INVESTIGATION OF THE FUNGI AFFECTING MICHIGAN CHESTNUT CULTIVARS AND THE EFFECT OF STORAGE CONDITIONS ON CHESTNUT DECAY DEVELOPMENT

By

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ABSTRACT

Species of the *Castanea* genus (chestnuts) are used in the production of chestnut fruit (nuts). This edible product is incorporated into various food products and has become popular among consumers with demand and consumption increasing. Few fungal pathogens are reported to cause nut decay and negatively impact nut production. To identify the fungal species affecting the yield and postharvest quality of nuts, 1,814 samples from 11 Michigan orchards were collected and assessed. Rot symptoms were observed from 16% of the samples with 20 fungal species isolated from nut kernels. *Gnomoniopsis* sp. 1 was most frequently isolated, while *Gnomoniopsis smithogilvyi* was associated with severe disease symptoms. Seven species were evaluated for pathogenicity and two were confirmed as nut rot pathogens. While the other species are known to cause rot, they were not significantly different from the control. To evaluate the effect of cold storage on disease development, nuts from the cultivars Labor Day, Colossal, and Benton Harbor were inoculated with *G. smithogilvyi*, the pathogen that causes brown rot on chestnut. Following inoculation, the nuts were stored for various periods of time at 4 °C in three experiments conducted from 2019 to 2021. The cultivar Colossal was most susceptible to brown rot and cold storage treatments suppressed rot symptoms. Cultivar susceptibility, the pathogens that cause rot of nuts, and postharvest storage conditions should be considered in the development of IPM strategies.

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INTRODUCTION

Castanea species (common name= chestnuts) are from the *Fagaceae* family and are used for fuel, building materials, agroforestry, and food (Beccaro et.al., 2020). *Castanea* spp. are cultivated around the globe for their fruit (i.e. nut), producing 4,065,134 metric tons of nuts in 2020 (FAO, 2022). With the growing interest in chestnuts as a non-nut and gluten free food product, production of nuts for consumption continues to rise in the United States (US) (Whetstone, 2016). As of 2017, the state of Michigan is the highest producer of nuts domestically followed by California, Iowa, Ohio, Florida, and Virginia (USDA, 2022). Three species of *Castanea* (*C. crenata*, *C. mollissima*, and *C. sativa*) are favored for nut production for human consumption either as fresh or processed (such as flour or chips used in beer making) (Beccaro et al., 2009; Chenlo et al. 2008; Whetstone, 2016).

Castanea nuts are unique compared to other harvested tree nuts because they have an unusually high amount of carbohydrate (46%) and water content (49%) compared to others. For example, almonds, have a low carbohydrate (20%) and water (6%) content (Barreca et al., 2020). Harvest, processing, and post-harvest storage are key components to maintain high quality nuts for consumers and minimize microbial post-harvest yield losses (Donis-Gonzalez, 2008; Ertan et al., 2015; Lee et al. 2016; Monarca et al., 2014; Wills and Golding, 2016; Zhu, 2016). Temperature and relative humidity comprise the basic components of cold storage design (Wills and Golding, 2016) and are critical to manage post-harvest to limit storage loss and maintain high of quality (Wills and Golding, 2016). Due to the relatively small size of chestnut orchards, an investment in complex, energetically, and time intensive post-harvest systems for handling of nuts is not viable (Fulbright et al., 2010). Generally post-harvest handling of nuts in Michigan

includes cold temperature storage but relative humidity sensors and maintenance mechanisms are lacking (Ekman, 2014).

A Michigan survey of nuts (2007) found that important pathogens inciting nut rot such as *Ciboria batschiana* and *Phomopsis castanea* which are common in Europe and Oceania, have not been detected in Michigan (Beccaro et al., 2020; Donis Gonzales et al., 2016). Other fungi including *Penicillium* spp., *Acrospeira mirabilis*, *Botryosphaeria ribis*, *Sclerotinia sclerotiorum*, *Botryotinia fuckeliana* (anamorph *Botrytis cinerea*) and *Gibberella* sp. (anamorph *Fusarium* sp.) have been isolated from the kernel and shell of nuts with up to 25% of the harvest affected in 2007 from Michigan chestnut orchards (Donis-González et. al., 2016). Recently, brown rot disease caused by *Gnomoniopsis smithogilvyi* was detected for the first time in Michigan (Sakalidis et al., 2019). Globally, this disease accounts for up to 90% of post-harvest crop loss and brown rot incidence. In Michigan, the incidence has increased since its first detection (Donis-Gonzalez et al., 2016; Kolp, 2018). Since Michigan's chestnut industry is still relatively small, any yield loss causes concern.

Gnomoniopsis smithogilvyi (= synonym *C. castanea*), an ascomycete fungus, is the causal agent of brown rot (BR) and affects chestnut production worldwide including Australia, New Zealand, Italy, India, and Switzerland (Dar and Rai., 2015; Dennert et al., 2015; Maresi et al., 2013; Sakalidis et al., 2019; Shuttleworth et al., 2013). Brown rot affects chestnuts post-harvest causing the degradation of the kernel of the nut. Symptoms include light to dark brown lesions of the nut's kernel resulting in dry and spongy tissue (Shuttleworth et al., 2013). As a result, a diseased nut may not be differentiated at harvest from a healthy nut without cutting it open rendering the nuts unsalable for the fresh market (Shuttleworth et al., 2013).

CASTANEA SPECIES

Chestnut trees belong to the *Castanea* genus and the *Fagaceae* family (Beccaro et al., 2019). *Castanea* is thought to have originated in what is now eastern Asia around 60 million years B.P. from a common ancestor shared with the *Quercus* genus (Lang et al., 2007; Manos et al., 2001). As the *Castanea* genus moved westward from eastern Asia, through Europe and to North America they subsequently diversified into the eight different species of *Castanea* seen today: *C. crenata* (Japan), *C. mollissima* (China), *C. seguinii* (China), *C. henryi* (China), *C. sativa* (Europe), *C. pumila var. ozarkensis* (North America), *C. dentata* (North America), *C. pumila var. pumila* (North America) (Lang et al., 2007). *Castanea* species have been important in the forests and regions of their speciation (Beccaro et al., 2019). Three of the species within the *Castanea* genus are used in the commercial production of nuts: *Castanea crenata* (Japanese chestnuts), *C. mollissima* (Chinese chestnuts), and *C. sativa* (sweet or European chestnuts) (Beccaro et al., 2019).

Castanea species were domesticated in Asia, Europe, and North America multiple times (Beccaro et al., 2019; Manos and Stanford, 2001; Rutter, 1991). In Europe, *C. sativa* was the only species to be cultivated and disseminated throughout central Europe and the Mediterranean (Beccaro et al., 2019). Generally, *C. sativa* grows between 400 and 1000 m above sea level (Beccaro et al., 2019). In Asia four species were domesticated: *C. mollissima*, *C. henryi*, and *C. seguinii*, and *C. crenata* (Beccaro et al., 2019). Their zone of origin falls in the areas of Japan, Korea, and North China and were also domesticated in South Korea and Taiwan (Beccaro et al., 2019; LaBonte et al., 2018). These Asian species favor mild summers and winters (Beccaro et al., 2019). Their growth is favored at 1300 m above sea level and in areas with abundant rainfall (Beccaro et al., 2019). In North America, two *Castanea* species evolved *C. dentata* and *C.*

pumila. *C. dentata*. The American chestnut was ubiquitous in Appalachian forests and favored for lumber due to it large upright growth. Its nuts were abundant and nutritional (Beccaro et al., 2019; Bolgiano and the American Chestnut Foundation, 2008). *C. pumila,* commonly called chinkapin, is used for timber or nut production in North America (Beccaro et al., 2019). Many chestnut cultivars exist today from breeding programs seeking pest resistance, specific nut properties, and environmental adaptation. These improvements are possible due to high levels of phenotypic and genotypic diversity within the *Castanea* genus (Beccaro et al., 2019; Bounous, 2001; Fulbright, 2003).

Chestnut cultivars are found beyond the natural geographical range of *Castanea* spp. and are used in agroforestry, food and lumber production, horticulture, and fuel (Beccaro et al., 2019). In the US, *C. dentata* was the dominant species in the Appalachian forests on the east coast (Fulbright, 2003) but was decimated by the introduced pathogen, *Cryphonectria parasitica*, the causal agent of chestnut blight (Bolgiano and the American Chestnut Foundation, 2008; Hepting, 1974; Ringling and Prospero, 2018). While oak and hickory have replaced the American chestnut, the chestnut remains in their former range as sprouts from old root systems (Hepting, 1974). In rare cases, pockets of mature American chestnut exist, and these trees often harbor a virus that decreases the virulence of *C. parasitica (*Hepting, 1974). The American chestnut has small nuts compared to the European or Asian species of chestnuts (Fulbright, 2003). Today, the use of chestnuts in a landscape or to produce nuts or has been continued with imports from Asia or Europe with species exhibiting tolerance to chestnut blight or improved characteristics for nut production (Fulbright, 2010). Thus, a variety of chestnut species have been selected for use in the US. (Fulbright, 2003; Fulbright et al., 2010; Vossen, 2000).

Tree height, tree vigor, leaf shape, nut shape, nut color, disease susceptibility, canopy shape, are among the differences noted among cultivated chestnut species (Beccaro et al. 2020). Disease susceptibility produced by crosses is determined for the overall tree not the susceptibility of the nuts (Beccaro, 2020). Typically, *C. mollissima* is considered the most disease tolerant chestnut species (Beccaro, 2020) and is considered resistant to chestnut blight but is susceptible to ink disease and Asian chestnut gall wasp (Beccaro, 2020). *C. crenata,* is considered the main sources of germplasm resistance to ink disease but is susceptible to the Asian chestnut gall wasp and chestnut blight (Beccaro, 2020). A third chestnut species used in commercial nut production is *C. sativa* which is susceptible to chestnut blight and the Asian chestnut gall wasp (Beccaro, 2020). However, there are reports that some *C. sativa* cultivars are tolerant of ink disease (Beccaro, 2020).

A mixture of chestnut species and their hybrids may be found in Michigan's production orchards (Fulbright et al., 2010). This diversity of genetics within an orchard can make management difficult due to the differing genetic resistance and phenotypic characteristics of each cultivar (Karlsson Green et al., 2020).

American chestnuts in Michigan include naturalized stands (Bolgiano and the American Chestnut Foundation, 2008). Starting in the mid-1800s, American chestnut and other species were planted in west Michigan where most commercial cultivation occurs (Fulbright et al., 2010). While these stands were decimated by chestnut blight, some stands remain as a result of protection by a native mycovirus that infects *C. parasitica* causing it to become less virulent (Rigling and Prospero, 2017). Planting American chestnuts for nut production is not viable due to the smaller nut size of its nut and its continued susceptibility to chestnut blight. Commercial nut production in Michigan started in the 1980s with the importing of Chinese chestnut seedlings

from open pollinated trees that exhibited favorable characteristics and resistance to chestnut blight; additional species/hybrids have been imported including those from Europe and Japan (Fulbright et al., 2010).

CASTANEA NUTS

Nuts are the sexual reproductive unit of chestnut trees (Beccaro et al. 2020; Rutter et al., 1991). Chestnuts may be vegetatively propagated through cuttings and grafting (Rutter et al., 1991). All chestnuts species are monoecious with both sexual reproductive structures on an individual tree (Rutter et al., 1991). While some chestnuts can self-fertilize, most must out cross with another genotype (Rutter et al., 1991). Chestnuts are also commonly protandrous, with the male catkins (male reproductive structures containing stamens) maturing prior to the maturation of the female flower (female reproductive structure containing the pistil) (Taiz and Zeiger, 1998). After the pollination of female flowers via insects or wind the fruit of the chestnut tree is produced (Rutter et al., 1991).

Nuts can be categorized into three primary layers including the shell (pericarp), the pellicle (seed coat), and the kernel (Beccaro et al., 2019). A chestnut's shell is porous and ridged with an outer physical barrier that encapsulates the pellicle and kernel that are enclosed within (Beccaro et al., 2020; Fulbright, 2003). The porousness of a chestnut's shell has implications for post-harvest quality due to water loss (or retention) and the breaching of this barrier by microorganisms (Beccaro et al., 2019; Rutter et al. 1991). The pellicle is a thin membrane that is located between the shell and kernel of a nut and is known to have antimicrobial properties (Tsurunaga and Takahashi, 2021).

The nut is protected during its development by the formation and growth of the bur, a spiny vegetative tissue that creates a physical barrier to pathogens and a deterrent to pests such as

small rodents, birds, and deer due to the spines that protrude from the burs surface (Beccaro et al., 2019; Bolgiano et al. 2007; Rutter et al. 1991). A mature nut can be characterized as an oval nut with the stylar end on which the flower was attached. The hilum end is where the nut connects to the mother tree (Beccaro et al., 2019).

The composition of a *Castanea* nut differs from many tree-nuts since the starch content of chestnuts is high (Fulbright, 2003). Conversely, the oil content of chestnuts is much lower than other tree-nuts (Fulbright, 2003). The composition of chestnut kernels is carbohydrate (41.2%), fat (1.9%), water, (50.10%) protein (1.9%), and minerals (1.18%)(Fulbright, 2003). Chestnuts unlike other tree nuts (e.g. almonds or pistachios) do not go through a kill step such as blanching or roasting (Fulbright, 2003). Instead, chestnuts are placed in storage where they continue to respire (Donis-Gonzalez, 2008; Ertan et al., 2015; Fulbright, 2003; Mignani and Vercesi, 2003) and maintain an active metabolism (Ertan et al., 2015; Wills and Golding, 2016). To "sweeten" chestnuts, they are placed in cold storage so that their starch breaks down into simple sugars (Amjad et al. 2019; Bufler and Horneburge, 2013; Sugawara et al., 1987; Wiblerley-Bradford et al., 2014; Wills and Golding, 2016; Zhu, 2016).

Nuts from the three *Castanea* species used in nut production can be differentiated based on their morphology (Beccaro et al. 2020). Generally, *C. sativa* nuts are darker in color compared to the two other species (especially *C. crenata*) and have thicker, dark vertical striping running from the stylar to hilum ends of the chestnuts (Bolgiano et al. 2007). *C. mollissima* nuts are also darker than *C. crenata* and *C. sativa* (Bolgiano et al. 2007). *C. sativa* and *C. mollissima* mature nuts range from less than 15 g to 20 g in fresh weight, whereas *C. crenata* nuts may be greater than 30 grams after harvest (Beccaro et al., 2020). *C. crenata* nuts have the lightest shell coloring of the three species and exhibit vertical strips running from hilum to stylar ends of the

nuts (Bolgiano et al. 2007). *C. crenata* also have the smallest and least dense pubescence covering its stylar end (Bolgiano et al. 2007).

COMMERCIAL NUT PRODUCTION IN MICHIGAN

Chestnuts in Michigan are harvested using various techniques and technologies. Growers may use U-pick (customers gather chestnuts from the orchard floor), hand harvesters (nut wizards), or mechanical harvesters to pick up nuts from the orchard floor (Beccaro et al., 2019; Lizotte, n.d.; Monarca et al., 2005, Monarca et al., 2014; Perry and Sibbett, 1998). Commercial growers typically place nuts into refrigerated storage after harvest prior to sorting (Fulbright, n.d.; Lizotte, n.d.).

DECAY CAUSING PATHOGENS

As of 2010, the major nut decay causing fungal pathogens, *Ciboria batschiana* and *Phomopsis castanea*, had been observed in Europe and Oceana but had not been detected in Michigan (Fulbright, et al., 2010). Since then, post-harvest loss of nuts due to decay have increased from an estimated 25% in an orchard in 2007 (Donis-Gonzalez et al., 2016) to 60% in 2021 (Unpublished data, Allie Watson). As chestnut production has increased in Michigan so has the reduction in marketable yield due to post-harvest nut decay (Donis-Gonzalez, 2016).

