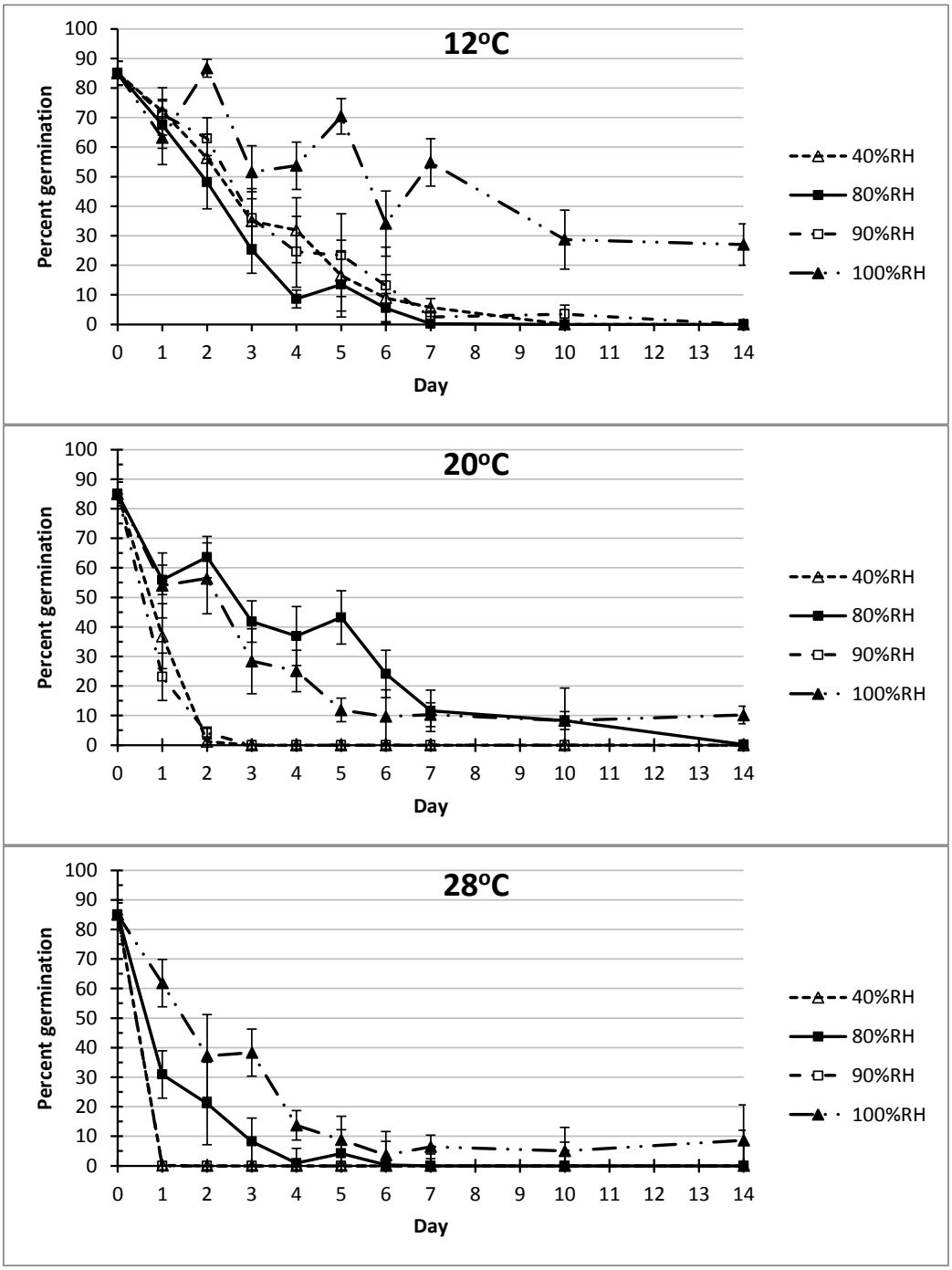
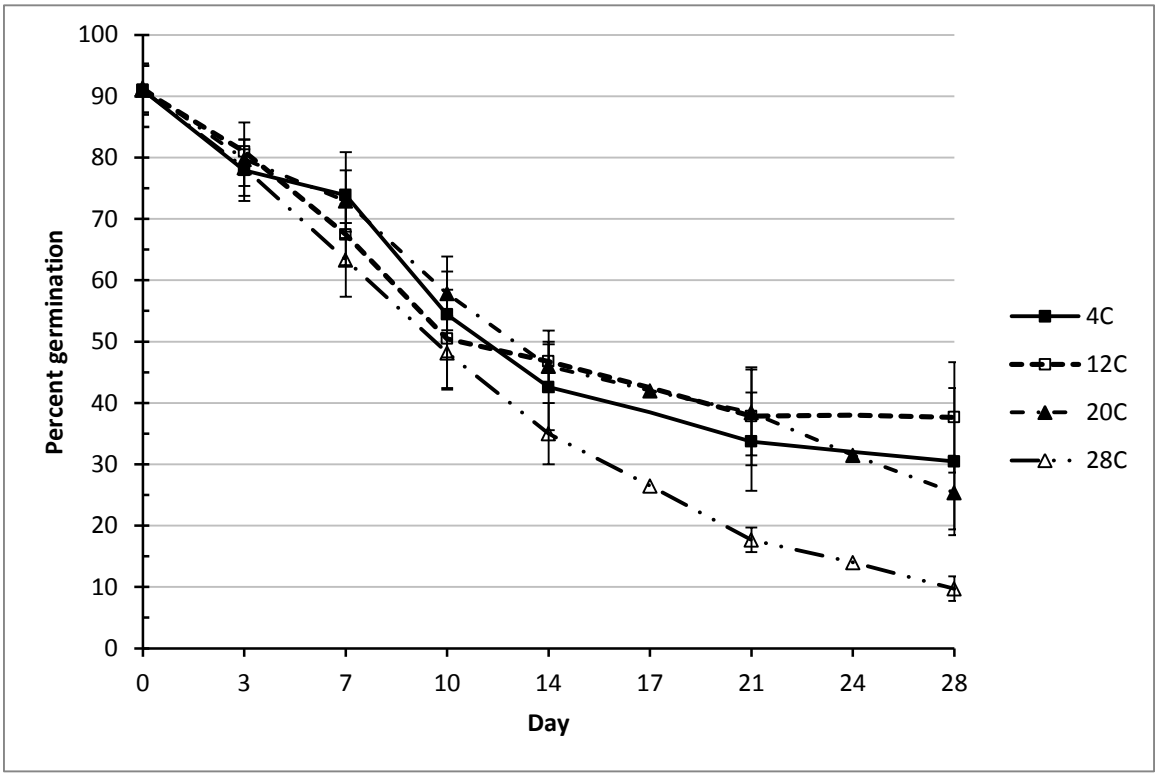


