Fungi isolated from black walnut branches in Indiana and Tennessee urban areas

A THESIS
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE

Jennifer Juzwik, advisor

March 2016

Acknowledgements

First and foremost, thanks to my advisor, Dr. Jennifer Juzwik, for accepting and guiding me as her advisee. She has provided expertise, understanding, patience and wit throughout my educational journey. I would also like to thank Dr. Robert Blanchette, for his encouragement and his insights as a member of my graduate committee, and for facilitating my graduate status during his active role as Director of Graduate Studies. Thanks to my graduate committee member Dr. Brian Aukema for his insightful observations, which aided the clarification of my objectives and my writing process. Also, a heartfelt thanks to Dr. Jim Bradeen, as he encouraged me to pursue this advanced degree from the very start of my coursework.

This thesis would not exist without the help I received in obtaining samples, sample processing, fungal isolation, and identification. Thanks to Dr. Bill Klingeman and Phil Flanagan at the University of Tennessee in Knoxville, to Matt Paschen at Purdue University in Indiana, to Mark Banik at the Wood Products Laboratory in Madison, to Jeanne Ciborowski and Stephanie Dahl of the BSL-2 at the University of Minnesota, and to the undergraduate assistants Antoine Tireau and Lijun Duan for their efforts and time towards this work.

A big thanks to Paul Castillo, for his versatile skills at so many points along the way. Thanks for all the support from people at the Forest Annex, who have encouraged me to undertake and continue my education.

Finally, I would like to thank my family – my parents, my siblings, my husband and my daughters for being my go-to role models, and for the lifelong support they have provided in countless ways.

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1.1. Introduction

Eastern black walnut (*Juglans nigra*) is a highly valued tree, both by commercial standards and by product aesthetics. Milled lumber and veneer produced from black walnut set this tree among the highest valued timber species produced in the United States (Conservation Commission of Missouri 2016). The estimated value of black walnut growing stock in the U.S. is greater than $539 billion (Newton et al. 2009). The wood itself is finely straight-grained and strong, responding beautifully to steam bending techniques. These qualities give black walnut high popularity in fine solid-wood cabinetry and gunstocks. Black walnut also is an important commodity for export to 67 countries, including those in Europe and Asia (Newton et al. 2009). This species is additionally valued for nut production and subsequent use in confections. The nuts also provide an important food source for wildlife.

The native range of *J. nigra* includes much of the eastern half of the U.S. (fig.1-1). Kansas is on the western fringe of the native range where the species is fairly abundant. The highest growing stock volume of *J. nigra* is found in Missouri (Oswalt et al., 2012). However, commercially important levels of black walnut also occur in a number of other states including Indiana, Kentucky and Tennessee.

J. nigra grows naturally as scattered individual trees or in small groups on a variety of sites. However, it grows best on well-drained bottomland or riparian sites in the

Appalachians and the Midwest. *J. nigra* is also found in urban and suburban landscapes. The species is grown for timber in plantations across the U.S. (Walnut Council 2016).

Although a large number of plant pathogenic microorganisms have been found on black walnut, there are only a few serious, fungus-caused diseases of the species. However, their impact has been considered significant. A number of insect species damage black walnut, but only a few are considered to be serious pests. Several serve as important vectors of walnut disease organisms. This chapter presents brief discussions of selected canker, root, and foliar diseases and selected insect pests, including bark and wood-boring insects, sucking insects, and defoliators of *J. nigra*.

1.2. Canker diseases

Canker diseases are a particularly destructive problem of hardwood trees. They are caused by biotic agents (mostly fungi) or abiotic conditions or injuries that cause damage to the bark and cambium. Microbial infection or abiotic injury results in a definite, localized area of dead tissue that may appear as sunken, raised, discolored or cracked (Shurtleff and Averre 1997). As biotic agents colonize living branch or stem tissue the cambium is killed. Multiple or coalescing cankers lead to girdling of branches and stems and often to tree death. Breakage at the canker ("tree-snap") may occur. Open cankers also provide an avenue of entry for decay fungi. Disfiguration or malformation of stem tissue may render butt logs unmerchantable. The most damaging types of cankers are those that grow unabated by host responses to invasion resulting in diffuse or perennial cankers.

1.2.1. Thousand cankers disease (TCD) – *Geosmithia morbida* (fungal pathogen) and *Pityophthorus juglandis* (insect vector)

Thousand cankers disease (TCD) currently represents the greatest threat to black walnut health, both within and outside the native range. The disease is a complex involving the fungus (*Geosmithia morbida*) that is transmitted by the walnut twig beetle (*Pityophthorus juglandis*). It was first recognized in 2007 (Tisserat et al. 2009) with the first report of the disease in its native range in 2010 (Seybold, Haugen, and Graves 2013). The insect pest may aggressively attack black walnut, introducing the pathogen along its entry tunnel or in excavated galleries. Thousands of insect attacks may lead to thousands of cankers on branches and stems. The first visual symptoms often observed are a thinning crown, leaf flagging and chlorosis, or individual dying branches. These symptoms resemble those of other diseases of black walnut or even of abiotic stress. If a very thin layer of the bark is removed, small, mostly circular cankers may be seen, usually associated with tiny insect holes, tunnels or galleries characteristic of *P. juglandis* (fig. 1-2). The cankers block the flow of nutrients (photosynthetic products) to the roots often contributing to the death of the tree. Small holes where the beetles have entered and exited the bark may be seen, especially on branches. Recent findings indicate *G. morbida* may be carried at least casually by insects other than *P. juglandis*, including ambrosia beetles (Juzwik et al. 2016 in press). The cankered tree may die 2 to 3 years after the first canopy symptoms are noticed. Disease development in the Eastern U.S. may be correlated with drought stress (Griffin 2015). Symptom progression in multiple TCD trees in Tennessee and Virginia appeared to be halted and other trees appeared to recover from the disease during a 2011-2013 monitoring study (Griffin, 2015). To date, there is

no practical treatment or control for TCD. State imposed quarantines restrict movement of walnut between states/counties in a number of eastern states.

1.2.2. Fusarium canker – *Fusarium* spp.

Fusarium cankers appear as breaks in the bark, or as sunken areas, that develop into elongate cankers (fig. 1-2). The wood beneath the canker is darkened and is often exposed as callus tissue forms at the margin of the perennial invasion of bark tissues by the pathogen. Sprouts often occur near the canker or base of tree, and may be the first visual indication of the disease. Injury includes leaf wilt and crown dieback. Young trees may be killed. Even when re-sprouting at ground level occurs, years of growth may be lost. Up to six *Fusarium* species have been associated with cankers and dieback, with *F. solani* being most frequently found (Carlson et al. 1993). Pruning wounds are a common entry point for *Fusarium* species that subsequently colonize the tree (Carlson et al. 1993). The fungus may also be introduced by ambrosia beetles, primarily *Xylosandrus germanus* (Kessler 1974). *Fusarium* canker may be controlled by the restriction of pruning to late winter. Cutting and burning infected trees may also contribute to disease control.

1.2.3. Perennial target canker – *Neonectria galligena*

Nectria canker first appears as a slightly sunken, elongated lesion. The surface of the outer bark is often discolored. The tree creates a callus ridge during the growing season as it attempts to contain the growth of the fungus. When the tree is not successful, the fungus will reinfect healthy wood beyond the callus ridge the following growing season. The damage has a target-like or concentric appearance (a perennial canker), because of the alternating fungal growth and the production of callus tissue (fig. 1-2).

Sexual fruiting bodies called perithecia (appearing as pink or cream colored cushions) are formed during spring and summer, and asexual fruiting structures called sporodochia (appearing as red to brown or black bumps) are formed in late summer to early fall. Wounds provide an entrance for ascospores and conidia dispersed from the perithecia and sporodochia, respectively, by wind and rain. Infection can occur during wet periods throughout the growing season. *N. galligena* occurs on many hardwood trees, so may be introduced by the fungus on neighboring trees. A single Nectria canker seldom girdles branches or stems, but coalescing of multiple cankers may lead to branch or stem death. Affected trees often break at the canker site and so are vulnerable to "stem snap". Preventative measures for the disease includes pruning during dry periods, and using pruning tools disinfected between each cut. This disease is most severe on stressed trees; thus, cultural practices that promote optimal growing conditions and maintain tree health are recommended.

1.3. Root rot diseases – *Phytophthora* spp (primarily *P. citricola*), *Cylindrocladium* spp. (primarily *C. scoparium*)

Phytophthora root rot, *Cylindrocladium* root rot are covered here.

Root diseases of *J. nigra* are mainly limited to nursery plantings, and are generally not observed in landscape or plantation settings. In bare-root nurseries, black walnut seedlings with root disease may be severely affected and experience significant levels of mortality. Symptoms initially include leaf yellowing and wilt. The stem of an infected seedling later turns black, as the causal agent grows through the roots to the root collar and into the lower stem. *Phytophthora* root rot may differ visually from *Cylindrocladium* root rot by its water soaked appearance in the blackened areas of

affected roots (fig. 1-3). This disease may develop during winter storage if moisture and temperature conditions are favorable. Because of this, northern nurseries should locate "heeling-in" beds in areas with good drainage. Infected seedlings may die rapidly upon transplant. Root rot will occur in patches, and is always more severe in low areas of nursery beds. The motile spores of *Phytophthora* species can move through wet soils in such areas. *Cylindrocladium* does not produce motile spores and normally develops from microsclerotia, small dormant structures in the soil. The microsclerotia may be visible to the naked eye and resemble small aggregated sand particles. These thick-walled fungus propagules are difficult to eliminate from soil by chemical fumigation. *Phytophthora* root rot is particularly prevalent in north central U.S. (Green 1975), whereas *Cylindrocladium* prefers warm soil conditions and thus is a more serious problem in southern nurseries, although some species occur in the north (Juzwik and Barnard 2012). Control methods for these root diseases include soil fumigation, soil sterilization using sunlight (solarization), and by avoiding, preventing and/or correcting high soil moisture level conditions (Kessler 1982).

1.4. Foliar diseases

Foliar diseases begin as spots and/or premature leaf yellowing. When severe, premature defoliation of diseased leaves occurs. Repeated defoliation can reduce growth and cause tree stress, thus, lowering host resistance to other pathogens or insect pests. Control is best achieved by choosing optimal planting sites, using good sanitation, mowing to control weed and grass competition, and applying fertilizers as needed.

1.4.1. Walnut anthracnose – *Gnomonia leptostyla*

Anthracnose symptoms appear as somewhat circular brown lesions (from pin-point size to 5 mm in diameter) on leaves followed by leaf yellowing and premature leaf drop when severe (Berry 1981) (fig 1-4). Numerous lesions can also lead to marginal browning and curling of leaflets. Symptoms are strikingly noticeable; however, the disease seldom causes much reduction in tree growth. Trees may be completely defoliated in August and September, but most tree growth has already occurred by this time. Extended wet periods promote fungal infections shortly after full leaf expansion (Kessler 1984). The disease progresses rapidly in mid-summer and results in premature defoliation by late August. Despite minimal impact to tree growth, premature leaf drop is one of the major causes of poor and irregular nut production (Beineke 1994). A wide range in susceptibility exists (Beineke and Masters 1973; Berry 1981). Control measures include raking and disposing of leaves and plant debris, thereby removing the fungal inoculum. Spring fertilization may reduce disease severity.

1.4.2. Bullseye leafspot – *Cristulariella spp.* particularly *C. moricola*

Bull's-eye or zonate leaf spot, like anthracnose, causes premature defoliation of black walnut trees. The spots are larger than those of anthracnose and usually appear in late July or August. The lesions have a concentric ring pattern with alternating light and dark tissue that gives them a target appearance. Leaflets eventually turn completely brown and curl and occasionally premature leaf drop occurs. *C. moricola* causes economically significant defoliation of black walnut. The disease range includes Ohio and southern Illinois. *Cristulariella* also attacks many other woody tree and shrub species

including maple and hickory (Sinclair and Lyon 2005). Cool, wet weather in mid-summer is conducive to epidemics of this disease. Control measures are the same as those for walnut anthracnose. Chemical control with a foliar-applied fungicide generally is not recommended.

1.4.3. Mycosphaerella leaf spot – *Mycosphaerella juglandis*

Angular lesions (up to 4 mm wide) result from infection of leaflets of *J. nigra* by sexual spores (ascospores) which are created in in fruiting structures (pseudothecia) produced during late spring on overwintered leaves. A repeating cycle of infection can then occur via spores (conidia) released from asexual fruiting structures (acervuli) on current season leaves (fig 1-4). The conidia are disseminated by splashing and dripping water. Severe infections result in premature yellowing and defoliation by August. This results in reduced growth and nut production. The disease is found in the Midwestern states and North Carolina. Control measures include removal of cast leaves, control of ground vegetation, and appropriate fertilization. Local extension offices should be contacted for current foliar fungicides and rates available for use on black walnut.

1.4.4. Bunch disease

Phytoplasma (fastidious bacteria) strains in a sub-group of *Prunus* X-disease group 16Sr III-G are responsible for bunch disease in black walnut.

Eastern black walnut is tolerant of infection by the phytoplasma responsible for bunch disease, although severe infections can occur. Disease symptoms in the species are primarily characterized by slow growth until pruning or felling occurs. Following these activities, a bunch of shoots (= broom) results from the proliferation of branches

from clustered buds or from buds in leaf axils that were induced by phytoplasma infection. The brooms on a large tree usually begin as sprouts formed along the main stem or main branches. Within the brooms, the leaflets are cupped, atypically narrow, and often chlorotic. The slow growth and subsequent broom formation may occur over decades in some trees. In addition, the nuts of diseased trees may fail to fill completely and shrivel into a black mass. Other nuts fall prematurely. Insects are thought to vector the phytoplasma but no species is known for bunch disease. Once the phloem is infected, the phytoplasma moves systemically within the host. Cold dieback is increased because of early leaf-out and late dormancy. Control measures include pruning out stem and branch sprouts. However, if brooms occur on the main stem or at the tree base, the tree should be removed and the root system killed.

1.5. Insect pests

1.5.1. Inner Bark Borers

This group includes the insects which cause damage of the greatest economic concern, especially the bark beetles (family Curculionidae: subfamily Scolytinae) and the metallic wood boring beetles (family Buprestidae). These insects feed on the inner bark of trees.