Fungi associated with decaying nuts in 2008 included *Penicillium* spp., *Acrospeira mirabilis, Botryosphaeria ribis, Sclerotinia sclerotiorum, Botryotinia fuckeliana* and *Gibberella* sp. (Donis-González's, 2008). These species have previously been reported as pathogens in other chestnut growing areas (Donis-González's, 2008). In 2018, the globally known chestnut pathogen that causes brown rot, *G. smithogilvyi*, was identified from symptomatic nuts grown in Michigan (Sakalidis et al., 2019). *G. smithogilvyi* was previously isolated from chestnut blight cankers on American chestnuts in Michigan in 2012 (Kolp, 2018).

GNOMONIOPSIS SMITHOGILVYI

Brown rot is a post-harvest disease affecting the kernel quality of chestnuts (Lione et al., 2019). *Gnomoniopsis smithogilvyi* is an *Ascomycete* fungus apart of the *Gnomoniopsis* genus. *G. smithogilvyi* was identified by Shuttleworth et. al. in 2012 (Crous et al., 2012). In the US, brown rot symptoms may be confused with internal kernel breakdown (IKB). IKB is caused by a genetic incompatibility that occurs when hybrid *C. sativa x C. crenata* cultivars are pollinized by *C. mollissima* leading to the breakdown of tissue in the nuts kernel (Fulbright et al., 2014). The incompatibility is characterized by fibrous brown feathering patterns appearing on the inside of a kernel (Fulbright et al., 2014). Brown rot conversely is caused by a fungus *Gnomoniopsis smithogilvyi*. Originally this rot was observed from Australian chestnut orchards (Shuttleworth et al., 2013). Key aspects of *G. smithogilvyis'* life cycle have been investigated (Dar et al., 2015; Dennert et al., 2015; Maresi et al., 2013; Sakalidis et al., 2019; Shuttleworth et al., 2013). *G. Smithogilvyi* overwinters in chestnut orchards on debris left on the ground after harvest including branches, nuts, and burs (Shuttleworth and Guest, 2017). In the spring, sexual spores are released into the canopy and initiate infection in branches, leaves, and flowers (Shuttleworth and Guest, 2017). This primary infection occurs during bloom and pollination (Shuttleworth and Guest, 2017). Secondary infections occur throughout the growing season via asexual spores' production in a tree canopies (Pasche et al. 2016, Shuttleworth and Guest, 2017). Decay is observed in low numbers in harvested nuts in the fall, with symptoms increasing over time in storage (Shuttleworth and Guest, 2017). In Australia, Shuttleworth and Guest (2017) found apothecia containing asci, sexual structures, overwintering on chestnut burrs in chestnut orchards. While originally characterized as a post-harvest decay of nuts, *G. smithogilvyi* causes cankers on trees, lesions on branches, branch dieback, and fruit mumification (Dar and Rai, 2015; Lewis et al.,

2017; Pasche et al. 2016). Brown rot has also been found in association or inside galls caused by the chestnut gall wasp which is endemic to Asia but an invasive species in Europe and North America (including Michigan) (Magro et al., 2010). Since the initial isolation of *G. smithogilvyi* in Michigan, in 2017, researchers and chestnut growers have furthered the understanding of its biology (Sakalidis et al., 2019). Silva-Campos et al. (2022) evaluated orchard fungicide treatments to prevent the fungal infection of the nuts and monitored the control of the conidial germination and mycelial growth of *G. smithogilvyi* in vitro. The fungicides pyraclostrobin and difenoconazole were identified as effective. Current management recommendations include the removing post-harvest debris to reduce *G. smithogilvyi* overwintering and the use of ground cover plantings to reduce ascospore movement into the canopy in the spring (Shuttleworth et al., 2013; Shuttleworth and Guest, 2017; Visentin et al., 2012). Post-harvest treatments using a hot water bath to kill pathogens in the nut and screening nuts in a buoyancy test to separate poor quality nuts from saleable nuts may also be used (Beccaro et al., 2019; Lizotte, n.d.; Ruocco et al. 2016). Cold storage is another tool to prevent fungal pathogen growth and improve nut taste (Donis-Gonzalez, 2008; Li et al., 2009; Megan and Lacey, 1984; Penagou et al., 2006; Wells and Golding, 2016; Vekiari et al., 2007).

CHAPTER 1: DIVERSITY OF FUNGI ASSOCIATED WITH POST-HARVEST NUT ROT IN CULTIVATED CHESTNUT IN MICHIGAN

ABSTRACT

Species of the *Castanea* genus (chestnuts) are used to produce chestnut fruit (nuts). Michigan is the largest producer of nuts in the United States and consumption is increasing. Post-harvest decay is a challenge for Michigan growers. Chestnut producers and researchers have observed an increase in nut decay and a negative impact on marketable yield. The objectives of this study were to 1) determine the prevalence of brown rot in Michigan chestnut orchards, 2) determine if *G. smithogilvyi* is the primary fungal species inciting post-harvest rot of Michigan's chestnuts, and 3) evaluate the diversity and pathogenicity of fungi associated with symptomatic nuts. Nuts (1,814) were collected from 11 chestnut orchards during 2021. Nuts were split in half and visually evaluated for internal rot symptoms in the kernel and categorized if symptoms observed were characteristic of brown rot. Tissue taken from the edge of rot lesions were plated onto petri dishes containing Potato Dextrose Agar (PDA) supplemented with ampicillin and streptomycin to isolate fungal species associated with symptomatic tissues. Pathogenicity trials were conducted. Twenty fungal species were obtained from nuts exhibiting brown rot symptoms (~50%). Fungal species commonly isolated and grown on PDA from symptomatic nuts were inoculated into 'Colossal' nuts and incubated at 20°C for 14 days. A putative new species *Gnomoniopsis* sp. 1 accounted for 30% of all isolations and was identified in all sampled orchards. *Cytospora vinacea* accounted for 17% of all isolations. *Gnomoniopsis smithogilvyi* was isolated from 8% of the samples. Integrated pest management programs should include strategies to limit nut decaying pathogens. Two species were confirmed to be chestnut pathogens from the pathogenicity study. Further research to elucidate the epidemiology and biology of these pathogens would also be helpful.

INTRODUCTION

Castanea species, referred to as chestnuts, are in the *Fagaceae* family and are used for fuel, building materials, agroforestry, and food (Beccaro et.al., 2020). *Castanea* spp. are cultivated around the globe for their fruit (nuts), producing 4,065,134 metric tons of nuts in 2020 (FAO, 2022). In 2020, the top producers (in metric tons) were China (1,743,354 metric tons), Spain (188,690), Bolivia (80,882), Turkey (76,045), and the Republic of Korea (54,352) (FAO, 2022). The demand for chestnut products is increasing, as producers use them as a key ingredient in gluten free products, pastries, and as a source of flavoring and sugar in beer (Mujić et. al. 2010; Paciulli et. al., 2018; Whetstone, 2016). With the growing interest in chestnuts as a food product, nut consumption continues to rise in the US (Whetstone, 2016). In the U.S, Michigan has planted the most chestnuts (675 acres) followed by California, Iowa, Ohio, Florida, and Virginia (USDA, 2022).

Cultivating chestnuts in Michigan has faced several challenges. As European settlers migrated in in the state in the mid-1800s, *C. dentata* (American chestnut) and other chestnut species such as *C. sativa* (sweet chestnut), *C. crenata* (Japanese chestnut), and *C. mollissima* (Chinese chestnut) were planted in western Michigan's lower peninsula (Fulbright, et al., 2010). In 1920, most American chestnut and sweet chestnut plantings in Michigan were decimated by the introduction of *Cryphonectria parasitica*, the fungal pathogen that causes chestnut blight (Fulbright, et al., 2010). To replace or plant new chestnut stands and orchards, chestnut enthusiasts and commercial growers have planted a wide variety of *Castanea* germplasm including Chinese (*C. mollissima*), European (*C. sativa*) and Japanese (*C. crenata*), and Euro-Japanese (*C. sativa* × *C. crenata*) hybrids (Fulbright, et al., 2010).

The most recent (2007) survey of nuts in Michigan found that major nut decay causing pathogens such as *Ciboria batschiana* and *Phomopsis castanea*, prevalent in Europe and Oceania, have not been detected in Michigan (Donis Gonzales et al., 2016). Fungi including *Penicillium* spp., *Acrospeira mirabilis*, *Botryosphaeria ribis*, *Sclerotinia sclerotiorum*, *Botryotinia fuckeliana* (anamorph *Botrytis cinerea*) and *Gibberella* sp. (anamorph *Fusarium* sp.) were isolated from the shell and kernel of nuts with up to 25% of the harvest affected in 2007 (Donis-González et. al., 2016). Recently, brown rot disease (caused by *Gnomoniopsis smithogilvyi*) was detected for the first time in Michigan (Sakalidis et al., 2019). Globally, this disease has accounted for up to 90% of post-harvest crop loss. In Michigan, incidence of brown rot has continued to increase since it was first detected (Donis-Gonzalez et al., 2016; Kolp, 2018,). Michigan's chestnut industry while growing, is still relatively small and yield loss caused by brown rot is of increasing concern.

Gnomoniopsis smithogilvyi (=synonym *C. castanea*) is the causal agent of brown rot and negatively affects chestnut production in Australia, New Zealand, Italy, India, the U.S. and Switzerland (Dar et al., 2015; Dennert et al., 2015; Maresi et al., 2013; Sakalidis et al. 2019; Shuttleworth et al., 2013). Infection by *G. smithogilvyi* results in the degradation of the kernel over time, with the formation of light to dark brown lesions leading to dry and spongy tissue (Shuttleworth et al., 2013).

In Michigan, there appears to be diversity among species associated with decaying chestnuts (Donis-González et. al., 2016; M.L. Sakalidis unpublished). Researchers in China have also found multiple species associated with decaying chestnuts (Jiang and Tian, 2019; Jiang et al. 2020). The diversity of *G. smithogilvyi* in Michigan (M.L. Sakalidis unpublished), along with the additional fungi associated with disease indicates that the post-harvest decay of nuts in Michigan

may be more complex than previously thought. The objectives of this study were to: 1) determine the prevalence of brown rot in Michigan chestnut orchards, 2) determine if *G. smithogilvyi* is the primary fungal species inciting post-harvest rot of Michigan's chestnuts, and 3) evaluate the diversity and pathogenicity of fungi associated with symptomatic nuts.

MATERIALS AND METHODS

Presence and diversity of fungi associated with brown rot symptoms

Collection of nuts

In 2020, 1,214 nuts were collected from Chestnut Growers Incorporated (Milford, Michigan), representing 10 commercial chestnut orchards predominately distributed along the west coast of Michigan's lower peninsula (**Figure 2.1. and Table 2.1.**). An additional 600 nuts were harvested from a 20-year-old Michigan State University (MSU) research orchard located in the center of Michigan's lower peninsula (**Figure 2.1. and Table 2.1.**). This research orchard is a 20-year-old experimental chestnut orchard located (N 42.87353992677423, W - 85.25872655981114) at MSU's AgBioResearch Clarksville Research Center in Ionia county (Clarksville, Michigan USA). The nuts represent a subsample of the 2020 yield total per orchard. Nuts from Chestnut Growers Incorporated (Milford, Michigan) and Clarksville Research Center were consistent with the description of the morphological characters of nuts from Euro-Japanese hybrid trees. Only intact and undamaged nuts were selected, placed into vented poly bags, and stored at 4°C at the Forest Pathology Laboratory at MSU prior to further processing.

Table 1.1. Incidence of symptomatic and asymptomatic nuts sampled from chestnut orchards in 2020 collected from nine counties in Michigan, USA.

		% and raw numbers of nuts sampled (raw no.)			
Orchard ID No.	**County**	**% asymptomatic**	**% general rot symptoms [b]**	**% BR symptoms only**	**Total**
1	Berrien	55.7% (54)	44.3% (43)	20.6% (20)	97
2	Oceana	41.5% (59)	58.5% (83)	16.9% (24)	142
3	Ingham	42.3% (47)	57.7% (64)	0.0% (0)	111
4	Antrim	42.2% (49)	57.8% (67)	7.8% (9)	116
5	Mason	64.0% (64)	36.0% (36)	36.0% (36)	100
6	Antrim	72.1% (80)	27.9% (31)	4.5% (5)	111
7	Van Buren	73.5% (72)	26.5% (26)	0.0% (0)	98
8	Antrim	61.4% (102)	38.6% (64)	4.8% (8)	166
9	Leelanau	91.9% (159)	8.1% (14)	1.2% (2)	173
10	Ottowa	89.0% (89)	11.0% (11)	9.0% (9)	100
11	Ionia [a]	24.3% (146)	75.7% (454)	29.2% (175)	600
Total		50.8% (921)	49.2% (893)	15.9% (288)	1814

[a] Single research orchard included in this study.

[b] Nuts exhibiting internal rot symptoms were considered symptomatic.

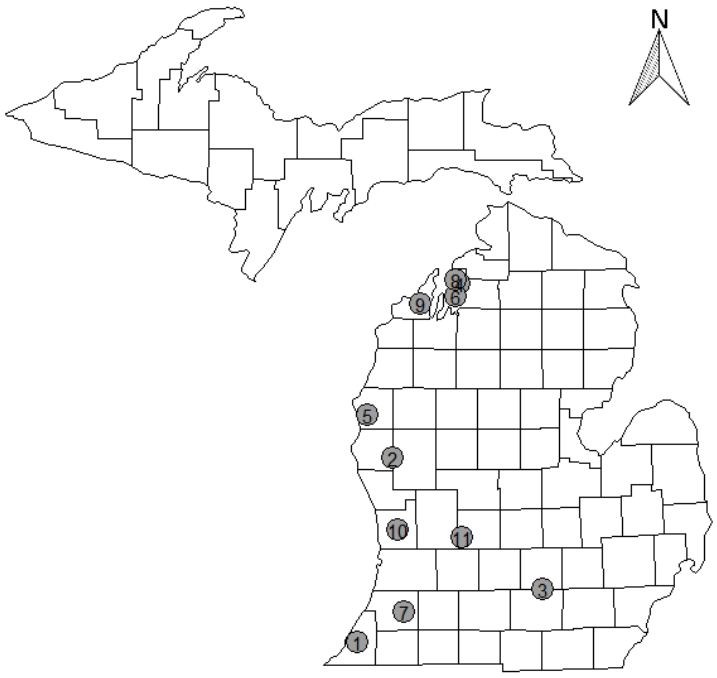

Figure 1.1. Distribution of commercial chestnuts orchards in Michigan's lower peninsula sampled in this study. Circles indicate location of orchards.

Disease Assessment

Nuts were split in half by making a vertical cut from the stylar to hilum end using a disinfected blade (**Figure 2.2.**) and visually evaluated for internal rot symptoms in the kernel. Nuts showing symptoms of BR were considered diseased (i.e., a symptomatic nut). These nuts were counted and disease incidence (%) calculated as the percentage of diseased nuts of the total number of nuts sampled (Shuttleworth 2013).