**Figure 3.5.** Germination of *Oc-j* conidia after storage in water suspension at four temperatures. Conidia were germinated on water agar plates at 20°C. Percentages are a mean of two isolates and three replications, bars represent standard error.



## Chapter 4. Conclusions

The list of North American tree species being seriously threatened by invasive, introduced diseases is long and new diseases continue to be found. Beginning with chestnut blight, white pine blister rust, and Dutch elm disease, the list has continued to grow with newer introductions of butternut canker, beech bark disease, and laurel wilt, among many others. In all of these cases, a disease-causing fungus or an insect-fungus complex has been imported from another continent and has taken hold in an environment without the usual checks and balances of the native ecosystem. Naive trees are left defenseless against the new enemy. The result has been eastern forests essentially devoid of chestnut trees, northern conifer forests with few white pines, and cities without their traditional elm-lined streets. If the trend continues, more species will be affected and some may disappear from our landscape.

Fortunately, there are reasons to be hopeful. After years of research and breeding, blight-resistant "nearly-American" chestnuts are beginning to be planted in test sites on the National Forests and early reports of growth and disease resistance are encouraging. There is little hope that the chestnut will ever achieve its former dominance in the eastern forest, but it may be restored as a component. On another front, years of selection pressure on the American elm by Dutch elm disease have revealed a handful of tree selections that are tolerant to the disease. Most of these selections are not truly resistant, but tolerant and they usually recover from a wilting episode after being inoculated by the fungus. Again, no one expects that the American elm will ever dominate our parks or city streets again, but it is beginning to be a viable landscape option.

Butternut has never had the place of dominance of either chestnut or elm, but as such should not be minimized. Its role, not just in the ecosystem, but its usefulness for food, wood and chemical products, should not be ignored. Research aimed at understanding butternut canker disease and finding resistance to it has lagged far behind that of chestnut blight and Dutch elm disease, both in time and resources. Gratefully, it has not been ignored. A number of forest-related agencies in several locations have been

actively engaged in searching for and propagating resistant selections of butternut, and research orchards are being planted. Some of the active programs include:

- Oconto River Seed Orchard, Wisconsin, run by the US Forest Service, Region 9, has a grafted orchard of 250 trees from 78 predominantly Wisconsin butternut selections (Scott Rogers, personal communication).
- The Ontario Ministry of Natural Resources, Canada has an active selection, grafting and seeding program, with 400 trees of 40 grafted selections to date on three sites and thousands of seedlings from healthy trees (Barb Boysen, personal communication).
- In Vermont, several federal, state and private agencies have teamed together to plant and maintain two grafted orchards with about 80 local selections. Also, a butternut seed planting project is planned over the next several years (Dale Bergdahl, personal communication).
- The Hardwood Tree Improvement Research Center based at Purdue University in Indiana in conjunction with the US Forest Service has a grafted orchard and is involved in inoculation, breeding, and selection. They have been involved with setting up grafted orchards in several other states (Missouri, Pennsylvania, Ohio, and West Virginia) with 22-35 selections each. There are butternut progeny seed tests in Indiana and Michigan. They are also involved in increasing resistance using hybrid butternuts and backcrossing to a more native form (Jim McKenna, personal communication).

The research presented here could have implications for these butternut selection and restoration efforts. The extract bioassay procedure described in Chapter 2 showed that at some periods during the growing season there was a statistically significant difference between selected and unselected butternut bark extracts in their inhibition of *Oc-j* germination. It also showed that this inhibition could be correlated to juglone content in some months. If the bioassay is found to correlate with actual field resistance, it could be an invaluable tool to save time and effort. With further study and refinement, a workable bioassay could be devised that would be useful in screening butternut trees in several

settings. It could be used as an initial screening of a potential selected tree, either before or in conjunction with scion wood collection. The bioassay could be especially helpful in the grafted orchards as these selected butternuts will need further screening to discern resistant accessions from escapes, or in seed trials to separate improved seedlings from wild type.

From the work described in Chapter 3, it was found that *Oc-j* conidia can germinate and grow under a wide range of temperatures, but are limited by extreme heat. The *Oc-j* viability study showed that conidia could survive surface drying and remain viable for two weeks or more under specific conditions. This information would also be useful in restoration efforts. It is vital to understand epidemiology and spread of the disease as these selected butternuts are challenged or re-planted in new locations, and it will be important to monitor insect populations and study their role as vectors.

The butternut tree will probably never again be the “common white walnut” of colonial days. But with continued research and concerted effort, it may continue as a vital part of our North American landscape.

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