1.5.1.1. Bark beetles – Walnut Twig Beetle – *Pityophthorus juglandis*

(Also see: Thousand Cankers Disease, p. 3, this chapter)

The walnut twig beetle is considered native to Arizona, New Mexico, and northern Mexico. However, it recently has been introduced to locations in the eastern United States on walnut burls, logs and slabs with bark attached. It has become

established in at least five eastern states (Maryland, Ohio, Pennsylvania, Tennessee, and Virginia) to date. The adults are small (1.5 to 1.9 mm long), yellowish brown in color, and are difficult to see *in situ* (Wood 1982) (fig. 1-5). They chew tunnels in the inner bark (phloem) that emanate from egg galleries. Entry and exit holes are very small. The insect may attack branches and main stems of black walnut in great numbers. Spores of the TCD fungus (*Geosmithia morbida*) are commonly carried by *P. juglandis* and are dislodged from external surfaces of the insect during attack. In Tennessee, adults emerge between late May and late June and in September and October (Nix 2013). Females chew chambers in which mating occurs (= nuptial chambers) in the inner bark. Eggs are laid in niches along galleries from which larval tunnels arise. Larvae pupate in early spring; however, adults may also overwinter in the bark. The beetles appear to have two generations per year in Tennessee. Removal and burying infested wood is currently used for control. Heat treatment kills the insect and the associated fungus, but treated logs may be re-infested (Audley et al. 2015).

1.5.2. Borer and Girdlers

Boring insects attack either soft plant parts (such as terminal buds) or bore directly into wood itself. Holes of sufficient depth and quantity can destroy timber value. Indirectly, boring insects can introduce fungus which may cause disease and potentially, death of the tree.

1.5.2.1. Ambrosia beetles

1.5.2.1.1. Black stem borer – *Xylosandrus germanus*

This exotic ambrosia beetle was first detected in the United States in 1932. It attacks over 200 plant species, including *J. nigra*. Furthermore, *X. germanus* is known to attack apparently healthy plants, as well as those that are dying or recently dead (Weber and McPherson1985; Barnd, Pijut and Ginzel 2008). Walnut trees less than 8 years old are most often attacked. Damage resulting from attack is usually not detected until numerous sprouts arise from the base of the tree or the trees are dead. *X. germanus* was one of the most abundant insect species colonizing artificially stressed black walnut in a recent study in Indiana and Missouri (Reed, et al. 2015) (fig. 1-5). *X. germanus* adult females are 2.0 to 2.3 mm in length, very dark brown to black in color, and strong fliers (Wood 1982). Males are smaller in length and rare in occurrence (Anderson and Hoffard 1978; Kessler 1974). Adult females attack hosts in May. Extruded frass that looks like a toothpick is indicative of their presence. The female adult's tunnel extends about 1 cm into the wood and then branches into several arms. The attacking adult may introduce the Fusarium canker fungus (*F. solani*) as it tunnels into a tree (Weber and McPherson 1985). Branch death above the attack site may result. Overlapping generations occur. Control of *X. germanus* includes removal and destruction of infested branches or logs. Some cultivars of *J. nigra* may be more resistant to beetle attack than others (Carlson et al. 1993).

1.5.2.1.2. Granulate ambrosia beetle – *Xylosandrus crassiusculus*

This exotic invasive insect is thought to have been introduced in the United States on solid wood packing material originating from the insect's native range (east Africa and Southeast Asia). The species was first detected in South Carolina in 1974. Since then it has proven to be a serious pest of ornamental, fruit and nut trees (Ranger et al. 2015). The species was one of the four most abundant insects colonizing artificially stressed walnut in a recent Midwestern study (Reed et al. 2015). The female adult is variable in length (2.1 – 2.9 mm) and longer than the dwarfed male (1.6 mm long), and reddish brown in color (Wood 1982) (fig. 1-5). The female attacks branches and main stems of susceptible hosts (including *J. nigra*) and excavates tunnels into the sapwood. *X. crassiusculus* has a symbiotic ambrosial fungus that it introduces to its constructed galleries. A toothpick-like spike of frass sticks out of attacked trees. Heavy infestations may lead to wilting, dieback, or death of the colonized tree. *X. crassiusculus* is thought to overwinter as an adult with the spring-mated females dispersing in late winter or early to mid-spring depending on latitude and climate (Cote 2005). There are at least 2 generations per year in the United States. The pest is difficult to control with chemical pesticides once it has infested a tree. Cultural practices that promote good growth and reduce tree stress are recommended for susceptible hosts. Heavily infested plants should be removed and destroyed (Cote 2005).

1.5.2.2. Flat headed apple tree borer – *Chrysobothris femorata*

The flat-headed apple tree borer is a native species common to the eastern U.S. and will attack black walnut trees. The adult is about 12 mm long, flattened and bullet-

shaped, and varies in color from dark metallic brown to dull gray (fig. 1-5). Females lay eggs in bark crevices. Larvae chew into the bark and develop long, often winding tunnels that become filled with frass. After pupation occurs, mature larvae bore into the heartwood, pupate and emerge beginning in spring. Emerged adults feed (maturation feeding) at the base of twigs, whereas the larvae tunnel into and feed in the phloem and outer sapwood. There is one generation per year. Weakened and stressed trees are most vulnerable to this pest. Controls include good pruning and proper fertilization. Additional control is achieved by wrapping the trunks to prevent females from laying eggs (Sadoff and Foster 2010).

1.5.3. Nut Feeders

1.5.3.1. Walnut curculio – *Conotrachelus retentus*

C. retentus is commonly found on black walnuts throughout eastern United States (Johnson and Lyon 1988). Both the adult and the larvae cause damage, but larval injury to branches, stems and nuts is most important. Larval damage to nuts results in poor nut fill and leads to early fruit drop (= "June drop"). This weevil species may cause loss of 60% or more of the nut crop (Weber et al. 1980). Weevil adults are light tan to reddish brown, ~ 5 mm in length, and have a short beak (Barnd, Pijut, and Ginzel 2008)(fig. 1-6). They generally appear when the fruit are beginning to ripen. The female lays eggs in cracks on the nut and the larvae bore into the nuts. The insects overwinter as larvae in the nut or in the soil where they pupate. Control is achieved by immediately discarding any immature nuts that fall during the growing season (Sadoff and Foster 2010).

1.5.3.2. Walnut husk fly – *Rhagoletis completa*, Walnut husk maggot – *Rhagoletis* sp.

Both the walnut husk fly and the walnut husk maggot fly occur commonly throughout the central United States. Both feed in the exocarp (= husk) and their damage is significant when it results in downgrading of nut kernels during commercial processing (Johnson and Lyon 1988). However, the taste and color of the nutmeat are not affected. Both sexes of the adult husk fly are characterized by three prominent dark bands on their wings (fig. 1-6). The male is darker in color than the yellow female (Beineke 1994). They are similar in size to common housefly. The female lays eggs in the exocarp of the maturing nuts. The developing larvae feed on the exocarp which then turns black and soft. The larvae mature in 3 to 5 weeks and then pupate in the soil. Control is achieved by immediately discarding infested nuts (Linit 1998).

1.5.3.3. Walnut shoot moth – *Acrobasis demotella*

The walnut shoot moth is closely related to the pecan leaf casebearer (*A. juglandis*). Both species occur throughout the eastern United States. The walnut shoot moth causes much more damage on *J. nigra* than the pecan casebearer (Soloman 1995). The larvae of the moth bores into unexpanded buds in the spring. The damage caused to terminal buds results in multiple forks and crooks in the main stem (Williams 1990). In contrast, the pecan casebearer is a leaf skeletonizer of *Carya* and *Juglans* species. Both species are nearly identical in appearance. The adult has just over a 2 cm wingspan, and is brownish grey in color with contrasting reddish brown patches (fig. 1-6). The larvae are purplish brown, with pale underside. The previously described habits serve to

differentiate the two species. Corrective pruning is used to control walnut shoot moth. Contact local extension for chemical control methods if necessary.

1.5.3.4. Walnut codling moth – *Cydia pomonella*

The walnut codling moth attacks nuts of *J. nigra* as well as fruit of various fruit trees. The larvae feed on the exocarp and unripe kernels leading to infestation of both early season and late season nuts (Barnd, Pijut, and Ginzel 2008). The adults are small (~12 mm long), and have mottled grey wings with a noticeable copper spot at the tip of each forewing (fig. 1-6). The adults mate around dusk. The female lays eggs near a developing fruit. Following hatch, the larvae bore into the nut. There are five larval instars and up to three generations may be produced per year depending on climate (Johnson and Lyon 1988). The final generation of the season pupates over the winter (Agnello and Cain 1996). The pest is controlled by removal and proper disposal of fallen nuts or other debris under the infested tree.

1.5.4. Fluid Feeders

These insects have highly modified sucking mouthparts which are generally inserted into the phloem of the host tree. The life cycles of these insects may be complex, and the damage to trees varies widely between species.

1.5.4.1. Aphids (plant lice) – *Monellia spp.* and *Monelliopsis spp.*

Aphids are common, widely distributed insect pests, and are found on the underside of walnut leaves. They are soft bodied, approximately 2 mm in length, and green or tan in color (fig. 1-7). Symptoms of aphid presence include leaf curl, yellowing, and possibly defoliation with growth reduction. Aphids often leave a sticky substance (=

"honeydew") on the leaf surface as they suck juices from leaves. Later, fungus growing on the honeydew may cause the leaf to blacken, and prevent the penetration of light, and reducing photosynthesis. A very serious infestation may result in branch dieback. Unless serious damage occurs, no control is recommended.

1.5.4.2. Walnut lace bug – *Corythuca juglandis*

The walnut lace bug is one of at least 27 species of *Corythuca* that feed on and damage deciduous trees and shrubs. It causes damage when the adults and nymphs suck the sap from the lower surfaces of walnut leaflets (Johnson and Lyon 1988). In general, even heavy infestations fail to cause permanent damage to walnut. The adult is very small (~ 5 mm long), light colored with darker markings, and the surface of wings and thorax appear an intricate, lacey network (fig. 1-7). The pest overwinters as adults in protected areas (e.g. bark crevices, branch crotches) on or near the host tree. Two generations are produced per year. For control of infestations on small trees, forceful water spray can be used to dislodge the nymphs feeding on the leaf undersurface. Debris and leaves around an infested tree can be removed and destroyed to reduce the population of overwintering adults. Sprays with insecticidal soaps or horticultural oils are also effective controls.

1.5.5. Defoliators

Defoliators, with chewing mouthparts, eat leaves. Defoliation may reduce vigor, slow growth, and increase susceptibility to other insects or diseases.

1.5.5.1. Fall webworm – *Hyphantria cunea*

The fall webworm is a native pest to the United States and feeds on *J. nigra* in addition to at least 88 other species of trees (Johnson and Lyon 1988). However, the

pest's feeding habit does vary from region to region. The insect is generally more of a nuisance than a threat to walnut tree health. However, abundant infestation and subsequent defoliation may reduce tree growth, but defoliated branches generally re-flush quickly. There are two races of the species, the blackheaded and the redheaded (Johnson and Lyon 1988). Fall webworm is recognized by its characteristic web, which encloses one or more branches of a tree and sometimes an entire small tree (fig. 1-8). Webs start at the outer tips of branches in mid-summer. Over the season the webs will become noticeably larger (= nests). For severe infestations on smaller trees, the nests of the fall webworm can be removed and destroyed (Johnson and Lyon 1988). Repeated defoliation several years in a row may require chemical control (Weber, Anderson, and Hoffard 1980).

1.5.1.2. Walnut caterpillar – *Datana integerrima*

The walnut caterpillar is found throughout most of the eastern U.S. The pest feeds on leaves in a cluster habit in which they will consume all leaves on one branch before moving to another. These large caterpillars (mature larvae are ~ 50 mm long) can defoliate trees and severely affect tree vigor and nut quantity and quality (fig. 1-8). Trees can withstand 2 or 3 consecutive years of heavy defoliation before they die. Outbreaks seldom last longer than 2 years in any one location. The larvae feed only on foliage of trees of the family Juglandaceae. One to two generations are produced per growing season. Controls include removing and destroying larvae when they are small (Farris, Appleby, and Weber 1982).

Figure 1-1. Native range (shaded area) of *Juglans nigra* (eastern black walnut) in the United States (lower 48 states).

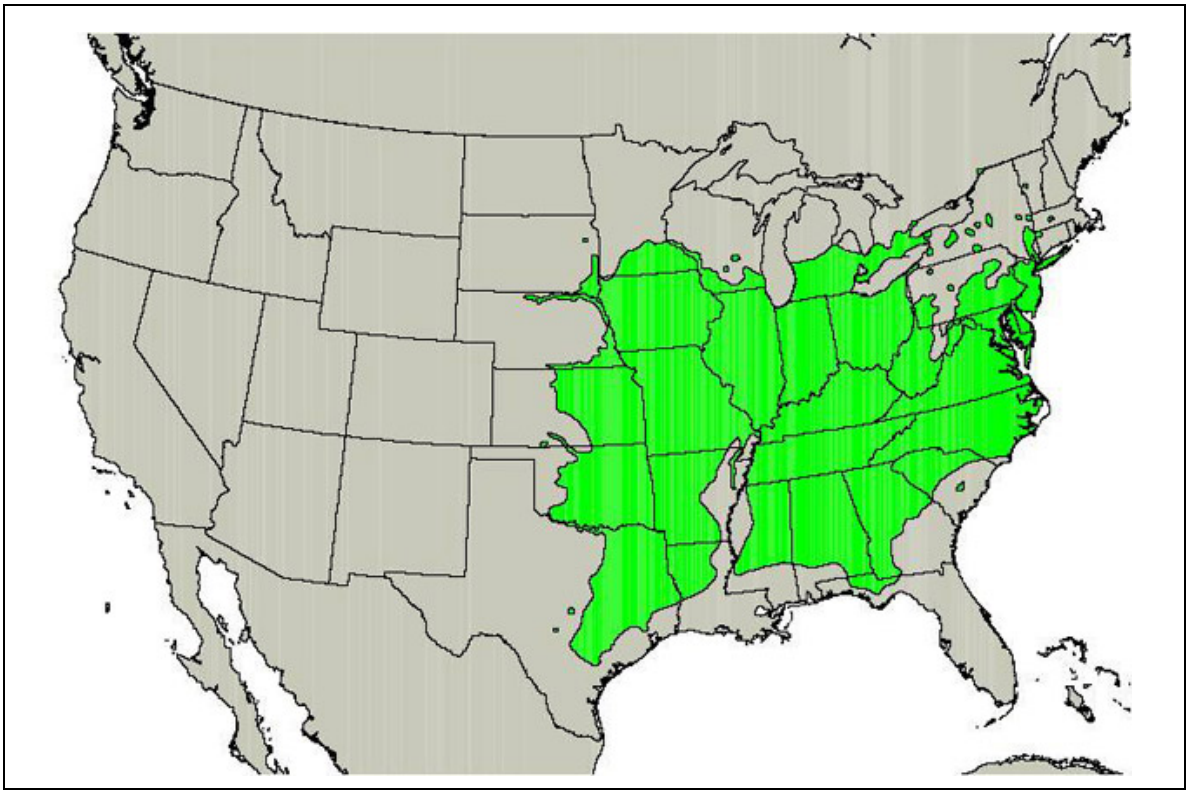


Photo: USGS.