Fungal isolation

Isolations were made from at least 12 symptomatic and three asymptomatic nuts per orchard. To expose fresh margins, the surface tissue of the kernel was removed and three to four thin pieces of tissue of approximately 2 mm^2 (**Figure 2.2.**) were placed onto a petri dish

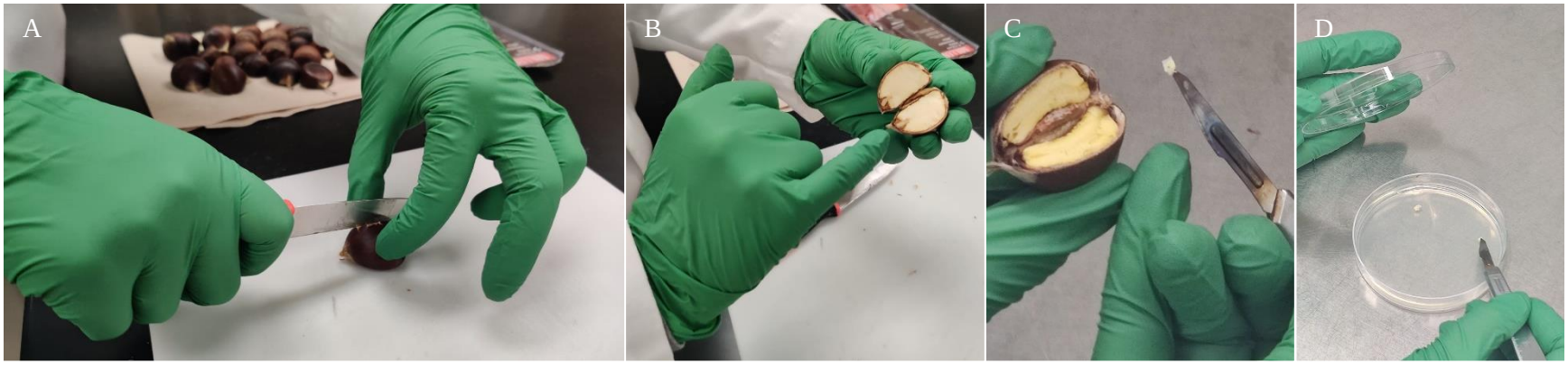

Figure 1.2. Evaluation of nuts for disease symptoms and sampling for isolation of potential fungal pathogens from nut kernels. (Panel A) Cutting open of nuts from stylar to hilum end using surface disinfected knife. (Panel B) Inspection of kernel tissue for symptoms of rot. (Panel C-D) Plating of nut tissue onto PDA using aseptic technique in laminar flow hood to isolate fungal species from.

(100 mm X 60 mm) containing Potato Dextrose Agar (PDA, 39 gr/l, Difco, New Jersey, USA) supplemented with ampicillin (100 mg/ml) and streptomycin (50 mg/ml). All Petri dishes were incubated at room temperature for seven days and subcultures were made from any fungal growth. All cultures were single-hyphal tipped until pure cultures were obtained. Pure cultures were stored long term as agar plugs, approximately 0.5 cm in diameter, in 2 ml polypropylene cryogenic vials (Cryovial silicon self-standing, Simport Scientific, Canada) containing 1 ml of 40% sterile solution of glycerol (VWR International, PA, USA) at -20 °C in the Forest Pathology Laboratory at MSU.

Molecular identification

DNA was extracted from all isolates using OMNI-Prep DNA Extraction Kit (G Biosciences, Missouri, USA) following the manufacturer's protocol with minor modification and stored at -20°C. Modification of the protocol included the use of 2% CTAB buffer to replace the kit's Lysis Buffer (Hamelin et al., 2000). To assist with the identification of fungal isolates the internal transcribed spacer region (ITSrDNA) was sequenced. Isolates belonging to the *Gnomoniopsis* genus were further characterized by the sequencing of two additional protein coding regions: the partial sequence of translation elongation factor 1-alpha (EF1- α) and beta-tubulin (β-tubulin). All primers used to amplify these regions are listed in (**Table 2.2.**).

The PCR mixture contained 5 µl of 25x colorless buffer (Promega Corp., Madison, WI), 4 µl of Mg^{2+} (100 µM) (Promega), 0.5 µl of dNTP (1:1:1:1 at 100µM) (Invitrogen, Carlsbad, CA), 1.0 µl of each primer (10µM), 0.13 µl of GoTaq Flexi (Promega; 5 U/µl), and DNA template adjusted to 50 ng for a total volume of 25 µl. Reactions for the amplification of ITSrDNA, EF1-α, and β-tubulin were run on an ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) using the following conditions: initial denaturation a

Table 1.2. Sequences and characteristics of primers used for the amplification of conserved gene regions from fungi isolated from chestnuts collected in Michigan, USA.

Gene Region	Primer Name	Primer Sequence (5'-3') [a]	Annealing Temperature (°C)	Approximate length of amplicon	Reference
ITSrDNA	ITS1F	CTT GGT CAT TTA GAG GAA GTA A	54 [b]	593	Gardes and Bruns, 1991
	ITS4	TCC TCC GCT TAT TGA TAT GC			White et. al., 1990
EF1- α	EF1-728F	CAT CGA GAA GTT CGA GAA GG	58 [c]	341	Carbone and Kohn, 1999
	EF119R	GGG AAG TAC CMG TGA TCA TGT			Walker et al., 2010
β-tubulin	BT2a	GGT AAC CAA ATC GGT GCT GCT TTC	58	517	Glass & Donaldson, 1995
	B2b	ACC CTC AGT GTA GTG ACC CTT GGC			

[a] Mixed base codes: M(A, C).

[b] Annealing temperature used by Gardes and Bruns in 1991: 55 °C.

[c] Annealing temperature used by Walker et. al. in 2010: 55 °C

95 °C for 2 min, then 40 cycles at 95 °C for 30 s, 54 °C (58 °C for EF1-α and β-tubulin) for 50 sec, and 72 °C for 1 min and a final extension of 72 °C for 5 min, with a holding temperature of 4 °C. Amplicons were resolved in a 0.75% agarose gel stained with SYBR Safe (Invitrogen, USA) and amplicon size was determined using 1 KB Plus DNA Ladder (Invitrogen, USA) by visualization with an Ultraviolet Fluorescence Analysis Cabinet (Spectronics Corporation, USA). PCR products were purified using the Monarch PCR & DNA Cleanup Kit (New England Biolabs Inc., MA, USA) according to manufacturer's protocol, and sequenced at the MSU Research and Technology Support Facility on an ABI 3730xl platform sequencer (Applied Biosystems, Foster City, CA). Forward and reverse sequences were aligned and manually edited using GeneiousPro Version 2020.2.4 (Biomatters, NZ) (http://www.geneious.com/). Initial identification of isolates was completed using BLAST® version 2.13.0 (NIH, USA) using greater than 98% pairwise identity (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search in GenBank® (NIH, USA) (https://www.ncbi.nlm.nih.gov/).

Pathogenicity Trial

The most common fungal species isolated from symptomatic nuts were included in the pathogenicity trial. In October 2021, 'Colossal' nuts were harvested from a research orchard at MSU's AgBioScience Research Station in Ionia county (Clarksville, Michigan USA), sorted based on buoyancy, disinfected, and placed in cold storage at 4°C. At harvest, 150 of the disinfected "sinker" nuts were evaluated for rot symptoms as described above to calculate the "baseline" of rot present at harvest. Sinking nuts or "sinkers" are considered to be of high quality with minimal defects.

Four days after harvest, 540 "sinker" nuts were removed from cold storage at 4°C and left at room temperature for 24 hours prior to inoculation. On the day of inoculation, all nuts were

surface disinfected with 70% ethanol (C_2H_5OH) solution for three minutes and allowed to air dry on sterile paper towels. An 11-gauge (3.0 mm outer diameter) bone biopsy needle was used to remove a section of the nut's shell and kernel at the mid-to hilum end (i.e., center) of the nut, and if asymptomatic inoculated with mycelia plug for each selected fungal isolate. A total of 60 nuts per isolate were inoculated. Mycelial plugs (2.4 mm in diameter) from 7-day-old cultures grown on PDA were created using the same size bone biopsy needle and placed into the hole created on the nut. Agar plugs of sterile, non-inoculated PDA were used as a negative control. An additional control of 60 of disinfected, nonwounded nuts (representing "natural infection") were included in this test as an additional negative control to assess the "baseline" of rot present at the end of the incubation period. Thirty nuts were inoculated by one of two lab personnel. The inoculation hole on every nut was sealed with waterproof silicon (Mfr. Model # GE500) (GE, CT, USA), which was left to dry before nuts were placed in one gallon polyethylene storage bags (Ziploc; S.C. Johnson, Racine, Wisconsin, USA) inside 28-quart clear plastic bins and stored at room temperature for 14-days. Plastic bins were blocked by isolate and personnel. Fourteen days post inoculation, all nuts were processed as described previously. The virulence of an isolate was measured by lesion size represented as the average disease severity for each isolate (Pariaud et. al., 2009). Disease severity was determined by rating nuts on a scale of 0-4 where 0 = no rot symptoms; 1= 1-25% of the surface area of the kernel of both nut halves with rot; 2 = 26-50% of the surface area of the kernel of both nut halves with rot; 3 = 51-75% of the surface area of the kernel of both nut halves with rot; and 4 = 76-100% of surface area of the kernel with rot (Sakalidis et al. 2019). Rot is defined as a lesion occurring on the kernel with or without sporulation and with or without a sign of the fungal isolates inoculated (i.e., mycelia).

Phylogenetic Analysis

Phylogenetic relationships were determined among isolates for three gene regions (ITSrDNA, EF1- α, and β-tubulin) individually and combined. Sequences were aligned using the MAFFT v7.450 plug in v7.450 in GeneiousPro Version 2020.2.4 (Biomatters, NZ). The MAFFT algorithm was set at Auto, Scoring Matrix at 200 PAM/k=2, a gap open penalty of 1.53, and an offset value of 0.123. The three genes were concatenated using GeneiousPro Version 2020.2.4 and aligned using the previously described MAFFT algorithm. Alignments of each protein coding gene region were manually trimmed on each end to remove uninformative and/or excess nucleotides. Maximum likelihood (ML) was calculated using raxmlGUI v2.0.7 (Edler et. al., 2021), with clade support being assessed with 1000 bootstraps. Phylogenetic trees were visualized using FigTree Version 1.4.4 (Andrew Rambaut, 2007; http://tree.bio.ed.ac.uk) and were rooted at their midpoint. GeneBank accession numbers are available in (**Table 2.3.)**.

Statistical Analysis

All statistical analyses were completed using JMP®, Version 16.0.0 (SAS Institute Inc., Cary, NC, 1989–2021). An analysis of variance (ANOVA, $p< 0.05$) was completed in JMP to determine if the average disease ratings were significantly different from each other. Following the ANOVA test, a Tukey HSD test was conducted to identify which average disease rating for each isolate included was significantly different from each other or the controls.

Table 1.3. Isolates considered in this study. For all isolates, the ITS clade is provided. GenBank numbers are provided for the three gene regions sequenced in this study (ITS, ß-tubulin and EF1-α). Dashes (-) indicate data is not available.

Species Name	Isolate Code [a, b]	Alternate Code [b]	GenBank Accession Number		
			ITSrDNA	EF1- α	β-tubulin
Diaporthe eres	CBS 138594*	-	KJ210529	KJ210550	KJ420799
Gnomoniopsis alderdunensis	CBS 125680*	-	GU320825	GU320801	GU320787
Gnomoniopsis castanopsidis	CFCC 54437*	-	MZ902909	MZ936385	-
Gnomoniopsis chamaemori	CBS 804.79	-	GU320817	GU320809	GU320777
Gnomoniopsis chinensis	CFCC 52286*	-	MG866032	MH545370	MH545366
Gnomoniopsis clavulata	CBS 121255	AR 4313	EU254818	GU320807	EU219211
Gnomoniopsis comari	CBS 806.79	-	EU254821	GU320810	EU219156
Gnomoniopsis daii	CFCC 54043*	CMF 002A	MN598671	MN605519	MN605517
Gnomoniopsis fagacearum	CFCC 54316*	-	MZ902916	MZ936392	MZ936408
Gnomoniopsis fructicola	-	AR 4275	EU254824	GU320792	EU219144
Gnomoniopsis guangdongensis	CFCC 54443*	-	MZ902918	MZ936394	MZ936410
Gnomoniopsis guttulata	-	MS 0312	EU254812	-	-
Gnomoniopsis hainanensis	CFCC 54376*	-	MZ902921	MZ936397	MZ936413
Gnomoniopsis idaeicola	CBS 125672	-	GU320823	GU320797	GU320781
Gnomoniopsis macounii	CBS 121468	AR 3866	EU254762	GU320804	EU219126
Gnomoniopsis occulta	CBS 125677	-	GU320828	GU320812	GU320785
Gnomoniopsis paraclavulata	CBS 121263	BPI 877448	EU254839	EU221939	EU219218
Gnomoniopsis racemula	CBS 121469*	AR 3892	EU254841	GU320803	EU219125
Gnomoniopsis rossmaniae	CFCC 54307*	-	MZ902923	MZ936399	MZ936415
Gnomoniopsis sanguisorbae	CBS 125299	-	GU320819	GU320806	GU320791

Table 1.3. (cont'd)

Species Name	Isolate Code [a, b]	Alternate Code [b]	GenBank Accession Number		
			ITSrDNA	EF1- α	β-tubulin
Gnomoniopsis silvicola	CFCC 54418	-	MZ902926	MZ936402	MZ936418
Gnomoniopsis smithogilvyi	CBS 130190*	-	JQ910642	JQ910645	JQ910639
	MIFCC502	-	-	-	-
	MIFCC503	-	-	-	-
	MIFCC504	-	-	-	-
	MIFCC505	-	-	-	-
	MIFCC506	-	-	-	-
	MIFCC507	-	-	-	-
	MIFCC508	-	-	-	-
	MIFCC509	-	-	-	-
	MIFCC510	-	-	-	-
	MIFCC511	-	-	-	-
	MIFCC512	-	-	-	-
	MIFCC513	-	-	-	-
	MIFCC514	-	-	-	-
	MIFCC515	-	-	-	-
	MIFCC516	-	-	-	-
	MIFCC517	-	-	-	-
	MIFCC518	-	-	-	-
	MIFCC519	-	-	-	-
	MIFCC520	-	-	-	-
	MIFCC521	-	-	-	-
Gnomoniopsis tormentillae	CBS 904.79	-	EU254856	GU320795	EU219165
Gnomoniopsis xunwuensis	CFCC 53115*	-	MK432667	MK578141	MK578067
	CFCC 53117	-	MK432669	MK578069	MK578143
Sirococcus castaneae	CBS 142041	Dca90	KX929744	KX929710	KX958443
	CBS 142042	Dca98	KX929751	KX929717	KX958450
Sirococcus conigenus	CBS 113.75*	-	EF512482	EF512544	EU219129
Sirococcus piceicola	CBS 119620*	-	EF512480	EF512542	EU219130
Sirococcus quercus	CBS 142126*	CPC 29512	KY173465	-	-
Sirococcus tsugae	CBS 119627	-	EF512478	EF512540	EU219143

Table 1.3. (cont'd)

[a]Fungal isolates sequenced in this study are presented in bold font and ex-type, epi-type or holotype species are indicated by an asterisk (*).

[b]MIFCC: Michigan Forestry Culture Collection, East Lansing, Michigan, United States of America; CBS: Centraalbureau voor Schimmelcultures Utrecht, Netherlands; CFCC: China Forestry Culture Collection Centre.

RESULTS

Fungal Diversity

Symptoms of rot incidence

Symptomatic nuts were found in all orchards sampled. Disease incidence varied among orchards. Nearly one-half (49%) of the 1,814 sampled nuts were symptomatic, with disease incidence ranging from 8% (orchard 9) to 76 % (orchard 11) with an average disease incidence of 40%. Orchards with rot incidence greater than the average included orchards 4 (58%), 3 (58%), and 2 (59%) (**Table 2.4**). Orchards with less than the average of rot incidence included orchards 10 (11%), 7 (27%), 8 (39%), 6 (28%), 5 (36%), 8 (39%), and 1 (44%) (**Table 2.1**).