Figure 1-2. Selected canker diseases of eastern black walnut

Thousand cankers disease

Ned Tisserat, Colorado State University
Invasive.org

Fusarium canker

Robert L. Anderson
USDA Forest Service
Bugwood.org

Perennial target canker

Robert L. Anderson
USDA Forest Service
Bugwood.org

Figure 1-3. Selected root rot diseases of eastern black walnut

Phytophthora root rot

Ralph J. Green, Jr.

Cylindrocladium root rot

Paul A. Mistreta
USDA Forest Service

Figure 1-4. Selected foliar diseases of eastern black walnut

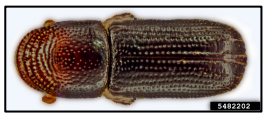
Walnut anthracnose

Shanyn Siegel, University of Illinois Extension

Mycosphaerella

USDA Forest Service

Figure 1-5. Selected bark beetle insect pests of eastern black walnut



Pityophthorus juglandis
(walnut twig beetle)

Steven Valley, Oregon Department of Agriculture, Bugwood. org

Xylosandrus germanus
Maja Jurc, University of Ljubljana, Bugwood.org

Xylosandrus crassiusculus
Javier Mercado, Bark Beetle Genera of the U.S., ASDA APHIS ITP, Bugwood.org

Chrysobothris femorata
(flat headed apple tree borer)

Steven Valley, Oregon Department of Agriculture, Bugwood. org

Figure 1-6. Selected nut feeding insect pests of eastern black walnut

Conotrachelus retentus

(Walnut curculio)

Hanna Royals, Museum Collections: Coleoptera, USDA APHIS ITP, Bugwood.org

Rhagoletis completa

(Walnut husk fly)

Natasha Wright, Cook's Pest Control, Bugwood.org

Acrobasis demotella

(Walnut shoot moth)

Ilona L. BugGuide.net

Cydia pomonella

(Walnut codling moth)

Ken Gray, Pacific Northwest Insect Management Handbook

Figure 1-7. Selected fluid feeding insect pests of eastern black walnut

Monellia sp.

(Aphids)

woennyy stock photo

Corythuca juglandis

(Walnut lace bug)

Whitney Cranshaw Bugwood.org

Figure 1-8. Selected defoliator insect pests of eastern black walnut

Hyphantria cunea
(fall webworm)

adult above

Mark Dreiling
Bugwood.org

Larvae below

Steven Katovich
USDA Forest Service
Bugwood.org

Datana integerrima
(walnut caterpillar)

adult above

larvae below

Lacy L. Hyche
Auburn University
Bugwood.org

Chapter 2. Fungi associated with observed damage on *Juglans nigra* branches from two locations (thousand cankers disease (TCD) and non-TCD areas)

2.1. Introduction

Thousand cankers disease (TCD) is an emerging disease lethal to eastern black walnut trees (*Juglans nigra*) (Tisserat and Cranshaw 2010). TCD has been documented both in the U.S. and in Europe, in northeastern Italy (Montecchio and Faccoli 2014). Initially, TCD was first described in Colorado (Tisserat et al. 2009). However, a decline of walnut had been previously reported in Utah (Murray 2008) and the principal insect involved (*Pityophthorus juglandis*) was also associated with dying *J. nigra* in New Mexico in 2001 (U.S. Department of Agriculture 2003). TCD was first confirmed in the native region of eastern black walnut in Tennessee in 2010 (Seybold, Haugen, and Graves 2013). Thus, the health of black walnut within its native range in the eastern U.S. is threatened by TCD, and the number of first reports is growing despite state quarantines. In the U.S., this disease is of great economic as well as ecological and aesthetic importance. Black walnut trees mature slowly, requiring approximately 20 years to produce full nut crops, and 60 years for quality lumber. The rapid progression of TCD (two to four years from first symptoms to death of the tree) is a real threat to sustainability of the species for commercial trade purposes (Newton et al. 2009).

TCD is characterized by thinning of the crown, chlorotic and flagging leaves followed by branch death and ultimately, death of the tree. Prior to discovery of the biotic agents involved in TCD, symptoms were mistaken for drought damage and tree decline (Zerillo et al. 2014). In the eastern U.S., late season scattered leaflet dropping, apparently

brought on by dry conditions, has been confused with TCD. Additionally, the progression of TCD in its native range has been shown to apparently vary with water stress. Some trees in the eastern U.S. have demonstrated a quiescent period in disease development, or even recovery, when precipitation levels are normal or higher (Griffin 2015).

TCD symptoms consistently result from aggressive attack by the walnut twig beetle (*P. juglandis*) and coalescing of numerous cankers caused by the fungus *Geosmithia morbida* (Kolarik et al., 2011) that is transmitted by the beetle. Small dark, two-toned, generally circular cankers, each of which may surround the entrance/exit hole or gallery of *P. juglandis* (walnut twig beetle) can be in the found in the inner bark of affected branches. The walnut twig beetle is considered to be the principal vector in the transmission of the canker pathogen (Tisserat and Cranshaw 2010) although a weevil species (*Stenomimus pallidus*) emerged from artificially stressed walnut has recently been found with *G. morbida* (Juzwik et al. 2015). In addition to *G. morbida*, there are many other fungi and insects which have been found on both visually healthy and TCD infected black walnut trees (Linit 1998; Reed et al. 2014; Sinclair and Lyon 2005). Among the fungi associated with walnut, *Fusarium* species are known to cause cankers and branch dieback in *J. nigra* in the eastern U.S. (Carlson et al. 1993; Tisserat 1987). Specifically related to TCD symptomatic trees, recent research has found distinct haplotypes of the *Fusarium solani* species complex (FSSC) to only be present in TCD areas compared to FSSC haplotypes in non-TCD areas (Montecchio et al. 2015). *Botryosphaeria dothidea* is also associated with cankers on *Juglans* spp and on many other woody perennials (Pijut 2005).

Despite the significance of *J. nigra* in the U.S., the array of fungi associated with inner bark damage has not been extensively studied, and the relationship to thousand cankers disease has not been established. The association of other pests and pathogens associated with the TCD complex is poorly understood. Furthermore, the contribution of other fungi to development of TCD on trees in the disease-positive, eastern states is unknown.

The above data gaps prompted this investigation of fungi colonizing branches of *J. nigra* in the eastern U.S. The main objectives of this study were to characterize damage types found in the bark of girdled and non-girdled branches of eastern black walnut, isolate for fungi from the different damage types observed, and identify any putative canker pathogens associated with each. Because interactions or spatial overlap of other insect pests or pathogens with *P. juglandis* and *G. morbida* could contribute to TCD symptom development, study trees were located in a TCD area (Tennessee) and compared with damage and fungi present on branches in a state not known to have TCD (Indiana) at the time of the study. The overall goal of this research is to improve the understanding of TCD etiology.

2.2. Materials and Methods

2.2.1. Study sites and trees.

Study sites were located in four urban communities in Indiana where TCD is not known to occur and in two suburbs of the greater Knoxville, Tennessee area where TCD is well established (fig. 2-1). In Indiana, eastern black walnut street, residential and park trees in Delphi, Lafayette/West Lafayette, Carmel and Franklin were selected. Indiana study trees (n = 17, 14 treated and 3 non-treated) ranged in diameter from 26 to 89 cm at

1.4 m height (dbh) on the main stem. In Tennessee, trees in urban parks and on a residential property were selected within the known range of TCD. Tennessee study trees (n = 12, 9 treated and 3 non-treated) ranged in diameter from 33 – 63.5 cm dbh on the main stem. Trees selected for treatment had visually healthy crowns while the three non-treated trees had crowns exhibiting TCD symptoms.

2.2.2. Branch treatments

In Indiana, two branches on opposite sides of the tree crown were selected for each study tree in each community. Heights of branches ranged from 6 to 15 m. An aerial lift was used to reach the branches. For one branch on each of four trees in each community, two encircling cuts 7 cm apart (= girdling) were made into the outer sapwood where the branch diameter was approximately 10 cm. The bark in between the cuts was removed and the branches tagged and marked with flagging. The other marked branch in the crown was tagged, but no cuts were made (= non-girdled). In three of the Indiana communities, two non-girdled branches in single "control" trees were also included for comparative purposes. In Tennessee, two branches on opposite sides of the tree crown were selected for each study tree. Height of branches ranged from 3 to 6 m. A stepladder was used to reach the branches. Girdling treatment as described for Indiana trees was used for all treated trees in Tennessee. Two non-girdled branches in each of three TCD symptomatic trees (= non-treated) were also included for comparative purposes.

2.2.3. Branch sampling

All branches used in this study were cut from the trees in early to mid September, 2012. The cuts were made on the trunk side of the branch, approximately 10 cm before

the girdled area. In Indiana, branches were harvested with a chain saw from the aerial lift. In Tennessee, branches were harvested with a pole pruner from the ground or using a ladder, as needed. Each branch was cut into segments of approximately 28 cm on site using a handsaw or a chain saw. Approximately ten segments per branch were grouped and labeled by branch. In Tennessee, segments were placed in plastic bags by branch at the site. In Indiana, segments were placed in labeled milk crates by branch at the site. All branch segments were then transported by vehicle to the laboratory and stored at 4ºC in plastic bags until subdivided.

In the laboratory, segments were examined for external damage including cankers, lesions, discoloration or damage attributed to insects. Approximately eight segments from each branch were selected for insect emergence while two branch segments with diameters ranging from 2.5 to 10 cm were selected from the set from each branch for canker characterization and fungal isolation. All segment ends were dipped in melted paraffin, and freshly cut branch stubs or mechanical wounds were sealed with melted paraffin. The segments designated for insect emergence were then placed in rearing containers (separate study, see appendix). The samples designated for pathological assessment were stored at -20ºC until overnight shipment to the Minnesota containment facility (BSL-2) in early October, 2012. Indiana sample sections were stored at 4ºC until transported to Minnesota and further processed in the laboratory.

2.2.4. Sample processing for pathological evaluation

In the laboratory, the bench area and tools were surface sterilized with 70% ethanol prior to sample observation. The outer surface of each branch segment was

examined for any signs of insect damage. Each sample was clamped into a vise in the biological safety cabinet (Tennessee samples) or laminar flow hood (Indiana samples), and portions of the outer bark of each sample were successively removed with a small drawknife. Signs of insect activity, and any insect life stages were noted. Details on damage, canker or stain observed were recorded, including the size (cm^2), color, margin, margin color, shape, single vs. coalesced cankers, and any additional distinctive qualities. Representative samples of damage, cankers, and stains were photographed. Tennessee sample images were obtained using a digital camera (Olympus C7070 Wide Zoom). Indiana sample images also were obtained using a digital camera (Canon Power Shot SY10 IS). Samples which appeared entirely necrotic were noted and not used for characterization or fungal isolation.

2.2.5. Fungal Isolation

A flame-sterilized scalpel was used to cut eight thin tissue samples (~3mm x 8mm) from bark at the margin of each representative canker, discolored area or insect damaged or colonized region. Four samples were placed on each of two 100 mm diameter petri plates containing ¼ strength potato-dextrose agar (PDA) amended with 100mg/litre streptomycin sulfate and 100 mg/litre chloramphenicol (Tisserat et al. 2009). Plates were labeled with representative damage area code, sample code, branch number, tree number, and date. The plates were incubated at 23ºC in a dark growth chamber at 50% relative humidity. Plates were checked weekly for actively growing fungi. Using a flame sterilized scalpel, a square approximately 3mm x 3mm in size was incised from the margin of each fungal colony phenotype which grew from the tissue samples, and transferred to ½ strength PDA (½ PDA) in 100 mm diameter plates. Fungal colonies were

grown under the same conditions as the original isolation plates. Subsequent transfers, using hyphal tipping, were made from resulting isolates until pure cultures were obtained. Pure isolates obtained from Indiana branches were stored at 4ºC until further processed. For pure isolates from Tennessee branches, extra precautions were taken before removal from the BSL-2 facility to the U.S. Forest Service Annex (St. Paul).

The Tennessee isolate cultures were bagged, and placed in rigid plastic containers with tight-fitting lids. The exterior of the containers was decontaminated with a 70% ethanol spray, and air-dried for thirty minutes prior to relocation to the Forest Service building.

2.2.6. Fungal Identification

Representative isolates were chosen separately for those obtained from Indiana trees and Tennessee trees. Groups of similar isolates within the two collections were based on morphological characteristics such as colony color (top and reverse), size, texture, and margin characteristics. Up to three representative isolates of each morphological colony type were transferred to ½ PDA slants in 8 ml glass scintillation vials with screw caps. Once fungal colonies covered half of the slant surface, the stock cultures were moved to a 4ºC incubator for long-term storage.

Tentative identification of several groups was made based on colony morphology and microscopic characterization. However, final identifications were based on DNA ITS region sequences obtained from each isolate. A small amount of mycelium was scraped from each isolate culture and placed in a 200 µl strip tube with 200 µl CTAB lysis buffer (containing 1.4 M NaCl, 0.1 M Tris-HCl, 20 mM EDTA, 2% CTAB). Samples were

frozen at-20ºC. in St. Paul, MN, until transferred by vehicle to the Forest Products Laboratory, Madison, WI.

DNA extraction and PCR reactions were performed on the isolates (Lindner and Banik 2009). Briefly, the samples were thawed at room temperature and incubated at 65ºC for 2 h. Tubes were then centrifuged at 10,000 rfc for 5 m. The supernatant (100µL) was transferred to clean strip tubes. Ice cold isopropanol (150µL) was added, and the tubes were inverted several times, then placed in -80ºC freezer for 10 min. Tubes were then centrifuged at 0ºC 10,000 rfc for 20 minutes. The supernatant was discarded and175µL ethanol was added to the pellet. Tubes were centrifuged at 10,000 rfc for 5 min. The supernatant was again discarded and the pellet was air dried for 5 minutes. Forty-five µL sterile, nanopure (MG) water, along with 135µL NaI solution and 2.5 µL of glass milk obtained from commercial DNA extraction kits (Gene Clene III, Qbiogene, www.qbiogene.com) were added. Tubes were agitated for 5 minutes, then centrifuged at 10,000 rfc for 8 s. The supernatant was discarded and the pellet was washed with 175 µL New Wash solution provided with the kit, above. Tubes were vortexed for 5 minutes and centrifuged for 8 seconds as above. The supernatant was discarded and the pellet air-dried for 15 minutes. Fifty µL MG water added and vortexed for 1 s then centrifuged at 10,000 for 35 s. The supernatant containing eluted DNA was transferred to 96 well PCR plates for storage at -80ºC.

PCR amplifications were performed in 15 µl reaction mixtures containing 0.3 µl DNA template, 0.3 µl each forward and reverse primers ITS 1F and ITS4, 8.025µl of MG water, 0.3 µl dNTP, 3 µl 5x green GoTaq reaction buffer and .075 µl GoTaq DNA

polymerase (Promega, Madison, WI). Amplification reactions were temperature-cycled in 96-well plates using a thermocycler (Bio-Rad, Hercules, CA, USA). Thermocycler conditions were as per Linder and Banik (2009). Three microliters of each amplified product were electrophoresed in 1.5% (wt/vol) agarose gel stained with ethidium bromide (0.5 μg/ml). Electrophoresis in Tris-borate-EDTA buffer was performed at 100 V for 15 minutes, removed from the bath and photographed under UV light illumination. All amplification products were stored at -20ºC for further processing. Amplification products observed under UV light as bands on the gel, as well as negative extraction controls were further prepared for direct sequencing. Sequencing reactions were performed following the BigDye terminator protocol (ABI Prism) with primer ITS5. Briefly, 1.8 μLwater, 2.5 μL 5x buffer, 0.5 μL betaine and 0.2 μL ITS5 primer (10x) were combined with 7 μL diluted DNA, for each sample.

Sequencing products were cleaned with magnetic beads (CleanSeq, Agencourt, Beckman-Coulter, Inc., Brea, CA, USA) following the manufacturer's protocol. Sequencing products were analyzed at Functional Biosciences, Inc. in Madison, WI from direct inject 96 well plates using ABI 3730xl equipment. DNA extraction and PCR for a portion of the isolates were done in St. Paul for logistic reasons. A different commercial kit was used (Genomic DNeasy Plant Kit™, Qiagen, Hilden, Germany) according to the manufacturer's instructions, in the St. Paul laboratory. PCR of these samples was performed in St. Paul, MN, in 25 μl reaction mixtures containing 1.0 μl DNA template, 0.5 μl each forward and reverse primers ITS 1F and ITS4, 13.0 μl of MG water, 10.0 μl Mastermix. Amplification reactions were temperature-cycled in strip tubes (8 - 200 μL tubes/strip) in a thermocycler (Bio-Rad, Hercules, CA, USA) with conditions described

in Lindner and Banik (2009). Three microliters of each amplified product were electrophoresed in 2% (wt/vol) agarose gel stained with ethidium bromide (0.5 μg/ml). Electrophoresis in Tris-borate-EDTA buffer was performed at 100 V for 15 minutes, removed from the bath and photographed under UV light illumination. All amplification products were stored at -20ºC for further processing. Amplification products observed under UV light as bands on the gel, as well as negative extraction controls were further prepared for direct sequencing. Sequencing reaction, Big Dye terminator clean-up, and ITS region sequencing of these samples was performed in Madison as previously stated.

2.2.7. Summarization and analyses

Insect related and non-insect related damage types were characterized for the Tennessee and Indiana branches. Damage frequencies were compared between trees by location and between trees by girdled and non-girdled branches, and between treated and non-treated trees.

Fungal ITS sequences were analyzed and edited using 4Peaks software (4Peaks by A. Griekspoor and Tom Groothuis, nucleobytes.com). Alignment with sequences in the NCBI database was performed using BLAST, UNITE and Clustal Omega. Aligned sequences were identified by match to the closest GenBank accessions. Putative pathogenic fungi were compared between damage types.

The preliminary nature of the fungal isolations from different damage types precluded formal statistical analysis of the frequency with which individual taxa were isolated. The damage areas on trees in a site varied by location, the controls were not taken from all sites, and isolation of individual fungi was dependent, in part, on the

relative competitive abilities of different taxa in culture plates. The occurrence of a canker type was scored as a single record regardless of the number of cankers developing on the branch segment. Fungal colonization frequency is defined as the percentage of fungus-positive damage type samples based on total number (n) of samples assayed within each damage type.

2.3. Results

2.3.1. Damage types

2.3.1.a. Indiana (non-TCD area)

The types of damage observed on branch segments from the 17 Indiana trees included spots, lesion or canker-like damage, stain, and discolored phloem tissue around insect associated damage (flat-headed wood borer (Coleoptera: Buprestidae) or weevil (Coleoptera: Curculionidae) tunnels) (fig. 2-2; table 2-1).

All damage types were found on segments from both girdled and non-girdled branches (fig. 2-3A). Frequencies of occurrence of damage types 1 through 5 were similar for both girdled and non-girdled branch samples. Flat-headed wood borer damage (type "B") was found on more non-girdled branches than on girdled branches. The reverse was observed for weevil-like damage (type "W"). Only spots (type 1), lesion or canker (type 2 and type 4 respectively), and flat-headed wood borer damage were observed on branch segments from non-treated trees (fig. 2-3B).

2.3.1.b. Tennessee (TCD area)

The types of damage observed on branch segments from the 12 Tennessee trees included stains, lesion or canker-like damage, and discolored phloem around insect associated damage (flat-headed wood borer or walnut twig beetle tunnels or galleries) (fig. 2-4; table 2-4).

There were differences in the damage types observed on girdled versus non-girdled branches of the treated trees (fig. 2-5A). Lesion or canker-like damage (types C, D, E), flat-headed wood borer damage (IB), and walnut twig beetle damage (IPj) were found on girdled branch segments. All types of damage, except for lesion or canker-like

damage type C, were observed on non-girdled branches. Walnut twig beetle damage was commonly found on both girdled and non-girdled branch segments of treated trees; however, it was more frequent on the girdled branches compared to the non-girdled branches (89% versus 63% of the segments, respectively). Lesion or canker-like damage types C and E, flat-headed wood borer damage (type IB), and walnut twig beetle damage (type IPj) were found on branches of non-treated, TCD-symptomatic trees (fig. 2-5B). Walnut twig beetle damage was detected on 69% of the branch segments examined, but highly elongated cankers (type E) were the most common of all damage types except for the insect-related types observed.

2.3.2. Fungi Isolated

2.3.2.a. Indiana

Fungi isolated from damaged areas on segments of Indiana tree branches included 24 Ascomycete species (from 16 genera) and 1 Basidiomycete. (table 2-5; figures 2-6A and 2-6B). *Diplodia seriata*, *Epicoccum nigrum*, *Fusarium solani*, and *Penicillium spp*. were isolated from branches obtained from all sites (data not shown). Fungi isolated from the control tree segments represented a subset (7) of the 17 genera isolated from treated segments.

2.3.2.b. Tennessee

Fungi isolated from damage observed on segments of branches from Tennessee trees included 64 Ascomycete species (representing 28 genera) and 6 Basidiomycete species (representing 4 genera), (Table 2-6, 2-7, figs 2-7A, 2-7B). Of these, 16 genera were isolated from both treated and non treated (TCD symptomatic) tree branch samples. The remaining 16 genera were isolated only from the treated trees. No genera were

isolated exclusively from only the TCD symptomatic samples. In Tennessee, several fungi were additionally isolated that were not closely matched (<97%) to existing entries in Genbank (table 2-8). These fungi were isolated only from the treated trees. All representative fungi of importance will be accessioned into Genbank.

2.3.3. Fungi by lifestyle

Fungi isolated from damage observed on the branch segments were categorized by putative lifestyle. The majority of the fungi isolated were considered to be saprotrophic or plurivorous (e.g. *Aspergillus* and *Penicillium* species). Biotrophic fungi isolated and of particular interest were categorized as woody plant pathogens, insect pathogens, fungal parasites, and sapstain fungi. Three of the fungi categorized as woody plant pathogens are known pathogens of walnut (fig. 2-8). *Botryosphaeria dothidea* (IN, TN), pathogenic on *Juglans* spp. was commonly isolated from canker-like damage (types C, D and E) and from xylem discoloration associated with insect colonization (Bruprestid and *P. juglandis*) in Tennessee. *Fusarium solani* (IN, TN), and *Geosmithia morbida* (TN), are confirmed pathogens of *J. nigra. F. solani* was isolated at low levels from six of the seven damage types characterized for Indiana branches and commonly from canker-like damage and discoloration associated with insect discoloration (Buprestid and *P. juglandis*) in Tennessee. *G. morbida*, the TCD-fungus, was isolated from three different types of cankers and from discoloration associated with *P. juglandis* galleries in Tennessee. The other woody plants pathogens isolated included *Diplodia seriata*, a known pathogen of many woody species (Phillips et al. 2012; Mohammadi et al. 2013),

which was isolated from branches in both Tennessee and Indiana. *Biscogniauxia atropunctata* was isolated from *P. juglandis* associated damage on one branch in Tennessee. This species causes dieback of stressed oaks and has been reported on a number of forest species, but not previously reported on *Juglans* (Sinclair and Lyon 2005). Two insect pathogenic fungi (not known to parasitize *P. juglandis*) were isolated from the branches, *Isaria farinosa* (IN) and *Fusarium larvarum* (TN) (Zimmermann 2008; Teetor-Barsch and Roberts 1983). *Trichoderma harzianum*, a fungal parasite (Elad, Chet, and Henis 1982), was isolated from branches in both states. One stain fungus, *Cladosporium cladosporoides*, was isolated from canker-like damage on branches from Tennessee.

2.4. Discussion

2.4.1. Mycological findings

Isolation of fungi from the margins of damage areas on black walnut branch bark enabled the identification of a diverse population of fungi, comprised primarily of ascomycetes.

Twelve genera of isolated fungi were common to both states. A greater number of fungal genera were isolated from Tennessee trees than from Indiana trees. This might be explained in part by the difference in moisture conditions between Indiana and Tennessee during the summer of 2012 (National Drought Mitigation Center 2012). Drought conditions in Indiana likely promoted faster desiccation of bark and wood tissue, potentially affecting viability of fungi in those tissues. Additionally in Tennessee, trees whose crowns appeared healthy were often colonized by *G. morbida*. Trees colonized by

pathogenic fungi may display a more diverse range of fungal isolates than disease-free trees (Boddy 2001; Kowalski and Andruch 2012).

Following categorization as plurivorous saprotrophs or as biotrophs, fungal taxa were further evaluated qualitatively. The plurivorous fungi (most notably *Penicillium* and *Aspergillus* spp.) were isolated from all damage types in both locations. These genera were assumed to be functioning as saprotrophs, although several *Penicillium* and *Aspergillus* spp are known pathogens of other plant species. *Fusarium* spp., (with the exception of *F. solani* and *F. larvarum*) were included here, as was *Diplodia seriata* the anamorph of *"Botryosphaeria" obtusa,* suspected to be pathogenic on *J. nigra.* The small numbers of basidiomycetes isolated were included in this category as well. Based on these results, the majority of fungi associated with black walnut in these locations are not host specific. Generally, species of the genera isolated are widespread saprobes associated with a wide range of plants.

The biotrophs were sub-categorized as insect pathogens, fungal pathogens or stain fungi. Woody plant pathogens are considered separately in 2.4.2.

The three entomopathogenic fungi which were isolated have been found extensively in both natural and managed systems. *Isaria farinosa* was isolated from weevil type damage in Indiana. The natural host range of *I. farinosa* contains a number of families including members of the Curculionidae (Zimmermann 2008). *Fusarium larvarum* was isolated only from IPj type damage. *Fusarium larvarum* was possibly introduced to the U.S. accidently along with the woolly adelgid (*Adelges piceae*) (Fuxa et al. 1998). Both the fungus and insect are found extensively in this part of Tennessee

(Johnson, K. 1980). The fungus may have other hosts, and is likely vectored by mites. *Lecanicillium* was isolated from both TCD and non-TCD states. The natural host range of *Lecanicillium* covers numerous insect orders. The potential mycopathogenic properties of this fungus are being explored as well (Goettel et al. 2008).

The fungal parasite *Trichoderma harzianum* was isolated from several damage types at both locations. *Trichoderma harzianum* is a common inhabitant of natural and managed systems, in the forest floor and in mulch wood chips (Smith, W. H. 1995). *Trichoderma harzianum* strains, as well as derived chemicals, are exploited commercially for biological control. The succession of other isolated fungi from this study, or their relative presence may have been affected by the consistent colonization of this fungus (Vanneste et al. 2002; Salahuddin AL-Saeedi and Moqdad AL-Ani 2014).

Two stain fungi common to soft and hardwoods were isolated. *Cladosporium* spp were isolated from the stain types A (TCD region) while *Epicoccum nigrum*, a fungus ubiquitous in the environment was isolated not only from stain B in TCD location, but also from other damage types in both regions. Each of these fungi is known to colonize *Juglans nigra* (Schmidt 2006). The stain types characterized in this study were superficial, extending no further than the inner bark.

2.4.2. Phytopathological aspects

Geosmithia morbida, *F. solani and B. dothidea* as well as *D. seriata* were isolated from branch segments and each fungal species was obtained from several damage types.

Geosmithia morbida (part of the TCD complex) was isolated only from the Tennessee branch segments, from damage type IPj, and additionally from types C, D and

E, which had no apparent insect damage. TCD was described in the U.S. in 2009 (Tisserat et al. 2009), followed by confirmation of TCD (2010) in the native range of black walnut in Tennessee (Seybold, 2013). Symptomatic TCD trees have since has been detected in Virginia, Pennsylvania, Maryland, and Ohio. The presence of TCD in other states is likely. Although *G. morbida* is considered to be a weak pathogen, when introduced by WTB the shear number of small cankers can coalesce to girdle a branch or the trunk of the host, killing the tree over the course of several years. *J. nigra* species are particularly susceptible to TCD. Both organisms (fungus, twig beetle) are generally found together. Thus, the identification of either organism has been used in diagnosing thousand cankers disease on eastern black walnut. In this study, the additional damage types from which *G. morbida* was isolated point to other means of dispersal of *G. morbida*. *Geosmithia morbida* has recently been isolated from the weevil *S. pallidus* and two ambrosia beetle species (Juzwik et al. 2015; Juzwik et al. 2016). The role of other insects as potential vectors of *G. morbida* is under current investigation (J. Juzwik, personal communication).

Fusarium solani was isolated from branches in both states, and within Tennessee it was isolated from several damage types on both TCD symptomatic and non-TCD symptomatic trees. Several *Fusarium* spp have been demonstrated to cause cankers on black walnut trees in the central U.S. (Carlson et al. 1993), most notably *F. solani* (Tisserat 1987). Infection can result in dark, elongate cankers which appear in the split bark of the host tree, usually on the lower portion of the tree near the ground. Cankers may completely girdle a tree, and may also occur on branches in the lower crown of young trees. The wood under the bark becomes necrotic. Sprouts are often produced

below the canker or at the base of the tree. Leaf wilt and subsequent dieback at the top of the tree also occurs. It has been suggested that *F. solani*, associated with trunk cankers in advanced stages of TCD plays a minor role in TCD canker development (Tisserat et al. 2009). The degree of relationship between *F. solani* and the TCD complex is presently undetermined. Recent work has identified possible *F. solani* association (phylogenetic species 25 within the *Fusarium solani* species complex) with early TCD development (Montecchio et al. 2015). Further studies are needed to elucidate the role of particular phylogenetic species with TCD development in the eastern U.S.