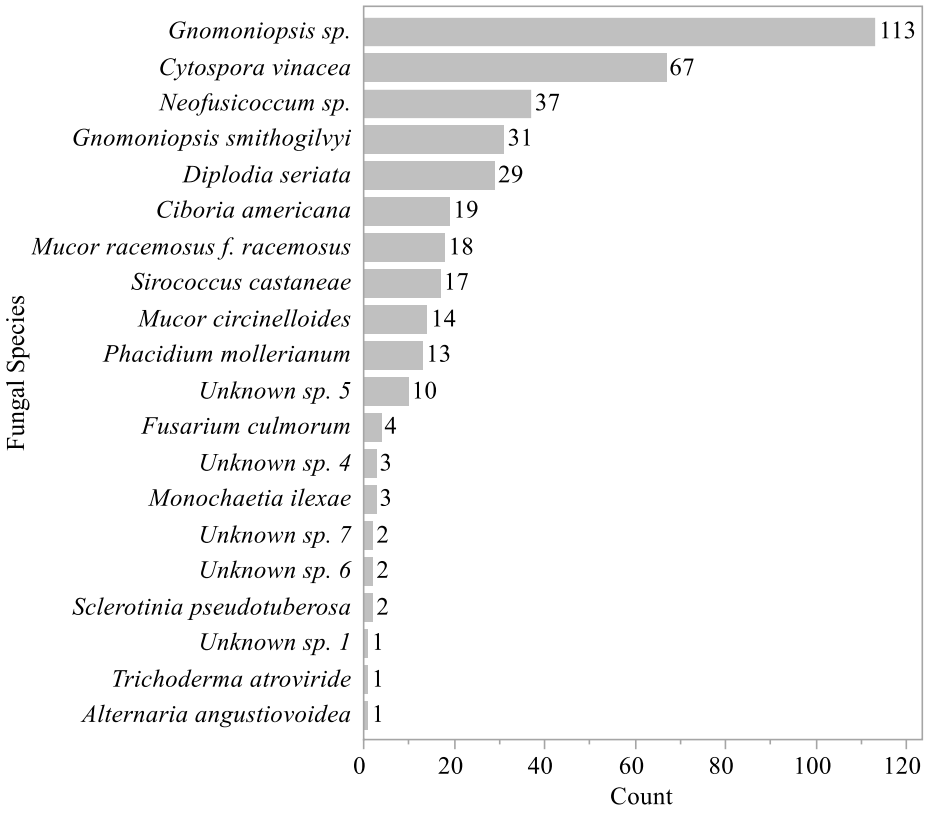

Figure 1.3. Overall diversity and frequency of fungal species isolated from symptomatic and asymptomatic nuts sampled from chestnut orchards in 2020.The number to the right of each bar is the isolation frequency for each fungal species (N= 222 isolates).

Brown rot disease incidence

Brown rot symptomatic nuts were observed in 9 orchards and disease incidence varied. Twelve percent of the 1,814 sampled nuts had brown rot symptoms with incidence ranging from 0% (orchards 3 and 7) to 36% (orchard 5). Orchards with above the average brown rot (12%) included orchards 2 (17%), 1 (21%), 11 (29%) and 5 (36%). Orchards with below average brown rot (12%) included orchards 9 (1%), 6 (5%), 8 (5%), 4 (8%), and 10 (9%) (**Table 1.1.**).

Fungal diversity and incidence from all sampled chestnuts

The 222 fungal isolates obtained from asymptomatic and symptomatic nuts were sorted into 20 morphological groups corresponding to 13 known fungal species (57%) and seven unknown fungal species (43%) (**Figure 1.3.**). Sixty-five percent (144/222) of the isolates belonged to *Gnomoniopsis*; 14% (31/222) were identified as *G. smithogilvyi*. The five most common fungal species that were isolated included *Gnomoniopsis* sp. 1 (29%), *Cytospora vinacea* (17%, GenBank accession no. MK554867.1, 100% match), *Neofusicoccum* sp. 1 (10%; GenBank accession no. MT197527.1, 100% match), *G. smithogilvyi* (8%, GenBank accession no. JQ910642 and 100% match), and *D. seriata* (7%, GenBank accession no. MT587370.1 and 100% match) (**Figure 1.3.**).

Fungal species present in symptomatic vs asymptomatic nuts

Of the 20 species isolated, 14 species were isolated from brown rot symptomatic nuts (70%) and none were obtained from asymptomatic nuts. The most commonly isolated species from BR symptomatic nuts were *Gnomoniopsis*

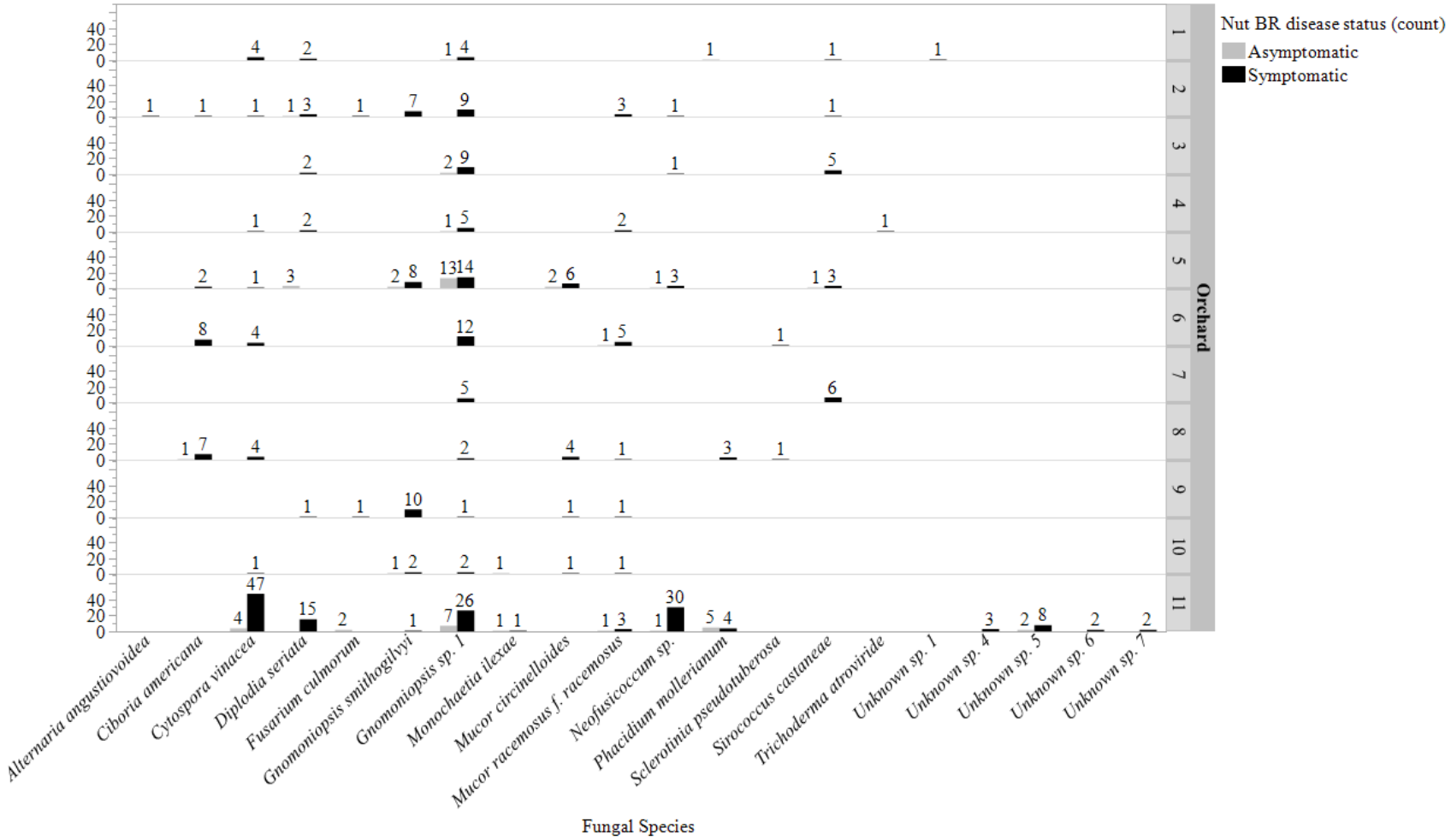

Figure 1.4. Per orchard diversity and frequency of fungal species found in sampled chestnut orchards in Michigan, USA. The number at the top of each bar is the isolation frequency for each species for nuts from each orchard (N= 222 isolates).

sp. 1 (27 %), followed by *Cytospora vinacea* (19%), *Neofusicoccum* sp. (11%) and *G. smithogilvyi* (8%). All of the four most commonly isolated fungal species from symptomatic nuts were also isolated in asymptomatic nuts, but at lower frequencies (**Figure 1.4.**).

Fungal Diversity within Chestnut Orchards

The number of fungal species isolated from symptomatic and asymptomatic nuts in each orchard ranged from 13 (orchard 11) to 2 (orchard 7) with an average of seven species isolated. Orchards 1, 3, 4, 6, 7, 9, and 10 were below the average and orchards 2, 5, 8, 13 were at or above the average (**Figure 1.4.**).

Fungal species incidence per orchard

Of the 20 species isolated, *Gnomoniopsis* sp. was found in all 11 sampled orchards, with 12 species found in more than one orchard. Seven species were found in a single orchard and included: *Alternaria angustivoidea*, *Trichoderma atroviride,* Unknown sp. 1, Unknown sp. 4, Unknown sp. 5, Unknown sp. 6, and Unknown sp. 7 (**Figure 1.4.**). Two of the 21 fungal species were isolated in only two orchards including *Monochaetia ilexae* and *Sclerotinia pseudotuberosa* (**Figure 1.4.**). Two fungal species were isolated from three orchards including *Phacidium mollerianum* and *Fusarium culmorum* (**Figure 1.4.**). Three fungal species were isolated in four orchards including *Ciboria americana*, *Mucor cirinelloides*, and *Neousicoccum* sp. (**Figure 1.4.**). Two fungal species were isolated in five orchards including *Gnomoniopsis smithogilvyi* and *Sirococcus* sp. (**Figure 1.4.**). Two fungal species were isolated in seven orchards including *D. seriata* and *Mucor racemosus f. racemosus* (**Figure 1.4.**). One fungal species was isolated in eight orchards, *Cytospora vinacea* (**Figure 1.4.**).

Pathogenicity Trial

Inoculated nuts were sampled to complete Koch's postulate. Isolates were obtained from lesions on the nut's kernel. *Diplodia seriata* and *Neofusicoccum* sp. produced lesions significantly ($p<0.05$) larger than the negative control (**Figure 1.5.**). Lesions observed in the pathogenicity trial ranged from small white lesions to gray with purple undertones. *Mucor racemosus f. racemosus, C. vinacea, Sirococcus castaneae, G. smithogilvyi isolate 205,* and *G. smithogilvyi isolate* 501 produced lesions significantly ($p<0.05$) smaller than the negative control (**Figure 1.5.**). The nuts inoculated with sterile PDA developed lesions at the inoculation site (**Figure 1.6.**). Lesions from PDA nuts were varied in color and texture (**Figure 1.6.**). *Mucor racemosus f. racemosus, Cytospora vinacea*, *Sirococcus castaneae*, *G. smithogilvyi isolate* 205, and *G. smithogilvyi isolate 501* incited white, dry, spongy lesions on the chestnut kernels after inoculation (**Figure 1.6.**). Nuts inoculated both with the *Neofusicoccum* sp. and *Diplodia seriata* isolates had slightly moist grey lesions with the *Neofusicoccum* sp. developing a purple tinge to the infected nut kernels (**Figure 1.6.**). *Mucor racemosus f. racemosus* resulted in the smallest kernel lesions even when compared to natural infection (**Figure 1.6.**).

Diplodia seriata and *Neofusicoccum* sp. were the most virulent isolates, causing the largest lesions on chestnut kernels. Nuts inoculated with *D. seriata* and *Neofusicoccum* sp. had an average disease severity of four 14 days after inoculation, and their average disease severities were not statistically different from each other (**Figure 1.5.**). Nuts inoculated with *Cytospora vinacea*, *Sirococcus* sp., *Gnomoniopsis smithogilvyi isolate* 205, *Gnomoniopsis smithogilvyi*

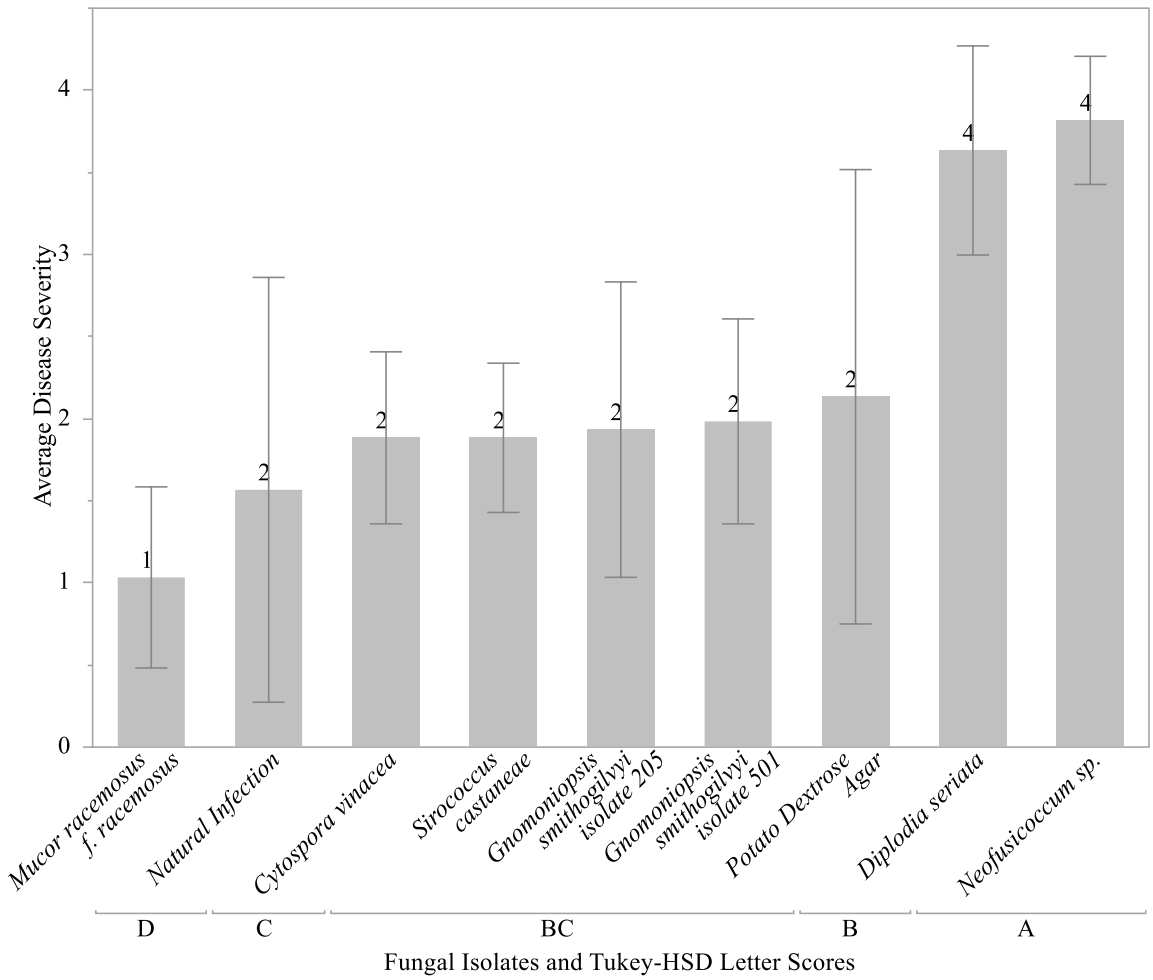

Figure 1.5. Average disease severity for seven fungal species inoculated onto cv 'Colossal' kernel. Disease severity was determined by rating nuts on a scale of 0-4, where 0 = no rot symptoms; 1= 1-25 % of the surface area of the kernel of both halves of the nut exhibited rot symptoms; 2 = 26-50 % of the surface area of the kernel of both halves of the nut exhibited rot symptoms; 3 = 51-75 % of the surface area of the kernel of both halves of the nut exhibited rot symptoms; and 4 = 76-100 % of surface area of kernel exhibited rot symptoms. The number at the top of each bar is average disease severity (n=60). Error bars are the standard deviation from the mean. Statistical significance indicated by letters below each isolate, Tukey-Kramer HSD ($\alpha = 0.05$). The two controls include Potato Dextrose Agar (PDA) (sterile, non-inoculated PDA) and Natural Infection (unwounded nuts).

isolate 501 had an average disease severity of two 14 days after inoculation, and their average disease severities were not statistically different from each other (**Figure 1.5.**). Nuts inoculated with *Mucor racemosus f. racemosus* had an average disease severity of one 14 days after inoculation severity, and its average disease severity was statistically different from all other isolates inoculated (**Figure 1.5.**). Potato Dextrose Agar (PDA) had an average disease severity rating of two 14 days after inoculation, and its mean lesion size was statistically different from all other inoculated isolates' (**Figure 1.5.**). Naturally infected nuts and had an average disease severity rating of two 14 days after inoculation, and its mean lesion size was statistically different from all other inoculated isolates' (**Figure 1.5.**). There was high variation in lesion size among the nuts inoculated with PDA and nuts that were naturally infected (**Figure 1.5.**).