Botryosphaeria dothidea was also isolated from both states. However, in Tennessee *B. dothidea* was isolated from several damage types on numerous branch segments. A single isolate only was obtained from one canker in Indiana. In contrast, *Diplodia seriata* ("*B." obtusa*) was isolated from a number of branch segments. *Botryosphaeria dothidea* is an opportunistic pathogen associated with cankers and branch dieback of many woody perennial plants including *Juglans* species (Pijut 2005; Mehl et al. 2013). In *Juglans* spp infection by *B. dothidea* results in Botryosphaeria canker, described first by Fawcett in 1915 as Melaxuma canker. Disease development is favored by hosts under physiological stress, and may appear as black cankers in the crotches and limbs of the host, or occasionally as sudden wilting and necrosis. *Botryosphaeria* spp have been isolated in addition to *G. morbida* from TCD confirmed trees in other studies (Hasey, Bostock, and Michailides unpublished data; Bostock 2014). *Juglans* spp show varying resistance to *Botryosphaeria*, although all host species (including *J. nigra*) show some degree of canker formation after inoculation (Smith, C.1934). While numerous susceptible hosts to *B. dothidea* in the eastern U.S. are well documented historically

(Shear, Stevens, and Wilcox 1925; Slippers et al. 2004), recent taxonomic changes necessitate a new look at *Botryosphaeria* host-pathogen coupling. *Botryosphaeria* taxonomy has undergone complete revision in the last decade, making reports of hosts prior to 2000 unreliable (Crous et al. 2006; Phillips et al. 2013). *Botryosphaeria dothidea* was considered synonymous with *B. ribis* until recently. Several distinct species within the genus were previously considered morphological variants within the *ribis* or *dothidea* species. Host colonization by various *Botryosphaeria* spp. must be teased apart and reorganized in light of new classifications. The complexity of *Botryosphaeria* colonization of woody perennials is enhanced when it occurs as a multi-fungal species infection complex (Ma et al. 2001; Chen, Morgan, and Michailides 2014). *Botryosphaeria* is presently interpreted as a latent endophyte, which emerges as a pathogen when the host becomes stressed (Slippers and Wingfield 2007). This may partially explain the appearance of several "*Botryosphaeria"* species simultaneously on the host. *Botryosphaeria dothidea* is a potential contributor to the development of TCD in affected *J. nigra*. Tangled within the *Botryosphaeria* debate, much controversy has surrounded the correct name for the fungus often termed "*Botryosphaeria" obtusa* (anamorph *Diplodia seriata*). At present, no correct name exists for the teleomorph of this fungus; however, *D. seriata* has been shown to be the anamorph of the fungus termed "*Botryosphaeria" obtusa* (Phillips, Crous, and Alves 2007). *Diplodia seriata* is a cosmopolitan, plurivorous fungus (Phillips et al. 2013). Pathogenicity of this species on woody perennial hosts is presently being explored and appears to differ greatly within species of host genera (Phillips et al. 2012). Research is needed regarding the ability of this species to cause disease in black walnut.

2.4.3. Additional factors

In addition to the involvement of *G. morbida* and the bark beetle *P. juglandis* in the development of thousand cankers disease, other fungal pathogens as well as environmental factors may also play a role. *Juglans nigra* is significantly affected by drought stress (Gauthier 2011) and this stress may exacerbate TCD disease severity (Griffin 2015). Drought stress often precipitates *F. solani* canker formation (Weber, Anderson, and Hoffard 1980) and as stated above, *Botryosphaeria* spp. pathogenicity may be influenced by host stress. This may contribute to disease progress and its ultimate impact on tree health.

2.5. Conclusions

In this survey, the isolates obtained represent a portion of the fungal community on black walnut from both within and outside TCD affected locations. Damage types observed in the bark were characterized, and the damage areas dictated the locations for attempts to isolate fungi. It is clear that in TCD locations, trees exhibited other damage types and were colonized by other pathogenic fungi in addition to typical TCD cankers caused by *G. morbida*. Additionally, *F. solani* and *B. dothidea* were isolated from damage on branches in both states. The results of this study indicate a need for further research into the role of these fungi in branch dieback, particularly when *P. juglandis* and *G. morbida* are present, and their relationship to development of TCD-like symptoms on black walnut in its native range.

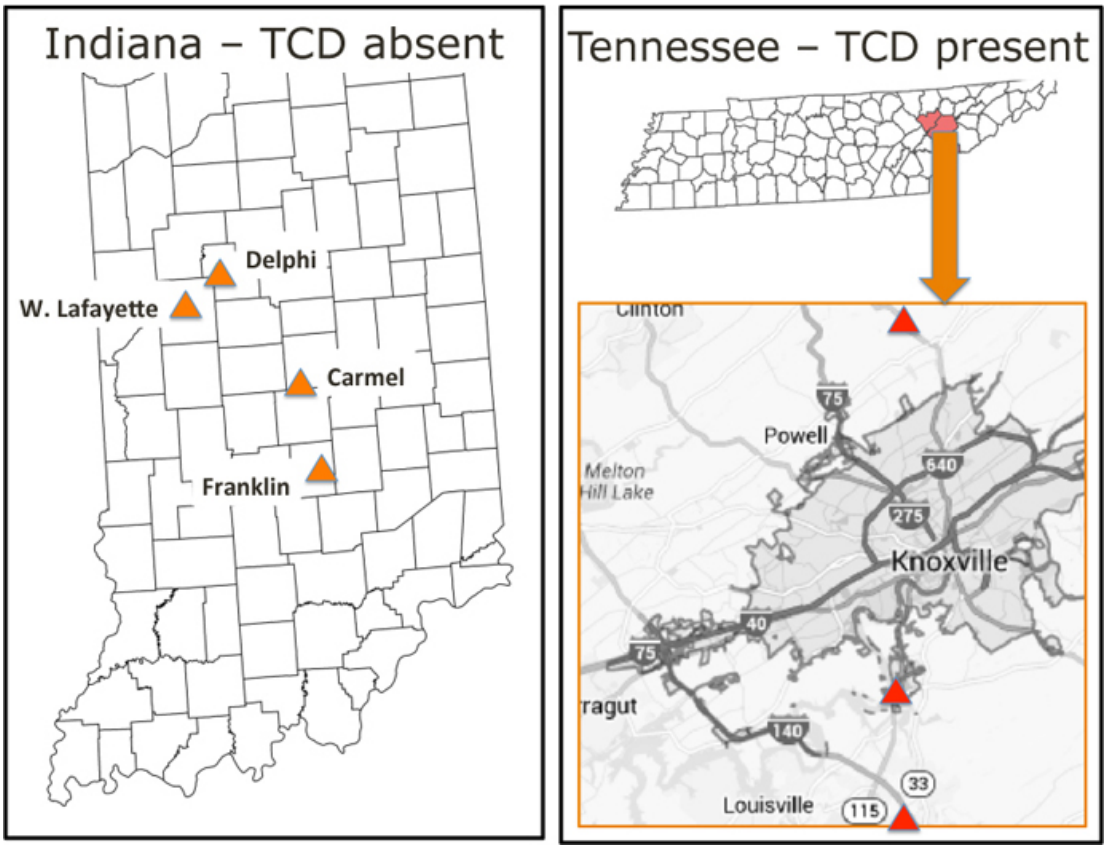


Figure 2-1. Geographical distribution of trees sampled from two different areas within the native region of eastern black walnut in the USA, 2012. (*left*) Healthy appearing trees were selected in each of four Indiana cities. (*right*) Healthy appearing and thousand cankers disease (TCD) affected trees were selected within three Knoxville, Tennessee suburbs.

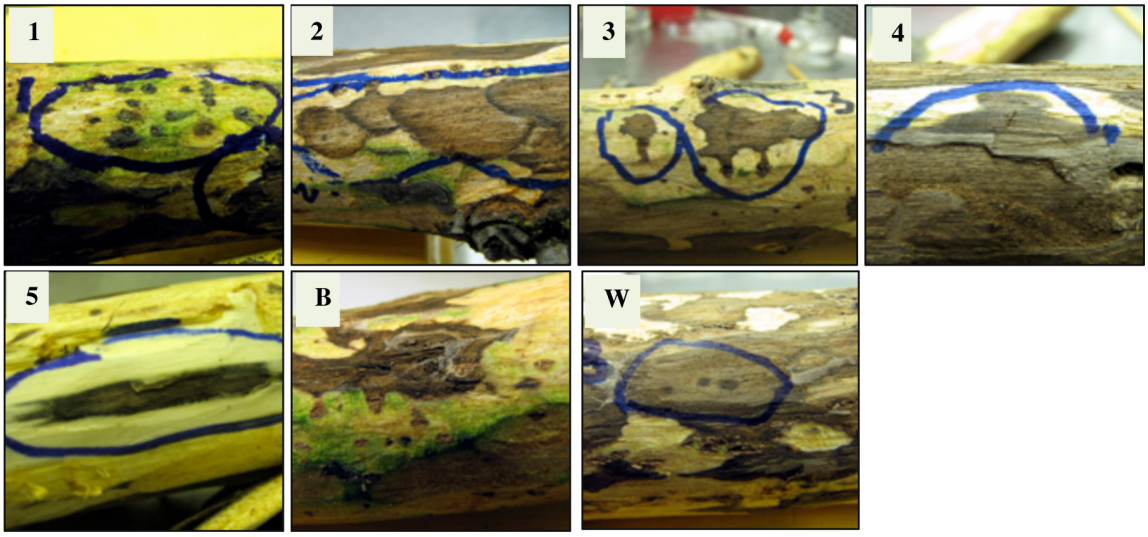


Figure 2-2. Types of damage observed on branch segments from study trees in Indiana, USA 2012. Types include spots (1), lesion or canker-like (2-4), stain (5) and discolored phloem tissue around flat-headed wood borer (B) or weevil (W) tunnels.

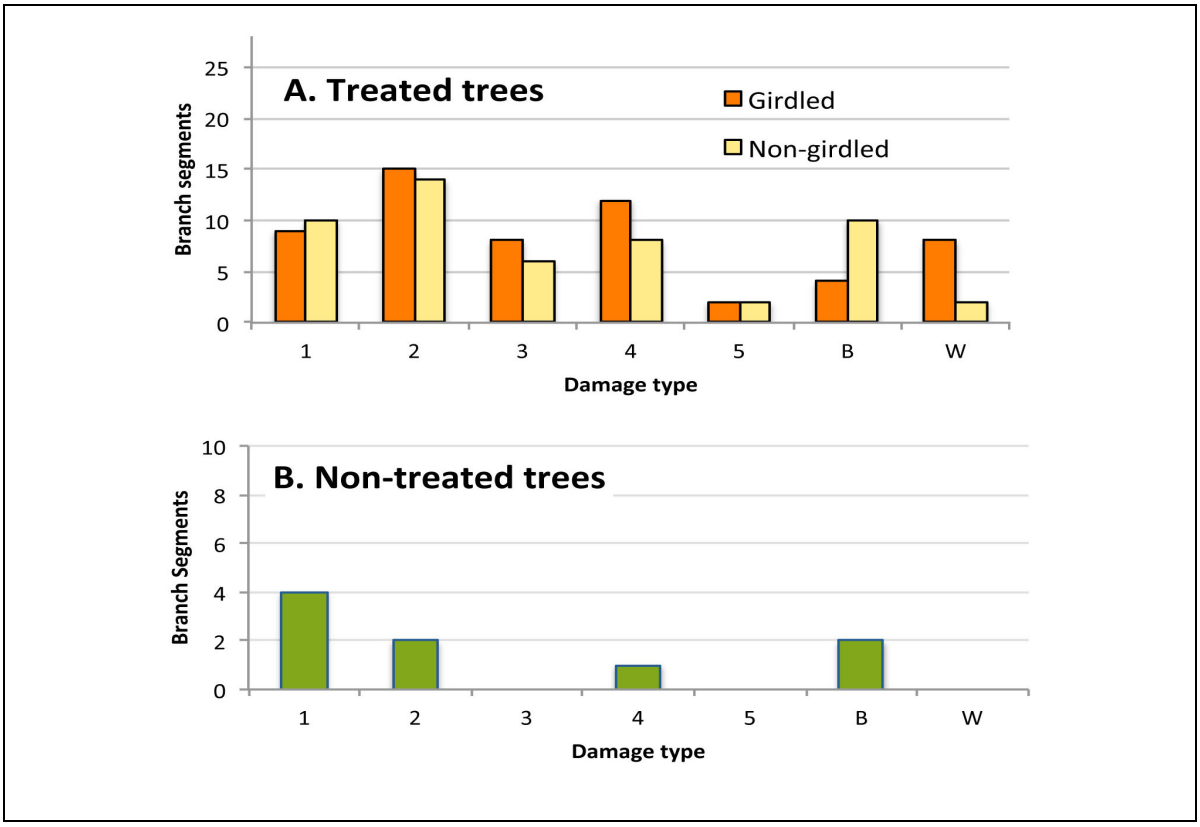


Figure 2-3. Damage types by frequency of occurrence (presence/absence) from branch segments of treated and non-treated branches on black walnut trees in Indiana, USA 2012. A, Girdled branch segments n = 28, non-girdled branch segments n = 28 (from fourteen trees) were evaluated. B, Branch segments n = 12 (from three trees) were evaluated.

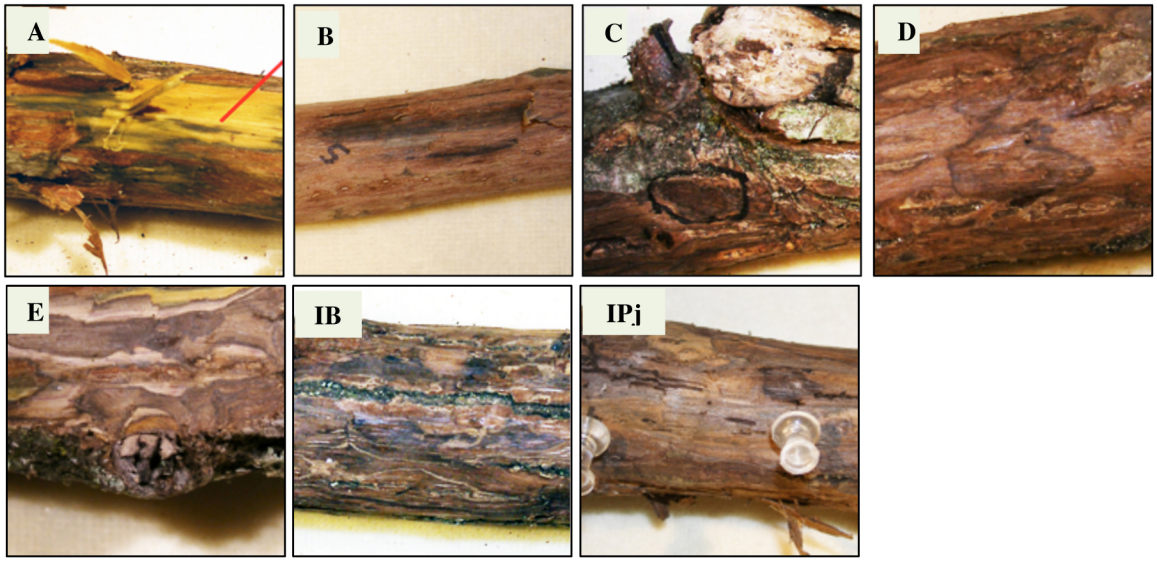


Figure 2-4. Types of damage observed on branch segments from study trees in Tennessee, USA 2012. Types include stains (A, B), lesion or canker-like (C-E) and discolored phloem around flat-headed wood borer (IB) or walnut twig beetle (IPj) tunnels or galleries.

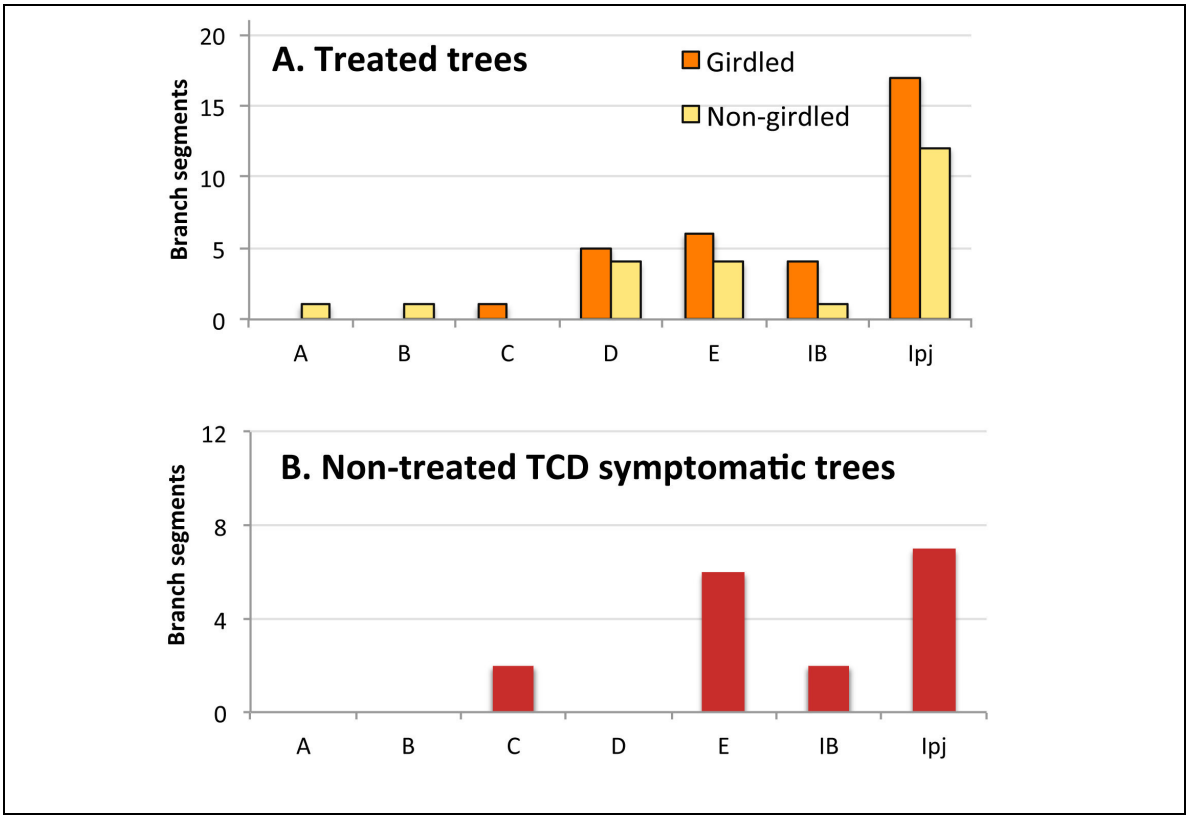


Figure 2-5. Damage types by frequency of occurrence (presence/absence) from branch segments of treated and non-treated (TCD symptomatic) eastern black walnut trees in Tennessee, USA 2012. A. Girdled branch segments n = 18, non-girdled branch segments n = 18 (nine trees total) were evaluated. B. Branch segments n = 12 (three trees total) were evaluated.

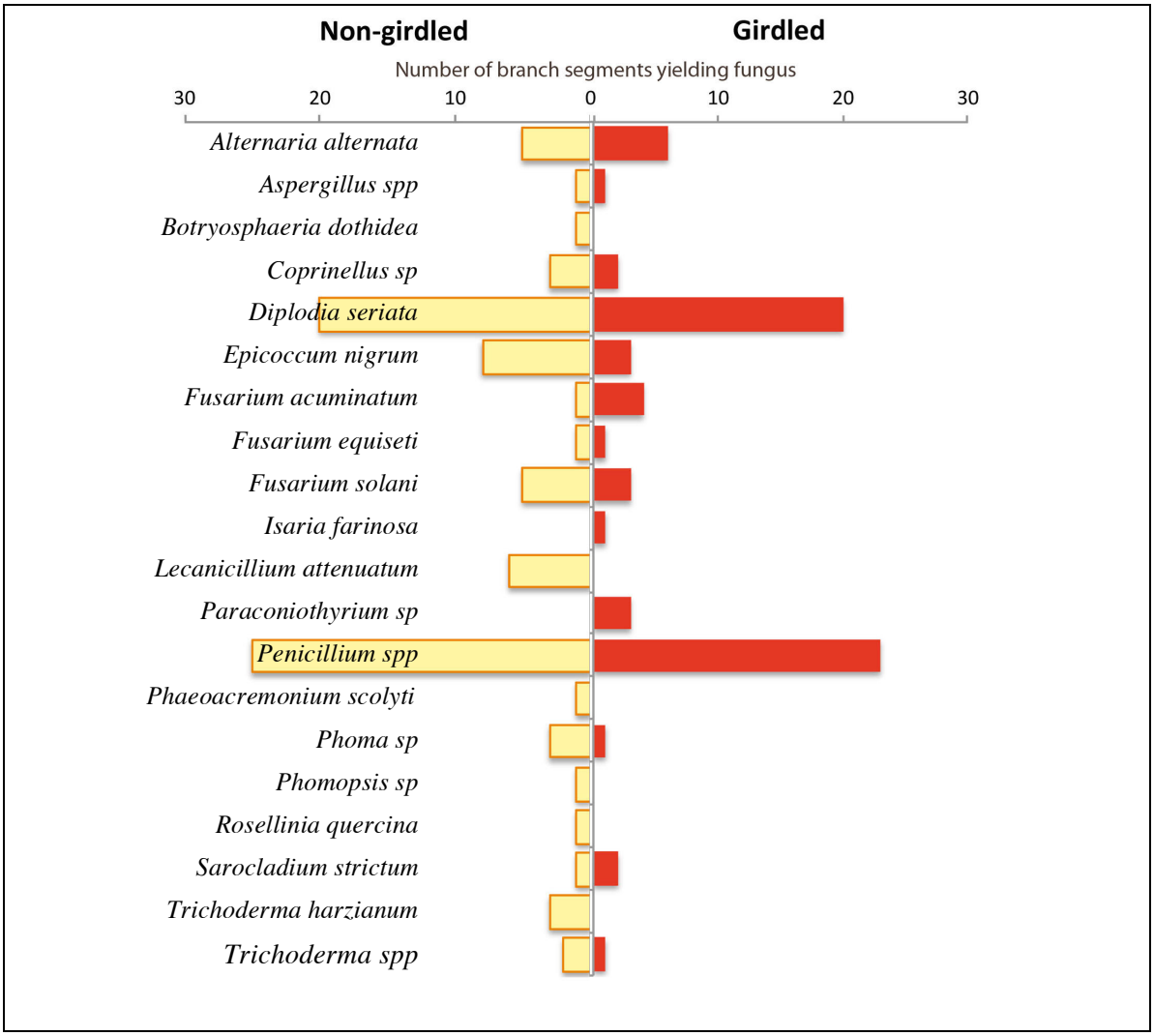


Figure 2-6a. Frequencies of fungi isolated from branch segments of treated eastern black walnut in four cities in Indiana, USA 2012. Number of trees=14. Number branch segments (two per branch) evaluated: two girdled and two non-girdled.

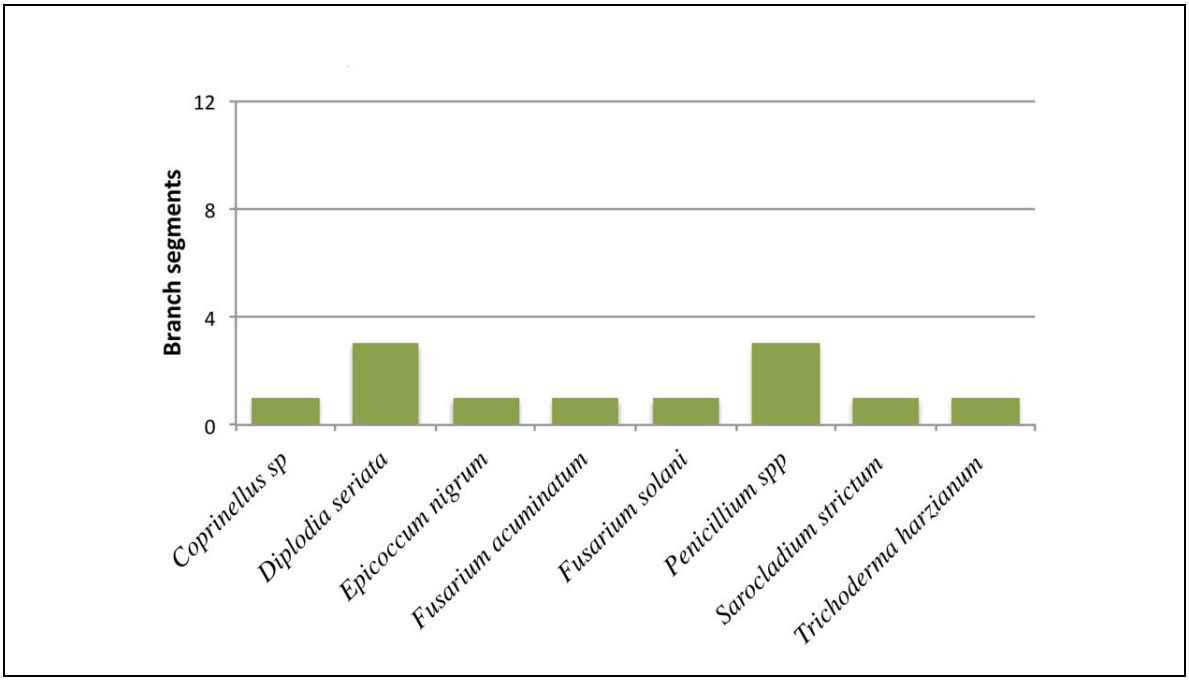


Figure 2-6b. Frequencies of fungi isolated from branch segments (n = 12) of three non-treated trees in four cities in Indiana, USA 2012. Two branch segments were evaluated for two branches from each tree.

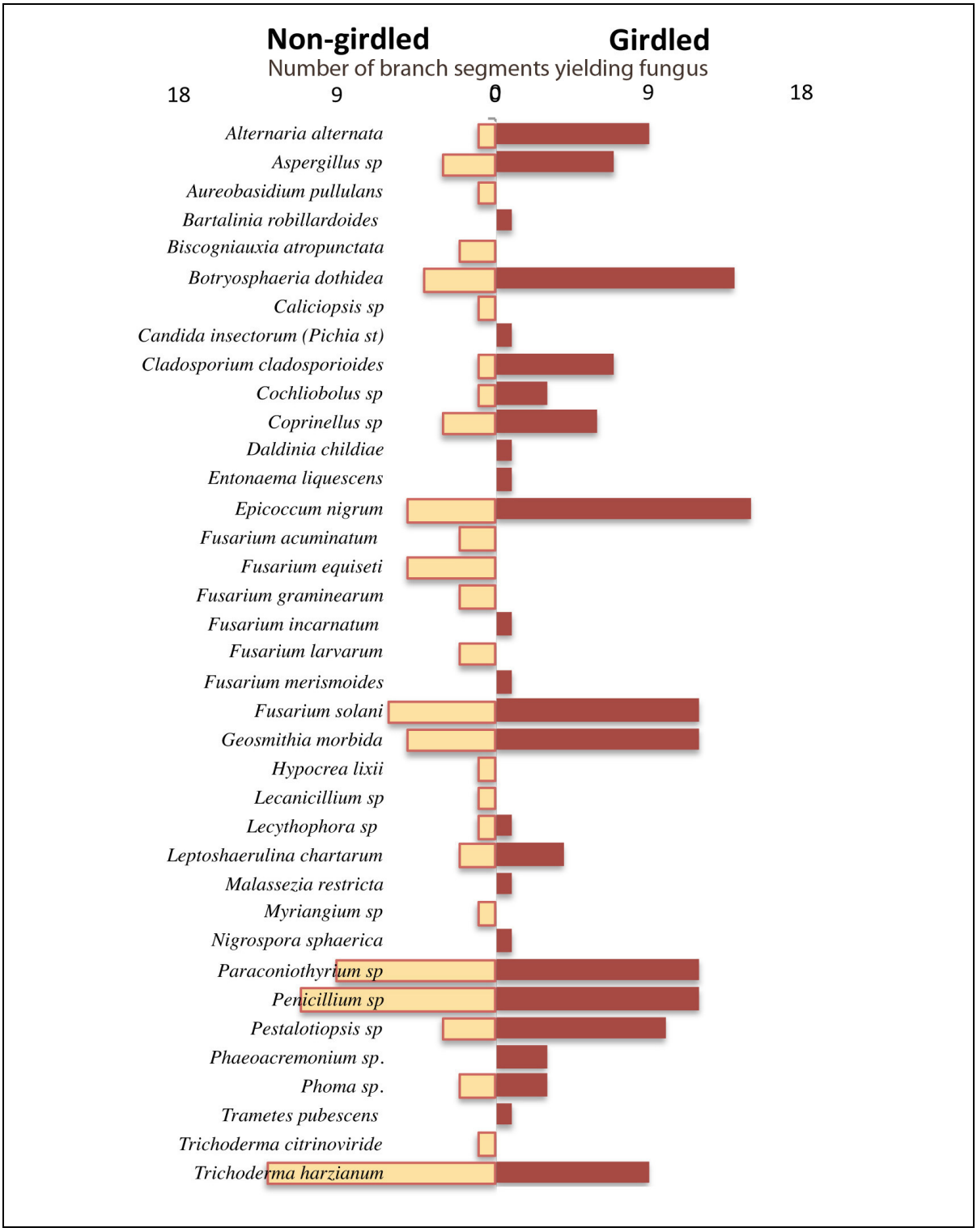


Figure 2-7a. Frequencies of fungi isolated from branch segments of treated eastern black walnut in Tennessee, USA 2012. Number of trees = 9. Number branch segments (two per branch) evaluated: two girdled and two non-girdled.