Phylogenetic Analysis

Sequences were trimmed to approximately 675, 1,208, and 865 characters in length for the ITSrDNA, EFR and, β-tubulin gene alignments respectively. Sequences from all three gene regions were obtained for all isolated fungi with the exception of MCP 163 for its β-tubulin gene region. The final concatenated three gene alignment included a length of 2,692 characters. ML analysis of the gene produced two different tree topologies for the isolates from this study. In all ML trees created separation of individual known species was maintained (**Figure 1.7., Figure 1.8., Figure 1.9., and Figure 1.10.**). Of the 16 isolates morphologically similar to *G. smithogilvyi,* 15 isolates from this study grouped with the type specimen of *G. smithogilvyi* (CBS 130190) into a single clade, showing 98% bootstrap support in the multigene gene ML tree (**Figure 1.7.**). An additional isolate, MCP 062, grouped with *G. smithogilvyi* in two of three

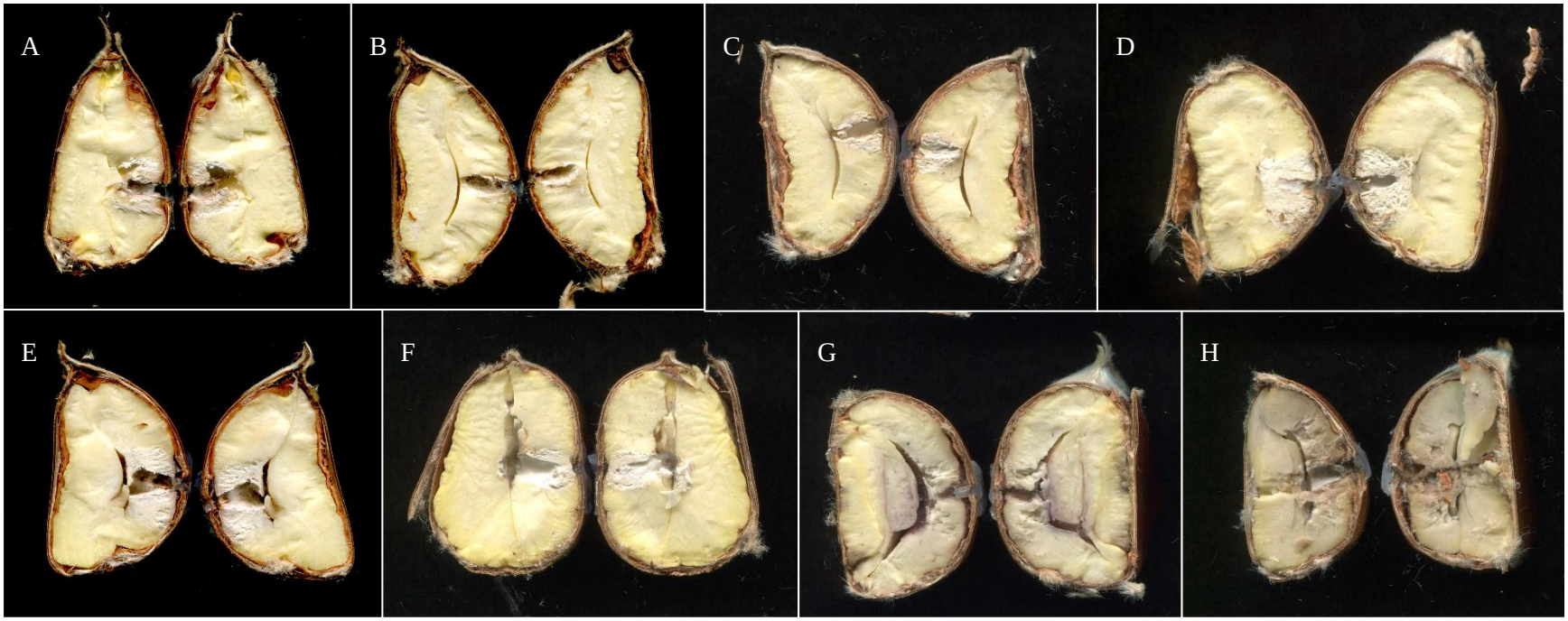

Figure 1.6. Internal kernel symptoms caused by seven fungal species on cv. 'Colossal' chestnuts at 14 days post inoculation. Panels (A: negative control, B-H: fungal species): (A) PDA, (B) *Mucor racemosus f. racemosus,* (C) *Cytospora vinacea,* (D) *Sirococcus castaneae,* (E) *Gnomoniopsis smithogilvyi isolate 205,* (F) *G. smithogilvyi isolate 501,* (G) *Neofusicoccum sp.,* (H) *Diplodia seriata.*

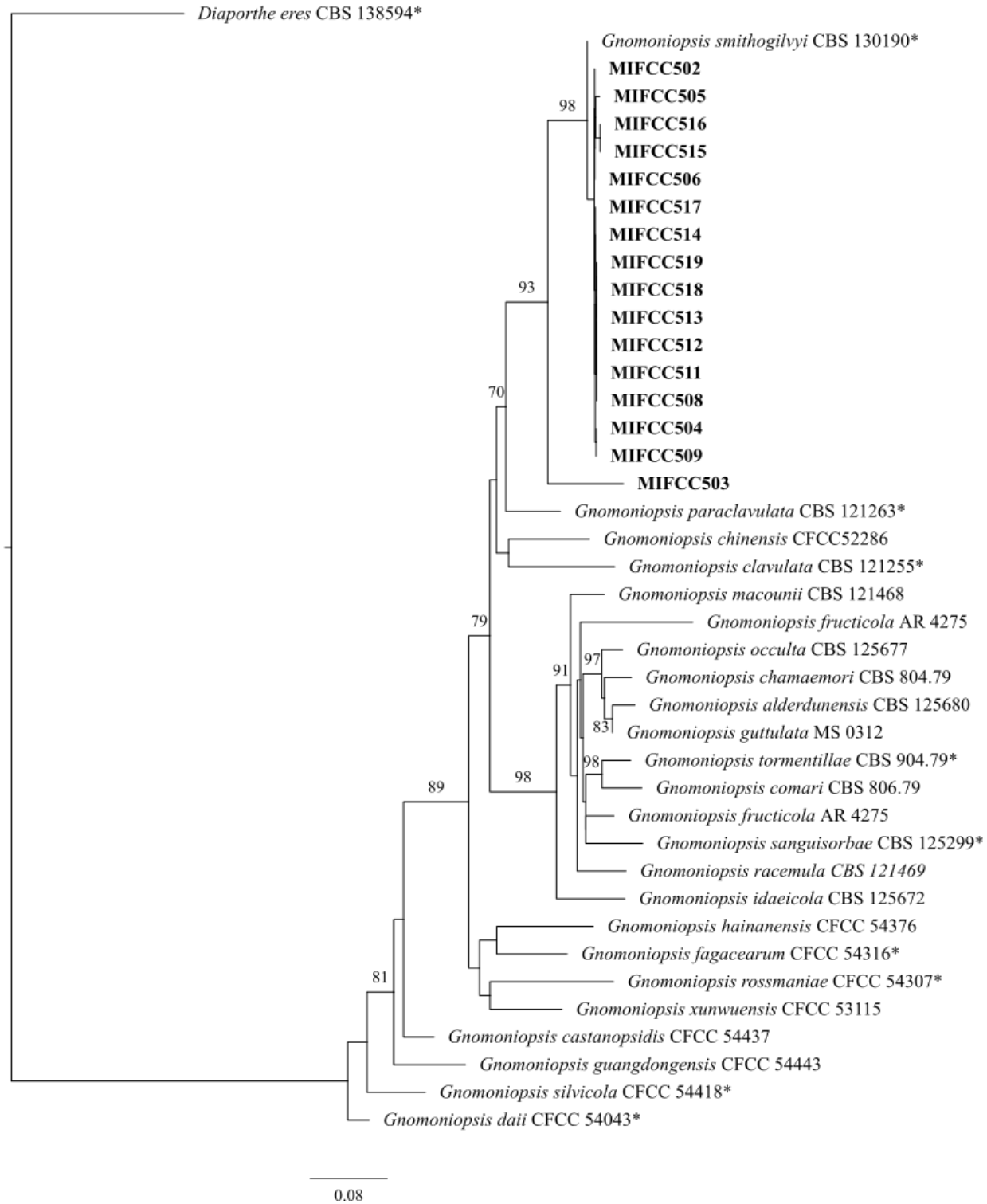

Figure 1.7. Combined 3-gene **(**ITSrDNA, EFR1-α, and β-tubulin) maximum likelihood (ML) phylogram of *Gnomoniopsis* species. Statistical support for the topology is presented when bootstrap values are ≥ 70%. The tree is rooted with *Diaporthe eres* (CBS 138594). Fungal isolates from this study are indicated by bold type face and ex-type, epi-type or holotype species are indicated by an asterisk (*).

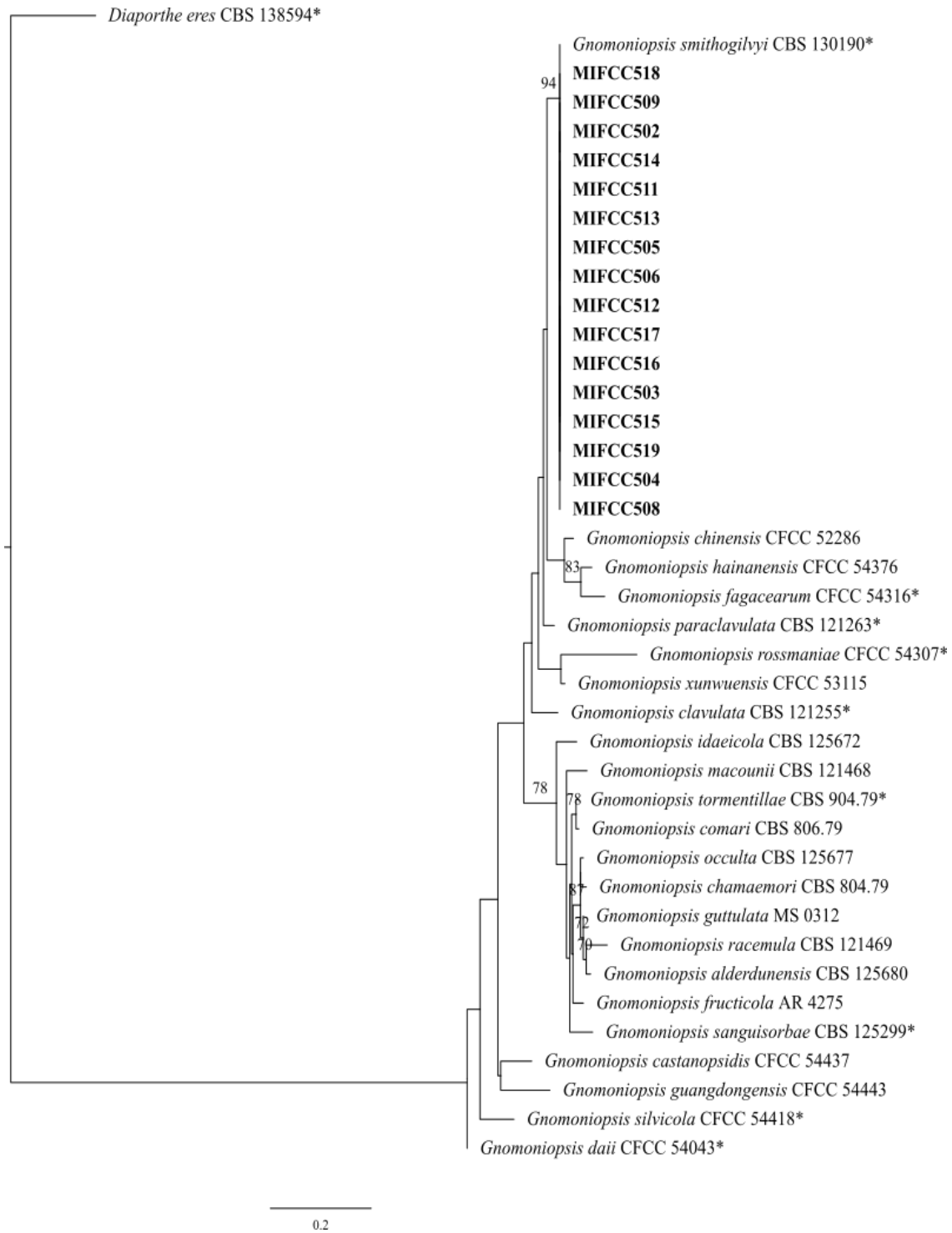

Figure 1.8. Single gene **(**ITSrDNA) maximum likelihood (ML) phylogram of *Gnomoniopsis* species. Statistical support for the topology is presented when bootstrap values are ≥ 70%. The tree is rooted with *Diaporthe eres* (CBS 138594). Fungal isolates from this study are indicated by bold type face and ex-type, epi-type or holotype species are indicated by an asterisk (*).

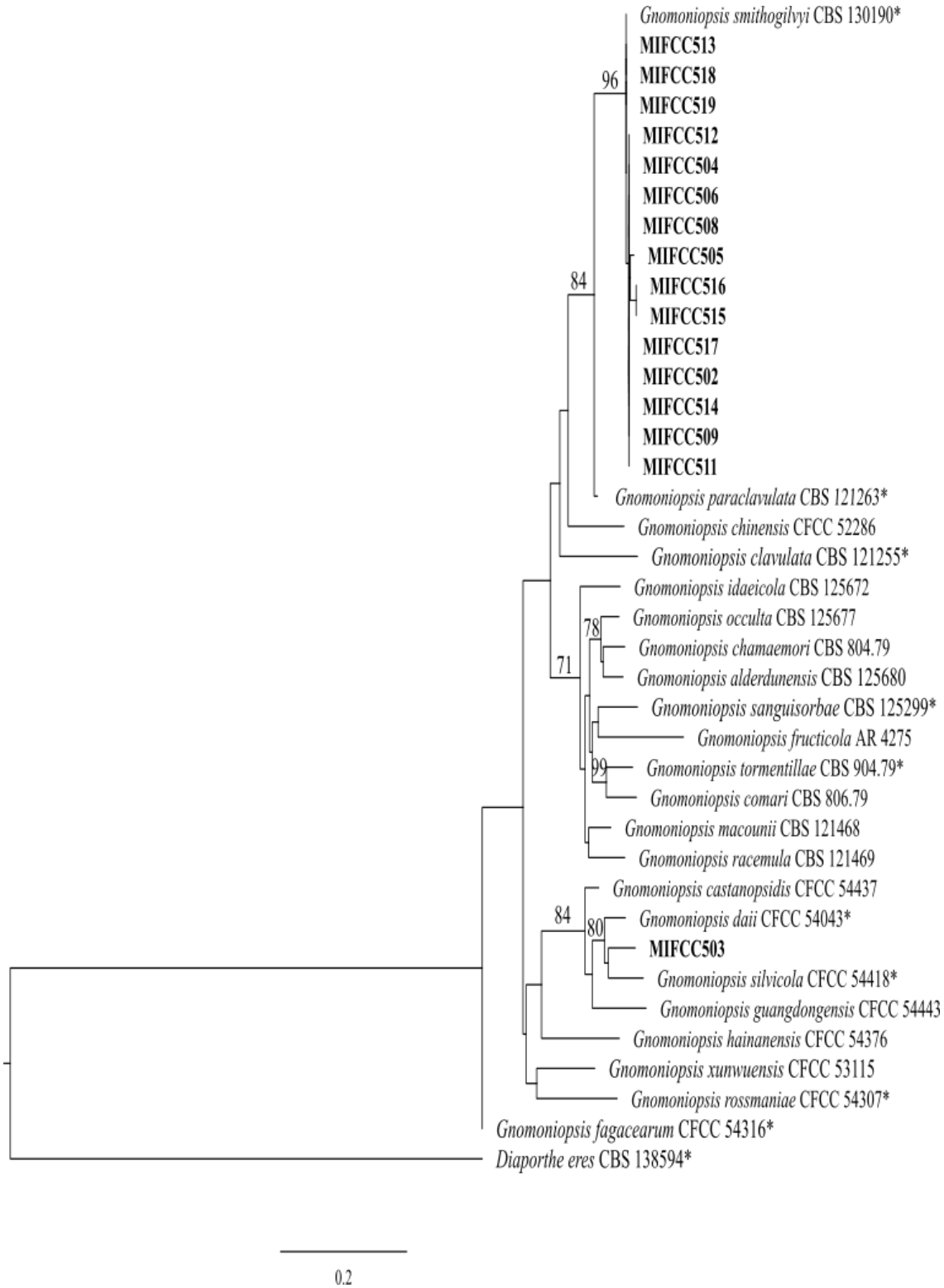

Figure 1.9. Single gene **(**EFR1-α) maximum likelihood (ML) phylogram of *Gnomoniopsis* species. Statistical support for the topology is presented when bootstrap values are ≥ 70%. The tree is rooted with *Diaporthe eres* (CBS 138594). Fungal isolates from this study are indicated by bold type face and ex-type, epi-type or holotype species are indicated by an asterisk (*).

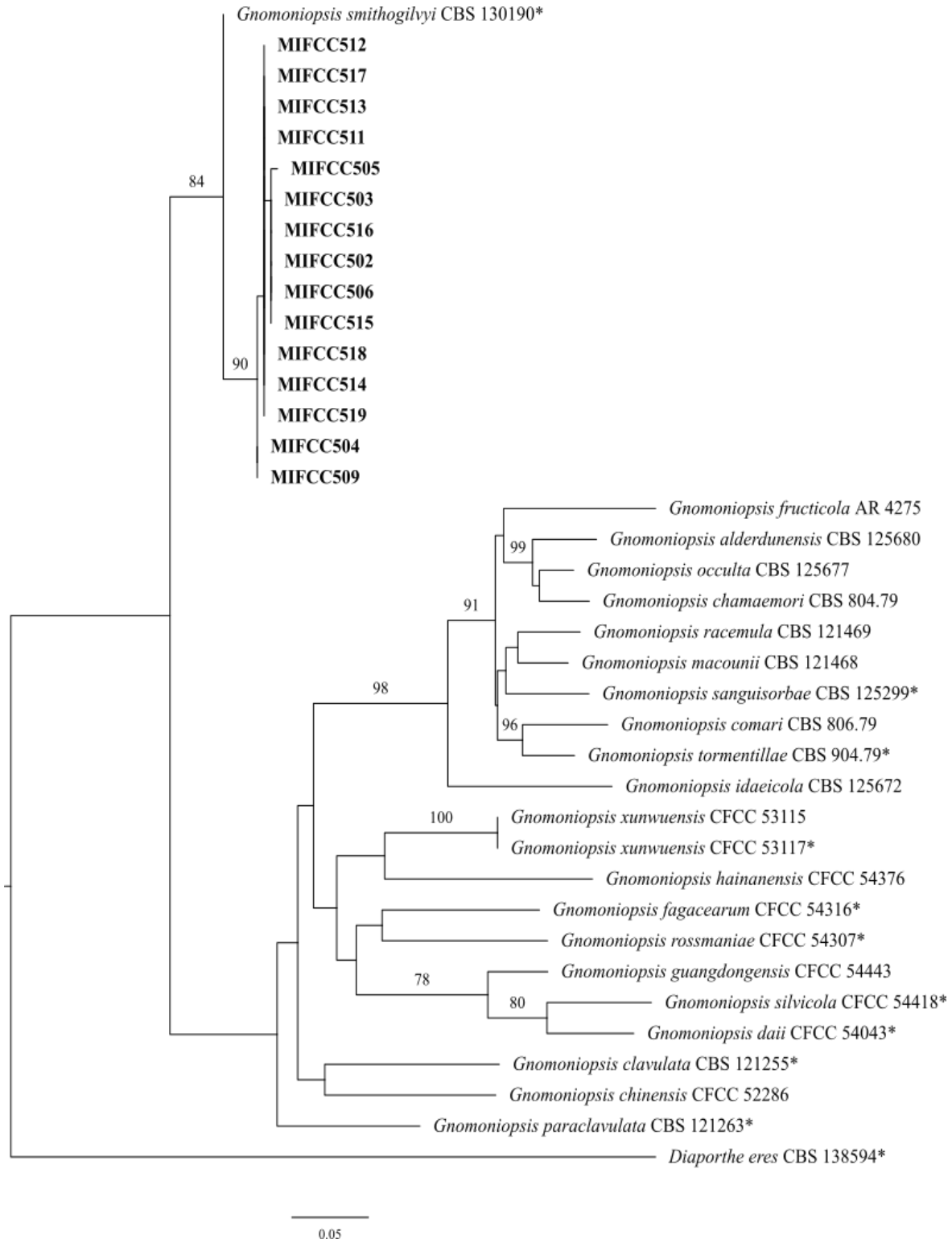

Figure 1.10. Single gene **(**β-tubulin) maximum likelihood (ML) phylogram of *Gnomoniopsis* species. Statistical support for the topology is presented when bootstrap values are ≥ 70%. The tree is rooted with *Diaporthe eres* (CBS 138594). Fungal isolates from this study are indicated by bold type face and ex-type, epi-type or holotype species are indicated by an asterisk (*).

single ML trees (**Figure 1.9. and Figure 1.10.**) but not the multigene ML tree or the ITSrDNA gene tree (**Figure 1.7. and Figure 1.8.**).

DISCUSSION

From the 2020 sampling of 1,814 harvested nuts, 49% exhibited rot symptoms; symptomatic nuts were obtained from all 11 orchards. Twelve percent of sampled nuts exhibited brown rot symptoms and were found in 8 orchards. The 222 fungal isolates obtained from the nuts corresponded to 13 known and seven unknown species from 13 genera. The most isolated species was *Gnomoniopsis* sp. 1 (a putative new species) accounting for 30% of all isolates. *Cytospora vinacea*, *Neofusicoccum* sp. 1, *G. smithogilvyi*, and *D. seriata* accounted for 17%, 10%, 8%, and 7% of isolates. *G. smithogilvyi* was obtained from 8% of all fungal isolations (14% of isolations from brown rot symptomatic nuts), and 5 orchards. Koch's postulates were confirmed in *D. seriata* and *Neofusicoccum* sp. 1, no other species exhibited symptoms significantly greater than the control.

Nearly 50% of sampled nuts were symptomatic at harvest; all orchards contained symptomatic nuts. These nuts are not marketable and would spoil at room temperature. Sampled nuts (12%) found in approximately two-thirds of the orchards exhibited BR symptoms. Other countries reported nut rot incidences 20% to 90%. In 2020, Michigan growers reported low incidence of nut decay. Previously, growers reported an incidence up to 60% (Dennert et al., 2015; Shuttleworth et al., 2013; Visentin et al., 2021).

G. smithogilvyi represented only 8% of the 222 isolations. The majority of *G. smithogilvyi* isolations were obtained from symptomatic nuts (BR or general decay symptoms). These data indicate either that *G. smithogilvyi* is not ubiquitous in Michigan or is not present at a high incidence. However, a putatively new *Gnomoniopsis* sp. was found in all orchards in about

one-third of all isolations, with approximately 78% of these isolated from symptomatic nuts. Understanding how *Gnomoniopsis* spp. contributes to postharvest rot in chestnuts and if many have the capacity to cause nut decay and tree cankers as has been reported elsewhere (Pasche et al., 2016).

While *G. smithogilvyi* was not the most commonly isolated fungal species, the genus *Gnomoniopsis* was the primary genus isolated from symptomatic and from asymptomatic nuts. Interestingly, some fungi isolated from nuts exhibiting BR symptoms were identified as either an undescribed species of *Gnomoniopsis* or as *Sirococcus castaneae*. The genus *Sirococcus* is a sister species to *Gnomoniopsis*. Walker et. al. (2010) postulated based on their multigene analysis of the *Gnomoniopsis* genus that species grouped within the *Sirococcus* genus should instead be grouped within the *Gnomoniopsis* genus. Our results suggest that multiple species within the genus *Gnomoniopsis* and closely related genera cause symptoms similar to those of BR (Jiang and Tian, 2019). With increasing work targeting the diversity of endophytes and epiphytes living in woody species phyllosphere, the number of species within the *Gnomoniopsis* genus is increasing each year (Crous et. al. 2012; Gong and Xin et. al., 2021; Jiang and Tian., 2019: Jiang et. al., 2020; Jiang et. al., 2021; Shuttleworth et. al. 2010). It will be important to establish the contribution and potential interactions between *Gnomoniopsis* spp. and closely related species to disease development in chestnuts.

As nuts in this study were collected from the receiving line, rather than harvesting directly from orchards, some variation in disease incidence, severity, and fungal species composition may be due to the variation among orchard practices (i.e., length of time from when they dropped from the tree and were harvested, refrigerated, and transported). Direct harvesting from orchards and reduced variability in storage and transportation timing and temperature may

be important for studies focused on orchard practices and or orchard location rather than fungal diversity in nuts collectively.

Fungal species from the genus *Gnomoniopsis* are not the only fungi to cause internal decay in nuts post-harvest (Bertuzzi et. al. 2015; Donis-González et. al., 2016; Jermini et. al., 2006; Seddaiu et. al., 2021; Vettraino et. al., 2005; Washington et. al., 1997; Wells and Payne, 1980; Wright, 1960). Other than *G. smithogilvyi, Ciboria batschiana* and *Phomopsis* spp. are of most common concern globally (Beccaro et al.,2020). An additional nineteen fungal species other than *G. smithogilvyi* were isolated in this study. Most (86 %) were isolated from symptomatic rather than asymptomatic nuts. *Sirococcus castaneae* and *D. seriata* have been reported as chestnut pathogens, while *Cytospora vinacea* has been reported as a pathogen on other hosts such as *Vitis* species (Dar and Rai, 2017; Lawrence et al, 2017; Meyer et al., 2017). Rare species, that were isolated once came from asymptomatic nuts. These species have previously been reported for use as biocontrol agents on *Solanum lycopersicum L.* (tomatoes) or for *Euphorbia esula* (leafy spurge) (Coppola et al., 2019; Yang, 1990). It is also important to note that far more microorganisms than just fungi affect post-harvest quality. Bacterial species have been shown to be present in and on nuts fruit and be associated with nut decay (Donis González et. al., 2016). This may indicate that these species that were isolated only from asymptomatic nuts are not pathogenic, rather they are endophytes living in or on chestnut fruit.

Two species isolated from symptomatic nuts in this study, were previously reported as pathogens of nuts in (Dar and Rai, 2017; Lawrence et al, 2017; Meyer et al., 2017). However, we did not identify any of the 16 species previously reported on Michigan nut kernels (Donis-Gonzalez et. al., 2016), we did however find three genera that Donis-Gonzalez (2016) previously isolated *Fusarium, Trichoderma* and *Sclerotinia*. The surrounding macro- and micro-biota, host

genetics, age of host, temperature of orchard, water availability and health of host influence the composition of fungal species present in chestnut trees' phyllosphere and their nuts (Laurent et. al., 2020; Cordier et. al., 2012). Donis-Gonzalez's 2010 study and this current study were designed and carried out differently in the where, how, and when nuts were harvested, handled, and sampled. Also, Michigan chestnut orchards are bordered by other tree, cereal, or vegetable crops, roads, and forests (Fulbright et. al., 2010). These factors may have influenced the composition of fungal species present in Michigan chestnuts in 2020. For example, *Cytospora vinacea* was isolated in a high quantity in orchard 11(31 %) relative to other orchards (2-29 %) may be because the chestnut trees in this plot were located next to a plot of *Vitis* spp. (common name= grapes) a host for *C. vinacea* (Lawrence et. al., 2017). This suggests that orchard planning and integrated pest management practices should take into consideration the composition of plant species surrounding a chestnut orchard as these nearby plant hosts impact the pathogens and pest populations in the orchard that is being managed.

The results of the pathogenicity trial support that fungal species ie *Diplodia* sp. and *Neofusicoccum* sp. tested were pathogenic on the chestnut cultivar Colossal; Koch's postulates were completed. All other species including known pathogens of nuts i.e., *G. smithogilvyi* and *Sirococcus castaneae* did not produce lesions significantly larger than the controls. We used two negative controls; one an intact nut reflected the natural incidence of rot present in the 2020 colossal nuts and the other was inoculation only with PDA. 25 % of the intact nuts and 13% of the inoculated nuts in the pathogenicity trial exhibited decay. Nut shells are compromised during inoculation and the decay that developed in the PDA control may be a result of saprophytic and pathogenic fungi present on or in the nuts shell. Other nut pathogenicity trials have also reported lesions in control nuts (Battilani et al., 2018; Dobry, 2021; Sakalidis et

al. 2019; Zhu Xiao-qing et al., 2009). While this demonstrates the importance of proper handling on nuts at- and post-harvest to ensure the structure and defensive properties of the nut's natural barriers against pathogens are maintained (Monarca et. al., 2005), this makes it challenging to conduct informative pathogenicity trials. Future trials may like to include a larger number of replicates, standardized spore concentrations for inoculations rather than mycelium plugs, nuts sourced from low rot orchards (although this can be variable from year to year), and/or additional surface sterilization methods prior to inoculation.

Post-harvest chestnut diseases result in significant loss. Results of this and other studies indicate that more than one species contributes to decay/rot symptoms in nuts. Symptoms previously attributed to *G. smithogilvyi* may be caused by additional fungi. Efforts to develop integrated pest management strategies for nut decay should include multiple pathogens and study of their epidemiology and biology.

CHAPTER 2: COLD STORAGE AND CULTIVAR EFFECT ON BROWN ROT DISEASE IN CHESTNUTS POST-HARVEST

ABSTRACT

Three species of *Castanea* (chestnuts) are favored for production of their fruit (nuts) which have a high starch content when compared to other tree nuts. Harvest, post-harvest processing and storage are key components to maintaining a high-quality, marketable nut. *Gnomoniopsis smithogilvyi*, the causal agent of brown rot on chestnut, is an increasing concern for Michigan growers. The objective of this study was to 1) evaluate cultivar susceptibility to *G. smithogilvyi* 2) determine the effect of storage conditions (temperature and time) on *G. smithogilvyi* growth, disease incidence and severity, and 3) identify the fungi associated with chestnut rot symptoms post-harvest. Three cultivars were evaluated for brown rot symptoms from the same orchard: 'Labor Day', 'Colossal', and 'Benton Harbor'. We evaluated cold (4 °C) storage treatments (no cold treatment, 1 month and two months cold storage) on the incidence and disease severity of brown rot symptoms on 'Labor Day', 'Colossal', and 'Benton Harbor' in 3,873 nuts. Pieces of tissue taken from the edge of brown rot lesions were plated onto petri dishes containing Potato Dextrose Agar supplemented with ampicillin and streptomycin to isolate fungal species associated with symptomatic tissues. 'Colossal' was most susceptible to brown rot; cold storage treatments suppressed disease incidence and severity. Fungal isolates (240) were obtained representing 20 fungal species. These results can positively impact the development of IPM strategies pre-harvest and post-harvest.