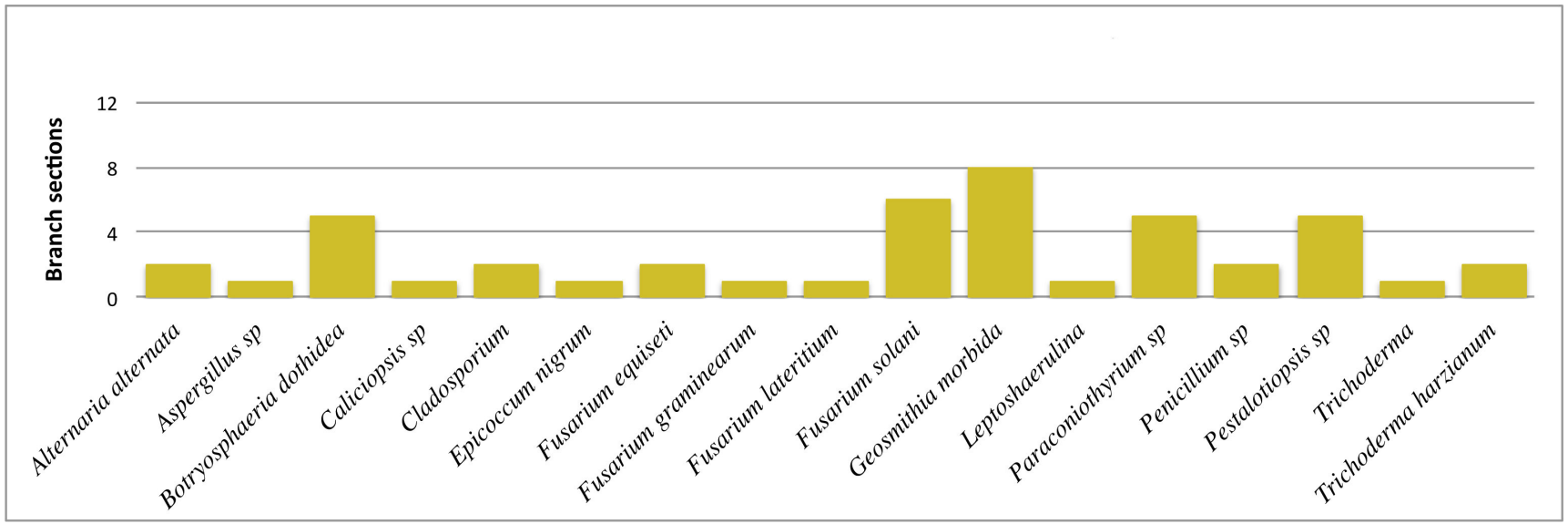


Figure 2-7b. Frequencies of fungi isolated from branch segments (n = 12) of three non-treated TCD symptomatic trees in Tennessee, USA 2012.

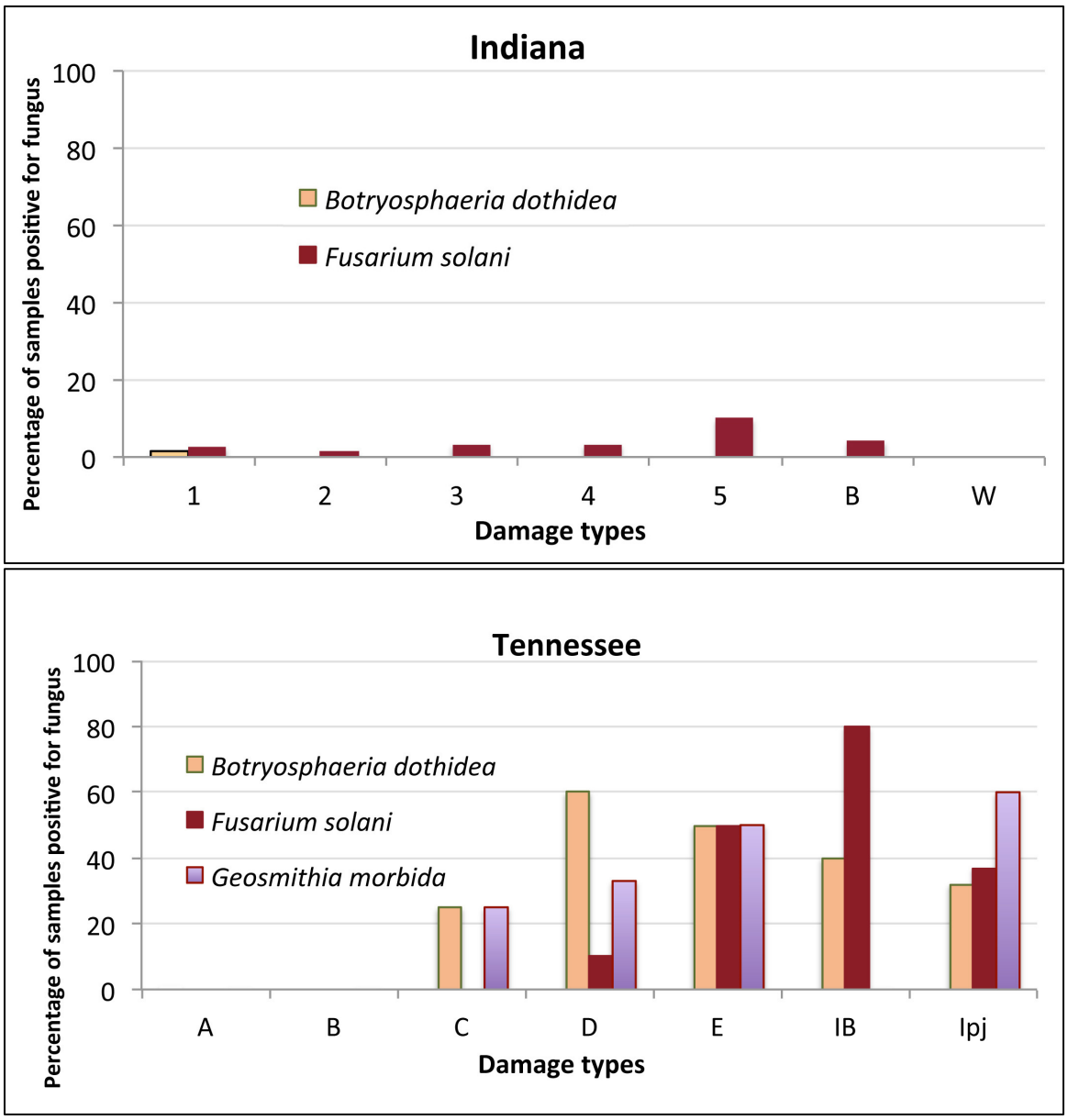


Figure 2-8. Percentage of fungus-positive samples based on isolates obtained from each damage type cultured from black walnut branch samples in Indiana and Tennessee, USA 2012. Number above each bar is number of that particular damage type sample size.

Table 2-1. Description of damage types on branch segments of *Juglans nigra* observed on four sites in Indiana, USA 2012.*

Damage type	Number characterized	Photo ref.**	Description				Damage area	
			Shape	Color	Margin	Other	mean (cm^2)	SE
Spots	37	1	Circular	Very dark brown	Same color	Observed in groups (multiples)	0.24	0.03
Lesion/canker	48	2	Somewhat circular	Medium brown	Darker brown margin	Often coalesced, appearance of overlapping scales	1.06	0.17
Lesion/canker	21	3	Irregular	Dark brown	Same color	Often colaesced, surrounding a lenticel	2.43	0.81
Lesion/canker	26	4	Elliptical, elongate	Chocolate brown	Same color	Sharply defined margin	2.49	0.54
Stain	4	5	Elongate	Dark brown	Dark grey	Diffuse margin	1.56	0.30
Insect (Buprestid)	27	6	Irregular	Dark brown	Same color	2mm diam. holes, tunnels running with grain, frass, surrounded by discoloration	1.62	0.38
Insect (weevil)	27	7	Somewhat circular	chocolate brown	Same color	Pin-hole size surrounded by discoloration	0.71	0.05

**Average branch segment 31 cm length by 4.2 cm diam., ** Photo number in composite figure 2-2 of damage types.*

Table 2-2. Presence of damage types on branch segments of *Juglans nigra* observed from four cities in Indiana, USA 2012, by location

Damage type photo ref.**	Location*				
	Carmel	Delphi	Franklin	W Lafayette	total
1	5	6	11	3	25
2	7	13	5	4	29
3	5	2	2	4	13
4	5	7	5	3	20
5	1	2	1	0	4
W	2	1	6	4	13
B	1	7	3	6	17
segment dead	0	2	0	2	4
No visible damage	4	0	3	4	11

**Segments each location n=16. **Photo number in composite fugure 2-2 of damage types.*

Table 2-3. Presence of damage types on branch segments of *Juglans nigra* observed from four cities in Indiana, USA 2012, by treatment (trt) and location.

		Damage types*						
Trt/No Trt	**Location**	**1**	**2**	**3**	**4**	**5**	**W**	**B**
Treated - girdled branch	Carmel	3	5	3	4	1	2	0
	Delphi	3	5	1	4	0	1	2
	Franklin	3	3	1	2	1	4	2
	W Lafayette	0	1	3	2	0	3	0
	G total(n=24)	**9**	**14**	**8**	**12**	**2**	**10**	**4**
Treated - non-girdled branch	Carmel	2	2	2	1	0	0	1
	Delphi	3	7	1	3	2	0	5
	Franklin	6	1	1	3	0	2	1
	W Lafayette	1	1	1	0	0	0	4
	N total(n=24)	**12**	**11**	**5**	**7**	**2**	**2**	**11**
No treatment	Carmel	0	0	0	0	0	0	0
	Delphi				no sample			
	Franklin	2	1	0	0	0	0	0
	W Lafayette	2	2	0	1	0	1	2
	C total(n=12)	**4**	**3**	**0**	**1**	**0**	**1**	**2**

**Damage type number corresponds to composite figure 2-2 of damage types.*

Table 2-4. Description of damage types observed on branch segments* of *Juglans nigra* from greater Knoxville, Tennessee USA 2012.

Damage type	Number characterized	Photo ref.**	Description				Damage area	
			Shape	Color	Margin	Other	mean (cm^2)	SE
Stain	3	A	Elongate	Dark olive	Same color	Diffuse margin	1.58	0.64
Stain	6	B	Elongate	Warm blackish	Same color	Diffuse margin	2.13	1.24
Lesion/canker	4	C	Somewhat circular	Chocolate brown	Same color	Monochromatic	0.75	0.39
Lesion/canker	11	D	Irregular	Medium brown	Dark brown	Concentric color variation (bullseye appearance)	2.3	1.85
Lesion/ canker	24	E	Highly elongate	Dark chocolate	Same color	Monochromatic, irregularily elongate	0.93	0.83
Insect (Buprestid)	6	F	Irregular	Dark brown	Same color	2mm diam. holes, tunnels running with grain, frass, surrounded by discoloration	3.5	2.28
Insect *(P. juglandis)*	87	G	Irregular, angular	Browns	Dark brown	1mm diam holes, tunnels, nuptial chambers, concentric color variation	2.08	2.02

**Average branch segment 26 cm length by 2.9 cm diameter. **Photo number in composite figure 2-5 of damage types.*

Table 2-5. Fungal species isolated from margin of damage areas on inner bark of *Juglans nigra* sections, from four sites in Indiana, USA 2012.

Closest GenBank match & accession number	Taxonomic placement	GenBank sequence	
		Number	Percent
Alternaria alternata (KF813070)	Ascomycota, Pleosporales	523/523	100
Ascomycota sp. H-8 (FJ375144)	Ascomycota	506/509	99
Aspergillus tubingensis isolate 3SGSK2.1II (KC920472)	Ascomycota, Eurotiales	391/391	100
Botryosphaeria dothidea isolate SC1 (KJ572119)	Ascomycota, Botryosphaeriales	291/297	98
Coprinellus radians isolate M105 (HM595561)	Basidiomycota, Agaricales	551/551	100
*Diplodia seriat*a strain xs-06 (KJ549774)	Ascomycota, Botryosphaeriales	538/539	99
Epicoccum nigrum strain 09174 (KC819619)	Ascomycota, Dothideomycetes	502/504	99
Fusarium acuminatum isolate Facu18 (KJ562375)	Ascomycota, Hypocreales	505/506	99
Fusarium equiseti strain HP7 (KJ677237)	Ascomycota, Hypocreales	394/394	100
Fusarium solani strain YQC-C5 (KF939490)	Ascomycota, Hypocreales	526/527	99
Fusarium sp. FL-2010c (HQ166535)	Ascomycota, Hypocreales	525/525	100
Isaria farinosa isolate HK7 (KC768083)	Ascomycota, Hypocreales	289/291	99
Paraconiothyrium sp. ATCC MYA-4697 (HQ999974)	Ascomycota, Pleosporales	543/543	100
Penicillium brevicompactum strain S3 (KJ45421)	Ascomycota, Eurotiales	544/545	98
Penicillium commune strain H09-122 (KC009831)	Ascomycota, Eurotiales	479/484	98
Phaeoacremonium scolyti strain CBS 113597 (KF764575)	Ascomycota, Diaporthales	470/476	99
Phoma aliena isolate E34	Ascomycota, Pleosporales	485/485	100
Phomopsis sp. UASWS0895 (KF525797)	Ascomycota, Diaporthales	538/543	99
Rosellinia quercina (AB017661.1)	Ascomycota, Xylariales	545/545	100
Sarocladium strictum strain SCAU-F-74 (KF881788)	Ascomycota, Hypocreales	313/313	100
Trichoderma arundinaceum strain NRRL 3199 (EU330932.1	Ascomycota, Hypocreales	504/504	100
Trichoderma brevicompactum strain VRU-Tb114	Ascomycota, Hypocreales	369/374	99
Trichoderma citrinoviride strain H09-105 (KC009820)	Ascomycota, Hypocreales	593/595	99
Trichoderma harzianum isolate UTHSC:02-2663 (KJ174168	Ascomycota, Hypocreales	581/581	100

Table 2-6. Ascomycota species isolated from margin of damage areas on inner bark of *Juglans nigra* sections, greater Knoxville area, Tennessee, USA 2012.