INTRODUCTION

Castanea species are used for agroforestry, building materials, fuel, and as a food source by humans and animals (Beccaro et al. 2009). Three species of *Castanea* (chestnut) are favored for production of the species fruit (nuts) for human consumption either as a fresh or processed product including gluten free flour, chips, and beer (Beccaro et al., 2009; Whetstone, 2016; Chenlo et al. 2008). Chestnut production is increasing in the U.S. and (Fulbright et al., 2010). In Europe chestnut production is an agroforestry practice that includes predominantly a single species (*C. sativa*). U.S. chestnut production is similar to that of Australia and resembles a standard fruit orchard with multiple cultivars. In Michigan the main chestnut species used for nut production are *C. sativa* x *C. crenata* hybrids, *C. crenata*, and *C. mollissima*.

Brown rot (BR) of chestnut is caused by *Gnomoniopsis smithogilvyi* (= synonym *C. castanea*), an ascomycete fungus. BR affects chestnut production worldwide including Australia, New Zealand, Italy, India, and Switzerland (Dar and Rai., 2015; Dennert et al., 2015; Maresi et al., 2013; Sakalidis et al., 2019; Shuttleworth et al., 2013). Brown rot affects chestnuts post-harvest in which the kernel of the nuts degrades over time prior to and following harvest (Crous et al., 2012). In Michigan, BR was first detected in 2016 and has had increasing impact in subsequent years. *G. smithogilvyi* infects chestnut flowers during bloom and becomes latent until harvest in the fall when symptoms develop on the nut kernel (Shuttleworth and Guest, 2017). *G. smithogilvyi* causes light to dark brown lesions on the kernel leading to dry and spongy tissue and degradation; symptoms increase over time (Shuttleworth and Guest, 2013). Destructive sampling is required to evaluate brown rot symptoms due to the cost of using other technologies such as nondestructive electronic sensing technology (Donis-González, 2013). Once the nuts have been cut open, they are no longer marketable for the fresh market (Shuttleworth and Guest,

2013). Currently, there is limited information on managing brown rot using chemical or biological control (Lione et al., 2019; Pasche et al. 2016; Silva-Campos et al., 2022). Orchard sanitation such as removing burs after nut harvest is the only proposed cultural approach to brown rot (Lione et al., 2019). Recommended post-harvest brown rot disease control measures include cold storage, sanitizing solutions, heat treatments and sorting of nuts (Lione et al., 2019).

Chestnuts have a high starch content compared to other tree nuts (Fulbright, 2003) and are similar to an apple or a potato in storage (Fulbright, 2003; Wills and Golding, 2016). To maintain optimum quality of fresh chestnuts, strategies that are commonly used include cold storage, washing of chestnuts, treatment with a sanitizing solution, fumigation, heat treatment, controlled atmosphere, biocontrol, and gamma radiation (Donis-Gonzales, 2008; Ertan et al., 2015; Lee et al., 2016; Panagou et al., 2006; Ruocco et al., 2016; Webber et al., 2021; Zhu, 2016). Due to the relatively small production size of chestnut orchards in Michigan, there have not been advancement in post-harvest systems (Fulbright et al., 2010). Although temperature is controlled, relative humidity and other conditions are not (Ekman, 2014).

Cold storage is standard for many crops (Wills and Golding, 2016). The two basic components of cold storage design include temperature and relative humidity (Wills and Golding, 2016). Optimization and maintenance of these components are critical to minimize post-harvest yield and quality loss (Wills and Golding, 2016). Temperature affects the respiration rate of the stored fruit and the metabolic rate of pathogens or pests (Gross, 2016; Wills and Golding, 2016). Relative humidity is critical to maintain nut quality of the nut. The nut's shell is a hard porous structure encapsulating the chestnut pellicle and kernel (Chenlo et al., 2010; Fulbright, 2003; Gross, 2016). Chestnuts are at high risk for desiccation and subsequent loss of solids and yield (Chenlo et al., 2010; Gross, 2016).

The composition and use of chestnuts also makes quality control challenging. They have a high amount of carbohydrate content (46%) and water content (49%) compared to almonds that have a low carbohydrate content (20%) and a low water content (6%) (Barreca et al., 2020; Senter et al., 1994). Unlike almonds or walnuts, chestnuts are not pasteurized or roasted, steps which are used to bring out flavors and reduce the risk of microbial contamination (Fulbright, 2003). Instead, chestnuts are typically stored and sold to consumers fresh which increases the risk of yield loss during post-harvest processing and storage and an increased risk of human pathogen contamination (Donis-Gonzales, 2008; Fulbright, 2003; Lee et al., 2016; Lizotte, n.d.; Perry et al., 1998).

Previous studies have been conducted on the effect of storage measures on nuts in Michigan and globally (Donis-Gonzales, 2008; Ertan et al., 2015; Lee et al., 2016; Panagou et al., 2006; Ruocco et al., 2016; Webber et al., 2021; Zhu, 2016). However, the development of BR resulting from *G. smithogilvyi* infection prior to, during, and after cold storage is not well understood. As of 2010, important nut decay causing fungal pathogens such as *Sclerotinia pseudotuberosa* and *Phomopsis castanea* have not been detected in Michigan (Fulbright, et al., 2010). Other fungi such as *Penicillium* spp., *Acrospeira mirabilis, Botryosphaeria ribis, Sclerotinia sclerotiorum, Botryotinia fuckeliana* (anamorph *Botrytis cinerea) and Gibberella* sp. (anamorph *Fusarium* sp.) have been isolated from nuts in Michigan (Donis-Gonzalez et.al., 2016). Since Donis-Gonzalez et. al.'s 2010 study, a new fungal pathogen causing post-harvest rot in Michigan nuts has been found in commercial chestnut orchards previously only seen in other continents chestnut producing regions, *G. smithogilvyi* (Sakalidis et al., 2019). The objective of this study was to 1) evaluate cultivar susceptibility to *G. smithogilvyi* 2) determine the effect of storage conditions (temperature and time) on *G. smithogilvyi* growth, disease

incidence and severity, and 3) identify the fungi associated with chestnut rot symptoms post-harvest.

MATERIALS AND METHODS

Sample collection and processing

From 2019 to 2021, nuts from 'Labor Day', 'Colossal', and 'Benton Harbor' were harvested from a 20-year-old experimental chestnut orchard located (GPS coordinates: 42.87353992677423, -85.25872655981114) at Michigan State University's (MSU) AgBioResearch Station in Ionia county (Clarksville, Michigan USA). The cultivars are planted in seven rows of 16 trees per row. Within each row, trees of the same cultivar were planted in blocks of 1-4 trees with 25 x 25 ft spacing between each tree. All available trees for each cultivar were sampled. These three cultivars were selected because they are commonly grown in Michigan commercial chestnut orchards and have three distinct genetic backgrounds: LD a Japanese seedling (*C. crenata*), CL a Euro-Japanese hybrid (*C. sativa* × *C. crenata*), and BH a Chinese selection (*C. mollissima*) (**Table 2.1.**).

Table 2.1. Number of chestnuts harvested from the Chinese, Japanese, or European-Japanese chestnut trees growing at the MSU's AgBioResearch Center, Clarksville (Ionia county), Michigan.

Cultivar	*Castanea* species	Genetic background	Number of trees sampled	Date of harvest		
				2019	2020	2021
'Labor Day'	*C. crenata*	Japanese	3	17-September	24-September	20-September
'Colossal'	*C. sativa* × *C. crenata*	European-Japanese	22	8-October	5-October	8-October
'Benton Harbor'	*C. mollissima*	Chinese	4	13-October	15-October	12-October

Nuts were harvested after peak chestnut fall for each cultivar. Peak chestnut fall is defined as the time at which 50% or more of the total yield crop for each tree have fallen to the ground (**Table 2.1.**). Nuts were harvested by hand using small or medium Nut Wizards® (Seeds and Such Inc, IN, USA) and placed into 22" x 18" mesh bags (ULINE, WI, USA). Nuts that fell within the area under the canopy of each tree were considered the yield of that tree. Areas where canopies from adjacent trees of different cultivars overlapped with any of the trees from the cultivars used in the study were excluded.

On the day of harvest, all nuts from a single cultivar were combined, mixed, and submerged in water to be sorted based on buoyancy. Sinking nuts or "sinkers" are considered to be of high quality with minimal drop in density commonly due to defects. Conversely, floating nuts or "floaters" are considered to be of poor-quality containing defects. Defects are nuts that did not develop properly, known as "flats", or are infested with insects or fungal microorganisms (Beccaro et al. 2020). "Sinker" nuts were used for the duration of the experiment while "floaters" and nuts with broken shells were discarded.

Sinker nuts were transported to the Forest Pathology Laboratory at Michigan State University (Ingham County, Michigan USA) at room temperature (approximately 22°C). To minimize the amount of external biological contaminants, the surface of the nuts was disinfected using a 10% commercial sodium hypochlorite (i.e., bleach) solution for five minutes, followed by a 70% ethanol (C_2H_5OH) solution for three minutes. Excess ethanol on the surface of the nuts was removed by blotting nuts on sterile paper towels and allowing them to air dry until shells appeared dry.

Storage Treatments

The storage experiment was conducted three times in the falls of 2019-2021. Nuts (150) of each cultivar were assessed for disease on the day of harvest to create a baseline of brown rot severity and incidence. Nuts (150) of each cultivar were placed in one gallon polyethylene storage bags (Ziploc; S.C. Johnson, Racine, Wisconsin, USA) inside one of three 28-quart clear plastic bins, one for each of the three storage treatments. Each polyethylene bag had four 1.0 cm holes punched to facilitate aeration. Each 28-quart clear plastic bins corresponding to a single storage treatment was placed at one of the three storage conditions: room temperature for two weeks (control), 4°C for 4 weeks and then room temperature for two weeks (1 month), and 4°C for 8 weeks and then room temperature for two weeks (2 months).The cold storage condition of 4°C was used in this experiment due to 4°C being the set point of the walk in refrigerator available for use during the experiment. After the cold treatment, nuts were removed from 4°C and left at room temperature for 14 days prior to disease assessment. On the day of disease assessment, the surface of the nuts was disinfected as previously described.

Cultivar susceptibility trial

In 2019 and 2021, 180 "sinker" nuts from each cultivar were inoculated with the *G. smithogilvyi* strain MIFCC 501 (Sakalidis et. al., 2019). Sinking nuts or "sinkers" are considered to be of high quality with minimal defects. An 11-gauge (3.0 mm outer diameter) bone biopsy needle was used to remove a section of the chestnut shell and kernel at the mid-to hilum end (center) of the nut. Mycelial plugs (2.4 mm in diameter) from 7-day-old cultures grown on PDA were created using the same size bone biopsy needle and placed into the hole created on the chestnut. Agar plugs of the same size from sterile, non-inoculated PDA, were used as negative control. A total of 180 of disinfected yet unwounded nuts ("natural infection") were included in

this test as an additional negative control to determine the "baseline" of rot present for each treatment. The inoculation hole on every nut was sealed with waterproof silicon (Mfr. Model # GE500) (GE, CT, USA), and all nuts were placed in one gallon polyethylene storage bags (Ziploc; S.C. Johnson, Racine, Wisconsin, USA) inside one of 28-quart clear plastic bins and stored at one of the three different storage treatments. Each polyethylene bag had four 1.0 cm holes punched to facilitate aeration. Each 28-quart clear plastic bins corresponding to a single storage treatment was placed at one of the three storage conditions: room temperature for two weeks (Control), 4 °C for 4 weeks and then room temperature for two weeks (1 month), and 4 °C for 8 weeks and then room temperature for two weeks (2 months).

Brown Rot Disease Assessment

To assess disease, nuts were split in half by making a vertical cut from the stylar to hilum end using a disinfected blade and visually evaluated for internal BR symptoms in the kernel. Disease incidence was calculated as the percentage of diseased nuts of the total number of nuts sampled (Shuttleworth and Guest, 2013). Disease severity was determined by rating nuts on a scale of 0-4, where 0 = no rot; 1= 1-25% of the surface area of the kernel nut halves with brown rot; 2 = 26-50% of the surface area of the kernel nut halves with brown rot; 3 = 51-75% of the surface area of the kernel nut halves with brown rot; and 4 = 76-100% of surface area of the kernel nut halves with brown rot (Sakalidis et al. 2019).

Effect of temperature on G. smithogilvyi growth in vitro

To determine the effect of temperature on the growth rate of *G. smithogilvyi* in culture, mycelial plugs (6.0 mm in diameter) of a seven-day-old isolate of *G. smithogilvyi* (MIFCC 501) were placed in the center of 100 x 150 cm Petri plates filled with 50 mL of PDA. Plates were incubated at 4° C for either zero (control), four, or eight weeks before being transferred to an

incubator at 20° C for one week, where the underside of each plate was scanned daily using an Epson Perfection V370 Photo scanner (Model J232D, Seiko Epson Corp., Japan) to measure culture growth. IMAGE J, Version 1.53f51 (National Institute of Health, USA) was used to calculate growth area from the scanned images (ten per treatment). Growth rate per day was normalized by taking the measured area of growth per day and dividing by the number hours between each measurement to represent the average daily growth rate. This experiment was conducted twice within a three-month period.

Fungal isolation

Tissue from at least three symptomatic nuts for each disease rating (0-4) was collected for fungal isolation. Three to four thin pieces of tissue of approximately 2 mm^2 were plated onto a petri dish (100 mm X 60 mm) containing Potato Dextrose Agar (PDA, 39 g/, Difco, New Jersey, USA) supplemented with ampicillin (100 mg/ml) and streptomycin (50 mg/ml). Petri dishes were maintained at room temperature monitored for fungal growth for 7-14 days. All petri dishes were incubated at room temperature for seven days. Where growth was present, a subsample of fungal mycelia was single hyphal tipped and transferred to another petri dish with PDA until pure cultures were obtained. Pure cultures grown on PDA were stored as agar plugs, approximately 0.5 cm in diameter, in 2 ml polypropylene cryogenic vials (Cryovial silicon self-standing, Simport Scientific, Canada) containing 1 ml of a 40 % sterile solution of glycerol (VWR International, PA, USA) at -20 °C long-term in the Forest Pathology Lab at MSU in Lansing, Michigan USA.

Molecular identification of fungal cultures

DNA was extracted from all isolates using OMNI-Prep DNA Extraction Kit (G Biosciences, Missouri, USA) following the manufacture's protocol with minor modification and stored at -20 °C. Modification of the protocol was the use of 2 % CTAB buffer to replace the kits

Lysis Buffer (Hamelin et al., 2000). To assist with the identification of fungal isolates the internal transcribed spacer region (ITSrDNA) was sequenced. The primers used during PCR to amplify the ITSrDNA region were ITS1F (Gardes and Bruns, 1993)/ ITSF4 (White et. al., 1990). The PCR reaction mixture contained 5 µl of 25x colorless buffer (Promega Corp., Madison, WI), 4 µl of Mg^{2+} (100 µM) (Promega), 0.5 µl of dNTP (1:1:1:1 at 100µM) (Invitrogen, Carlsbad, CA), 1.0 µl of each primer (10µM), 0.13 µl of GoTaq Flexi (Promega; 5 U/µl), and DNA template adjusted to 50 ng in a total volume of 25 µl. Reactions for the amplification of ITSrDNA were run on an ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) using the following conditions: initial denaturation a 95 °C for 2 min, then 40 cycles at 95 °C for 30 s, 54 °C for 50 sec, and 72 °C for 1 min and a final extension of 72 °C for 5 min, with a holding temperature of 4 °C. Amplicons were resolved in a 0.75 % agarose gel stained with SYBER Safe (Invitrogen, USA) and amplicon size was determined using 1 KB Plus DNA Ladder (Invitrogen, USA) by visualization with an Ultraviolet Fluorescence Analysis Cabinet (Spectronics Corporation, USA). PCR products were purified using the Monarch PCR & DNA Cleanup Kit (New England Biolabs Inc., MA, USA) according to manufacturer's protocol, and sequenced at the MSU Research and Technology Support Facility on an ABI 3730xl platform sequencer (Applied Biosystems, Foster City, CA). Forward and reverse sequences were aligned and manually edited using GeneiousPro Version 2020.2.4 (Biomatters, NZ) (http://www.geneious.com/). Initial identification of isolates was completed using BLAST® version 2.13.0 (NIH, USA) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search in GenBank® (NIH, USA)

Statistical Analysis

All statistical analyses were completed using JMP®, Version 16.0.0 (SAS Institute Inc., Cary, NC, 1989–2021). An analysis of variance (ANOVA, $p< 0.05$) was completed in JMP to determine if the average severity ratings were significantly different from each other. Following the ANOVA test, a Tukey HSD test was conducted to identify which average disease severity rating for each isolate and treatment were significantly different from each other or the controls. To examine fungal growth rate under different cold storage durations a multiple measure two-way ANOVA was used to analyze the difference between treatments' average daily hourly growth rate.