Closest GenBank match & accession number	Taxonomic placement	GenBank sequence	
		Number	Percent
Alternaria alternata (AY154682.1)	Pleosporales	536/536	99
Aspergillus sydowii isolate ASAU-1 (KJ524907.1)	Eurotiales	528/529	99
Aspergillus tubingensis isolate 3SGSK2 (KC920472)	Eurotiales	486/490	99
Aspergillus versicolor strain IHB F 1902 (KF381083.1)	Eurotiales	520/522	99
Aureobasidium pullulans strain Y11 (KC897669)	Dothideales	501/501	100
Bartalinia robillardoides (KF656706)	Xylariales	516/517	99
Biscogniauxia atropunctata isolate YMJ 128 (JX507799)	Xylariales	510/519	98
Botryosphaeria dothidea strain J11 (KF876691.1)	Botryosphaeriales	540/542	99
Candida insectorum strain MUCL 37920 (EU343839)	Sassharomycetales	461/467	99
Cladosporium cladosporioides strain 08SK037 (KF938462)	Capnodiales	514/514	100
Cladosporium silenes strain CMT48 (JQ754032)	Capnodiales	512/512	100
Cladosporium sphaerospermum isolate A1-5 (KF986434)	Capnodiales	349/352	99
Cladosporium velox isolate 040	Capnodiales	427/430	99
Cochliobolus geniculatus strain AL11s2 (KJ188717)	Pleosporales	440/441	99
Cochliobolus lunatus strain C99.2 (JQ936202)	Pleosporales	473/473	100
Diplodia seriata strain xs-06 (KJ549774.1)	Botryosphaeriales	530/530	100
Entonaema liquescens isolate agtS279 (AY616686)	Xylariales	453/458	99
Epicoccum nigrum strain MJ32 (HQ328049)	Dothideomycetes	515/515	100
Fusarium acuminatum isolate Facu18 (KJ562375)	Hypocreales	483/483	100
Fusarium acuminatum strain CHS-3 (KJ082098)	Hypocreales	524/524	100
Fusarium cf. solani 8780 (JX270175.1)	Hypocreales	467/467	100
Fusarium cf. solani CBS 2012 strain CBS 102824 (JX435197)	Hypocreales	525/526	99
Fusarium equiseti isolate JG22 (KJ412501)	Hypocreales	504/504	100
Fusarium incarnatum strain FI-00602 (KJ572780)	Hypocreales	501/502	99
Fusarium larvarum (FN868469)	Hypocreales	523/526	99
Fusarium larvarum var. larvarum strain F-266,785 (EU860064.1)	Hypocreales	523/527	99

Table 2-6, cont'd.

Closest GenBank match & accession number	Taxonomic placement	GenBank sequence	
		Number	Percent
Fusarium lateritium isolate T59 (FJ459977)	Hypocreales	497/497	100
Fusarium merismoides var. violaceum strain F-167,589 (EU860060)	Hypocreales	498/502	99
Fusarium solani isolate 12.4 (EU727450)	Hypocreales	529/530	99
Fusarium sp. NRRL 52776 (JF40929)	Hypocreales	502/504	99
Geosmithia morbida strain ATCC MYA-4903 (KC113640)	Eurotiales	533/539	99
Hyalodendriella sp. FF39 (FJ379833)	Helotiales	500/508	98
Lecanicillium sp. O_3_BESC_246b (KC007329)	Hypocreales	478/483	99
Lecythophora sp. YP363 (JX910080)	Coniochaetales	490/494	99
Leptosphaerulina chartarum strain DH08111quan1 (GU073119)	Pleosporales	540/540	100
Microcera larvarum strain A.R. 4580 (KC291751)	Nectriaceae	416/416	100
Nigrospora sphaerica strain LH-2 (KC519729.1)	Trichosphaeriales	519/519	100
Paraconiothyrium brasiliense strain 1-53 (JF502455.1)	Pleosporales	565/566	99
Paraconiothyrium hawaiiensis strain SGSGf29 (EU715661)	Pleosporales	494/497	99
Paraconiothyrium sp. ATCC MYA-4697 (HQ999974)	Pleosporales	565/567	100
Penicillium brevicompactum strain S3 (KJ145421.1)	Eurotiales	536/542	98
Penicillium chrysogenum isolate ER (KJ185377.1)	Eurotiales	510/512	99
Penicillium commune isolate NJP03 *HQ710533)	Eurotiales	465/475	98
Penicillium kloeckeri strain KUC1286 (HM469393)	Eurotiales	435/439	99
Penicillium olsonii isolate CAB74 (JX310568)	Eurotiales	437/438	99
Penicillium spinulosum (KF646101.1)	Eurotiales	538/539	99
Pestalotiopsis caudata (JN198508)	Xylariales	546/547	99
Pestalotiopsis hainanensis strain CNU060362	Xylariales	539/539	100
Pestalotiopsis microspora isolate T62 (FJ459945)	Xylariales	511/513	99
Pestalotiopsis sp. 1 AE-2013 strain F5069 (KF746154)	Xylariales	514/514	100
Phaeoacremonium iranianum strain PARC188 (KF764531.1)	Diaporthales	498/499	99
Phaeoacremonium sp. DoF19 (JQ388264.1)	Diaporthales	555/555	100

Table 2-6, cont'd.

Closest GenBank match & accession number	Taxonomic placement	GenBank sequence	
		Number	Percent
Phoma fungicola strain 7F91 (KC357254)	Pleosporales	484/488	99
*Phoma medicagini*s strain H5-B (KF293741)	Pleosporales	472/477	99
Pichia mexicana,Candida insectorum (FM199966)	Sassharomycetales	573/578	99
Pleosporales sp. 24_PH (HQ207041)	Pleosporales	450/450	100
Trichoderma citrinoviride strain AUKAR04 (KF698728.1)	Hypocreales	592/592	100
Trichoderma harzianum strain ML14-1 (KJ619614)	Hypocreales	545/547	99
Trichoderma harzianum strain VRU-Th107 (KJ000326.1)	Hypocreales	577/577	100

Table 2-7. Basidiomycota species isolated from margin of damage areas on inner bark of *Juglans nigra* branch sections, greater Knoxville area, Tennessee, USA 2012.

Closest GenBank match & accession number	Taxonomic placement	GenBank sequence	
		Number	Percent
Coprinellus micaceus strain SZMC-NL-3888 (JN943115)	Agaricales	647/652	99
Coprinellus radians isolate M105 (HM595561)	Agaricales	624/627	99
Coprinellus santhothrix isolate NEFU33 (KJ028784)	Agaricales	628/631	99
Hannaella kunmingensis strain ATT082	Tremellales	449/449	100
Malassezia restricta strain ATCC MYA-4611	Malasseziales	496/500	99
Trametes pubescens voucher HHB13585sp (JN164947)	Polyporales	577/577	100

Table 2-8. Fungal species isolated from margin of damage areas on inner bark of *Juglans nigra* branch sections, greater Knoxville area, Tennessee, USA 2012, with less than 97% match to existing entries in BLAST database.

ID	Closest GenBank match & accession number	isolate frequency	GenBank sequence	
			Number	Percent
Fungal sp. 1	Fungal sp. UI-6 strain PA1_3E1d(FJ438385.1)	1	467/516	91
Fungal sp. 2	*Hyalodendriella betula*e strain CZ301C (FJ755263.1)	3	453/485	93
Fungal sp. 3	*Phaeosphaeria* sp. 20081120 18S(HQ324780.1)	2	308/363	85
Fungal sp. 4	*Candida nitratophila strain* CBS 2027 (JX965188.1)	1	601/669	90

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Appendix 1: Tables of assayed insects emerged from eastern black walnut tree branch sections from Indiana and Tennessee in 2012, and fungi isolated from the insects.

Table 1. Assayed insects emerged from twelve study tree branch sections, from four cities in Indiana, USA 2012.

Species	No. assayed
Acamptus rigidus	2
Himatium errans	15
Orchestes alni	21
Sitophilus zeamais	5
Xyloborinus saxeseni	3
Xylosandrus germanus	2
Total assayed	**48**

Table 2. Fungal isolates obtained (ascomycetes) from insects assayed (see appendix table 1) in Indiana, USA 2012.

		Genbank sequence	
Closest Genbank match & accession number	**Taxonomic placement**	**number**	**percent**
Cladosporium sp. JS1043 (AM176680)	Capnodiales	494/498	99
Aspergillus niger strain SR/II/50 (KJ648618)	Eurotiales	359/360	99
Penicillium commune strain H09-104 (KC009819)	Eurotiales	551/552	99
Aspergillus flavus strain TP1F19 (KJ639912)	Eurotiales	357/360	99
Penicillium sumatrense strain SX12 (KC329625)	Eurotiales	447/450	99
Chaetomium funicola strain CBS 128488 (JX280786)	Sordariales	466/468	99
Penicillium sclerotiorum strain ZZ07-5 (JN581573)	Eurotiales	507/509	99
Lecanicillium attenuatum strain CHLB5 (KJ160143)	Hypocreales	516/516	100
Myriodontium sp. NCP02/09 (JX243811)	Onygenales	555/556	99
Aspergillus ustus (DQ649067)	Eurotiales	538/538	100
Aspergillus ochraceus strain 1219 (KF435031)	Eurotiales	528/528	100
Aspergillus ochraceus strain CD1128 (KF651178.1)	Eurotiales	369/377	98
Mycosphaerella sp. M15 (HM595519)	Capnodiales	500/502	99
Aspergillus calidoustus (HG931699)	Eurotiales	529/531	99
Petriella guttulata (FN394728.1)	Microascales	356/357	97
Penicillium commune strain H09-122 (KC009831.1)	Eurotiales	534/534	100
Diplodia seriata strain xs-06 (KJ549774)	Botryosphaeriales	536/536	100
Penicillium brevicompactum strain EPA 462 (AY373899)	Eurotiales	541/541	100
Aspergillus versicolor isolate NRRL 239 (EF652449)	Eurotiales	539/540	99
Aspergillus versicolor strain HT-M36 (KJ527011)	Eurotiales	531/531	100
Aspergillus versicolor strain UBOCC-A-101087 (KF225023)	Eurotiales	524/524	100
Trichoderma harzianum strain NF83	Hypocreales	577/577	100
Aspergillus dimorphicus (FR727119)	Eurotiales	551/551	100
Aspergillus sydowii (AM883157)	Eurotiales	531/531	100
Penicillium canescens strain CV0198 (JX140832)	Eurotiales	542/542	100
Aspergillus pseudodeflectus isolate NRRL 278 (EF652456)	Eurotiales	535/535	100
Aspergillus iizukae (FR733809)	Eurotiales	548/548	100
Trichoderma harzianum isolate TR100 (HQ608121)	Hypocreales	577/577	100
Penicillium biourgeianum strain UASWS0822 (JX139727)	Eurotiales	543/543	100
Pleosporales sp. SVIMS-MICRO F1 (KF922736)	Pleosporales	419/450)	93
Penicillium rugulosum strain D4 (GU566230)	Eurotiales	561/562	99
Penicillium kloeckeri strain DR49 (KC311844)	Eurotiales	424/424	100
Aspergillus versicolor strain QH40 (JF911763)	Eurotiales	527/527	100

Table 3. Fungal species obtained from assayed insects (see Appendix table 1) by percentage of insects positive for each species in Indiana, USA 2012.

fungus	percent of insects
Aspergillus spp	53
Chaetemonium sp	31
Cladosporium spp	9
Diplodia seriata	9
Lecanicillium sp	2
Myriodontum sp	16
Penicillium spp	91
Petriella guttalata	2
Pseudocercosporella fraxini	7

Table 4. Assayed walnut twig beetles (*Pityophthorus juglandis*) from three treated (TCD asymptomatic) trees in greater Knoxville region, Tennessee, USA 2012.

	P. juglandis	
Tree	**Female**	**Male**
1	32	13
2	24	21
3	23	20
total	**79**	**54**

Table 5. Fungal isolates obtained (ascomycetes) from *Pityophthorus juglandis* (see Appendix table 4) emerged from three treated trees (TCD asymptomatic) in Tennessee, USA 2012.

		Genbank sequence	
Closest Genbank match & accession number	**Taxonomic placement**	**number**	**percent**
Aspergillus iizukae (FR733809)	Eurotiales	309/311	99
Aspergillus nomius strain NF51 (KJ588245)	Eurotiales	556/556	100
*Aspergillus sydowi*i isolate ASAU-1 (KJ524907.1)	Eurotiales	523/524	99
Aspergillus versicolor strain HT-M36 (KJ527011)	Eurotiales	326/326	100
Aspergillus versicolor strain IHB F 1902	Eurotiales	479/479	100
Beauveria bassiana isolate (KJ573511)	Hypocreales	526/528	99
Cladosporium sphaerospermum strain UFMGCB (KC811048)	Capnodiales	359/360	99
Cladosporium velox isolate 040 (HM37870)	Capnodiales	432/438	99
Fusarium solani strain YQC-C5 (KF939490)	Hypocreales	329/330	99
Geosmithia morbida strain ATCC MYA-4903	Eurotiales	396/398	99
Hypoxylon perforatum voucher YMJ 66 (JQ009308)	Xylariales	574/581	99
Ophiostoma cf. abietinum 764RJ (JX028564)	Ophiostomatales	543/553	98
Penicillium brevicompactum strain S3 (KJ145421.1)	Eurotiales	542/543	99
Penicillium chrysogenum strain H09-114 (KC009826.1)	Eurotiales	382/382	100
Penicillium citrinum strain TP1F10 (kj639911)	Eurotiales	409/417	98
Penicillium commune strain LCC18 (KF990135.1)	Eurotiales	459/460	99
Penicillium crustosum strain 08CK030 (KF938473)	Eurotiales	454/456	99
Penicillium glabrum strain SFCF20120803-53 (KF313078.1)	Eurotiales	536/537	99
Penicillium guanacastense strain JP-NJ2 (KF991208)	Eurotiales	540/540	100
Penicillium kloeckeri strain DR49 (KC311844)	Eurotiales	454/459	99
Penicillium ochrochloron strain PFR8 (KF358720.1)	Eurotiales	370/377	98
Penicillium sclerotiorum (JN198418)	Eurotiales	479/480	99
Penicillium sclerotiorum strain DAOM 239931 (JN626130.1)	Eurotiales	534/537	99
Penicillium spinulosum (KF646101.1)	Eurotiales	522/534	98
Penicillium sumatrense strain SX12 (KC329625.1)	Eurotiales	453/459	99
Pichia hampshirensis strain ATCC 62897 (FJ381695)	Saccharomycetales	558/565	99
Pichia mexicana 18S rRNA (FM199966)	Saccharomycetales	597/600	99
Trichoderma citrinoviride isolate UTHSC:10-1704 (KJ174201)	Hypocreales	491/492	99
Trichoderma harzianum isolate TR100 (HQ608121.1)	Hypocreales	578/578	100
Trichoderma harzianum isolate UTHSC:02-2663 (KJ174168)	Hypocreales	584/584	100
Trichoderma harzianum strain ML16-1 (KJ619615)	Hypocreales	399/400	99

Table 6. Fungal species obtained from *Pityophthorus juglandis* by percentage of male and female insects positive for that species. Males n=54, females n=79. Tennessee, USA, 2012.

Fungus	females	males	both sexes
Aspergillus spp	32	35	33
Cladosporium spp	33	24	29
Fusarium solani	5	6	5
Geosmithia morbida	61	65	62
Ophiostoma sp	2	2	2
Penicillium spp	54	61	57
Hypoxylon spp	11	15	14
Pichia spp	75	74	74
Trichoderma spp	12	15	13
Beauveria bassiana	1	2	2