RESULTS

Disease incidence and severity at harvest

Nuts (1,1706) were evaluated with 698, 500, and 508 nuts evaluated for BR symptoms from the cultivars Benton Harbor, Colossal, and Labor Day respectively (**Table 2.2.**)

Annual brown rot disease incidence at harvest ranged from 4-6% and an average of 5% (**Figure 2.1.**). 'Colossal' exhibited significantly higher annual and overall disease incidence (9-12%, average of 10%) than 'Benton Harbor' (1-4%, average of 2%) or 'Labor Day' (1-5%, average of 3%) (**Figure 2.1.**). Disease incidence of Benton Harbor and Labor Day did not differ significantly from each other.

Annual brown rot disease severity at harvest ranged from 0-1% (**Figure 2.2.**). 'Colossal' exhibited significantly higher annual and overall disease severity (1-2%) than 'Benton Harbor' (0-1 %) or 'Labor Day' (0-1%) (**Figure 2.2.**). 'Benton Harbor' and 'Labor Day' disease severity were not significantly different from each other.

Table 2.2. Number of naturally infected nuts (unwounded nuts) and artificially inoculated nuts evaluated for brown rot symptoms over the course of the storage treatments in 2019, 2020, and 2021. A total of 7,180 nuts were evaluated.

Cultivar	Storage Treatment	Inoculum	Year			Total
			2019	2020	2021	
'Labor Day'	Control	*G. smithogilvy* isolate 501	59	-	60	119
		Natural infection	194	150	150	494
		PDA	52	-	60	112
	1 Month	*G. smithogilvy* isolate 501	55	-	60	115
		Natural infection	196	149	150	495
		PDA	54	-	80	134
	2 Months	*G. smithogilvy* isolate 501	60	-	60	120
		Natural infection	195	150	150	495
		PDA	59	-	60	119
'Colossal'	Control	*G. smithogilvy* isolate 501	118	-	60	178
		Natural infection	135	150	150	435
		PDA	118	-	60	178
	1 Month	*G. smithogilvy* isolate 501	120	-	60	180
		Natural infection	200	150	150	500
		PDA	119	-	60	179
	2 Months	*G. smithogilvy* isolate 501	102	-	60	162
		Natural infection	154	150	150	454
		PDA	92	-	60	152
'Benton Harbor'	Control	*G. smithogilvy* isolate 501	119	-	60	179
		Natural infection	203	150	150	503
		PDA	122	-	60	182
	1 Month	*G. smithogilvy* isolate 501	112	-	60	172
		Natural infection	195	150	150	495
		PDA	119	-	60	179
	2 Months	*G. smithogilvy* isolate 501	117	-	60	177
		Natural infection	197	150	150	497
		PDA	115	-	60	175
		Total	3381	1349	2450	7180

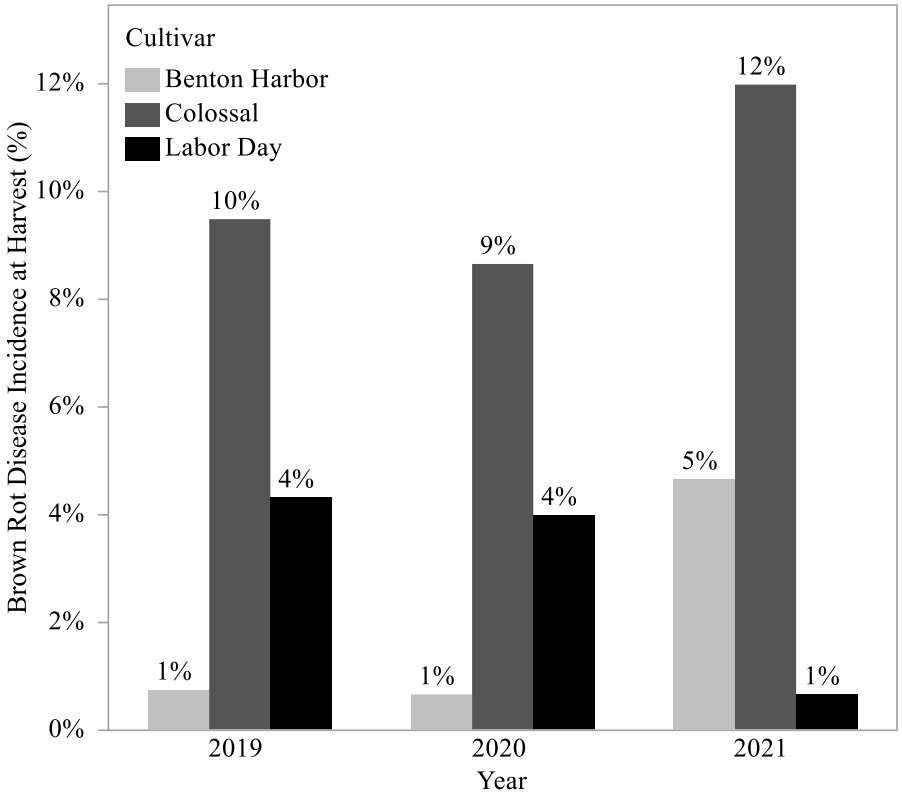

Figure 2.1. Annual (2019-2021) brown rot disease incidence on the day of harvest for 1,706 nuts (~ 150 nuts/cultivar/year) collected from chestnut cultivars cv. 'Colossal', 'Benton Harbor' and "Labor Day".

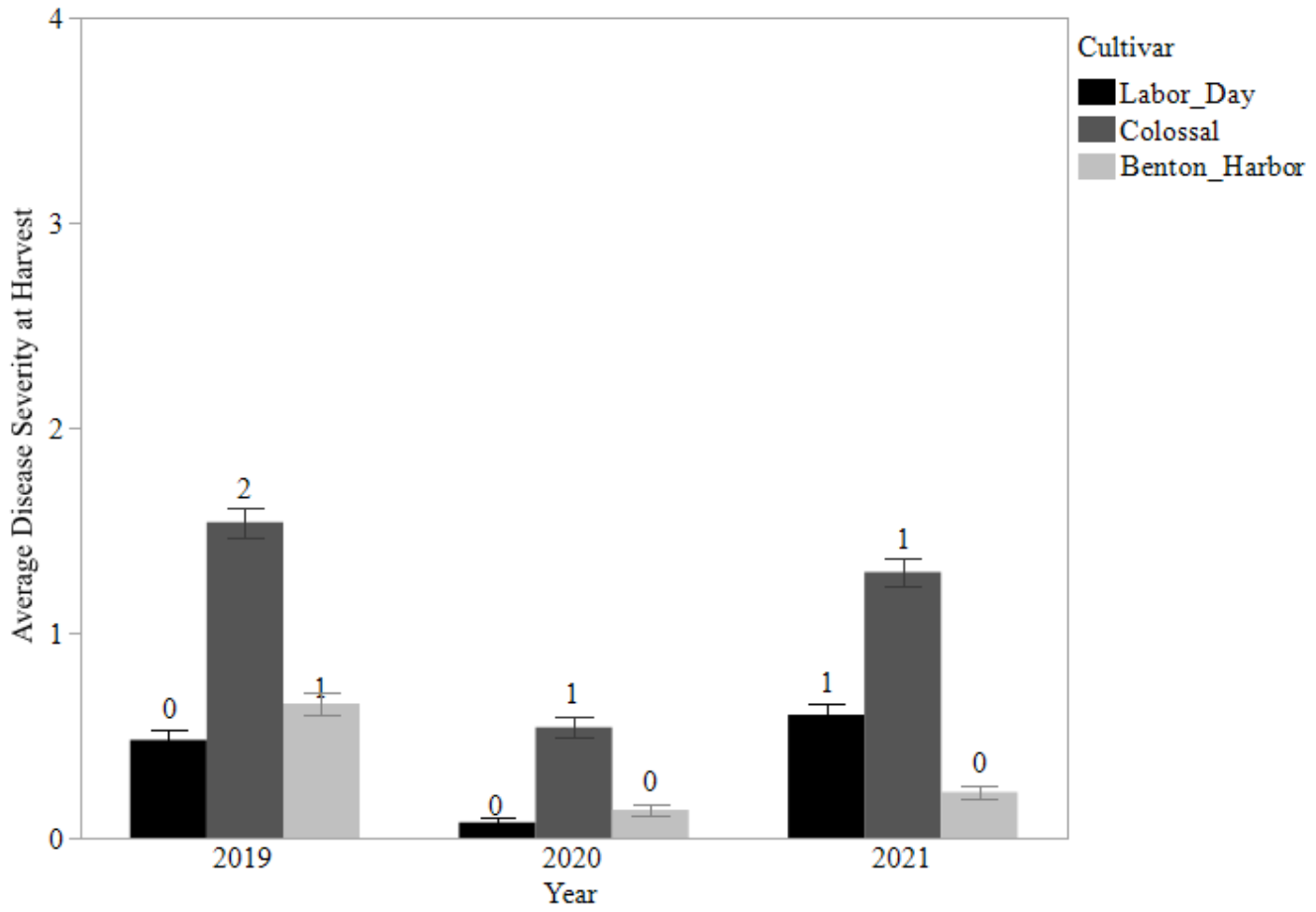

Figure 2.2. Annual (2019-2021) brown rot disease severity on the day of harvest for 1,432 nuts (~ 200 nuts/cultivar/year) collected from chestnut cultivars cv. 'Colossal', "Benton Harbor" and "Labor Day". Disease severity was determined by rating nuts on a scale of 0-4, where 0 = no rot symptoms; 1= 1-25 % of the surface area of the kernel of both halves of the nut exhibited BR symptoms; 2 = 26-50 % of the surface area of the kernel of both halves of the nut exhibited BR symptoms; 3 = 51-75 % of the surface area of the kernel of both halves of the nut exhibited BR symptoms; and 4 = 76-100 % of surface area of kernel exhibited BR symptoms. Each error bar is constructed using one standard error from the mean.

Effect of storage conditions (temperature and time) on natural infection disease incidence and severity

Disease incidence and severity were significantly higher after a period of cold storage than after harvest (**Figure 2.2., Figure 2.3., and Figure 2.4.**). A longer cold storage did not consistently lead to higher disease incidence or severity (**Figure 2.3. and Figure 2.4.)**. Instead, when there was an initial high disease incidence and severity at harvest, cold storage led to a relatively large reduction in disease incidence and severity, although still more than the amount immediately after harvest. Whereas when disease incidence and severity were relatively low at harvest, cold storage resulted in a minimal decrease or in some cases increase in disease incidence and severity **(Figure 2.3. and Figure 2.4.)**.

Disease incidence and severity after cold treatments

Natural infection

Annual brown rot disease incidence without cold treatment (control) ranged from 13-57 %, average of 34 % (**Figure 2.4. and Figure 2.5.**). 'Colossal' consistently exhibited significantly higher annual and overall disease incidence (28- 99%, average of 63%) than 'Benton Harbor' (8-48.77 %, average of 33%) or 'Labor Day' (3-25%, average of 18%) (**Figure 2.4. and Figure 2.5.**). 'Benton Harbor' and 'Labor Day' disease incidence were significantly different from each other.

Annual brown rot average disease severity without cold treatment (control) ranged from 0-2 (**Figure 2.6.**). With 'Colossal' consistently exhibiting significantly higher annual and overall disease severity (1-3) than 'Benton Harbor' (0-2) or 'Labor Day' (0-1) (**Figure 2.6.**). 'Benton Harbor' and 'Labor Day' disease incidence were not significantly different from each other.

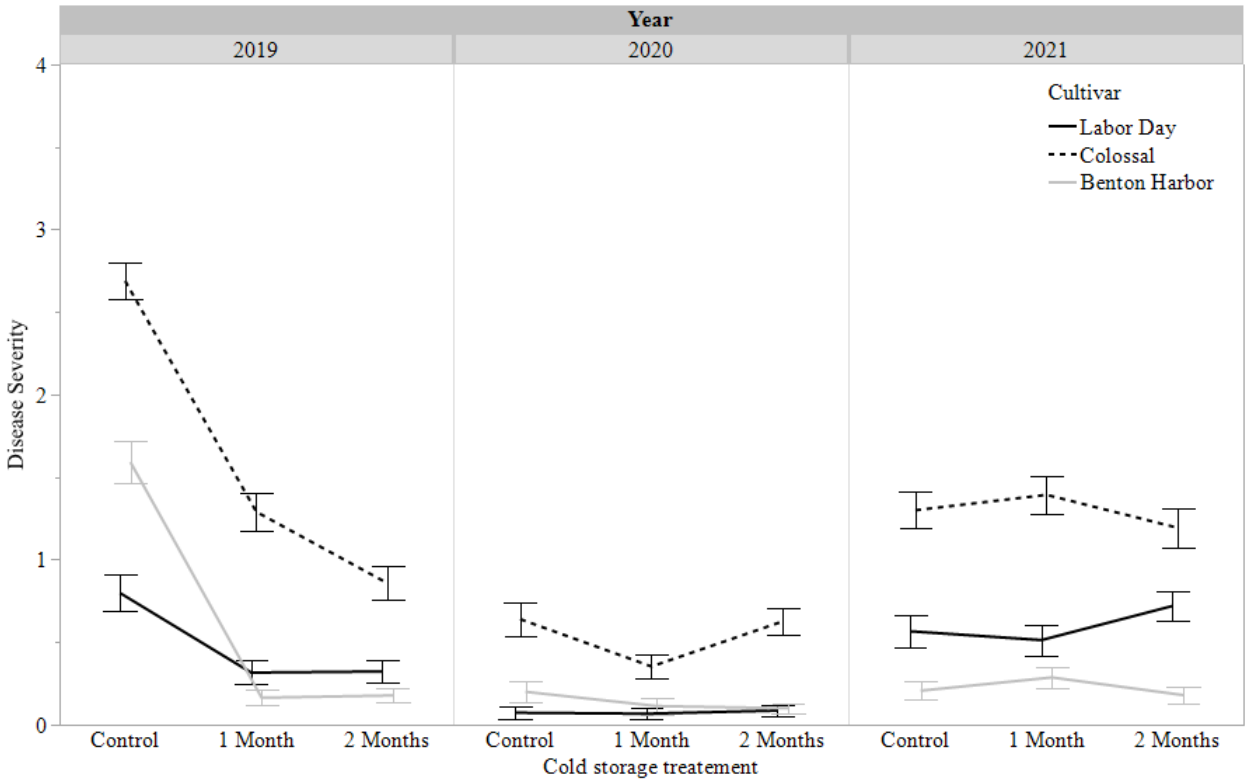

Figure 2.3. Average brown rot disease severity for cv 'Colossal', "Benton Harbor" and "Labor Day" for each of the three storage treatments in: 2019, 2020, and 2021 with naturally infected nuts. Each error bar is constructed using one standard error from the mean. The three storage conditions included: room temperature for two weeks (Control), 4 °C for 4 weeks and then room temperature for two weeks (1 Month), and 4 °C for 8 weeks and then room temperature for two weeks (2 Months). Disease severity was determined by rating nuts on a scale of 0-4, where 0 = no rot symptoms; 1= 1-25 % of the surface area of the kernel of both halves of the nut exhibited BR symptoms; 2 = 26-50 % of the surface area of the kernel of both halves of the nut exhibited BR symptoms; 3 = 51-75 % of the surface area of the kernel of both halves of the nut exhibited BR symptoms; and 4 = 76-100 % of surface area of kernel exhibited BR symptoms. Each error bar is constructed using one standard error from the mean.

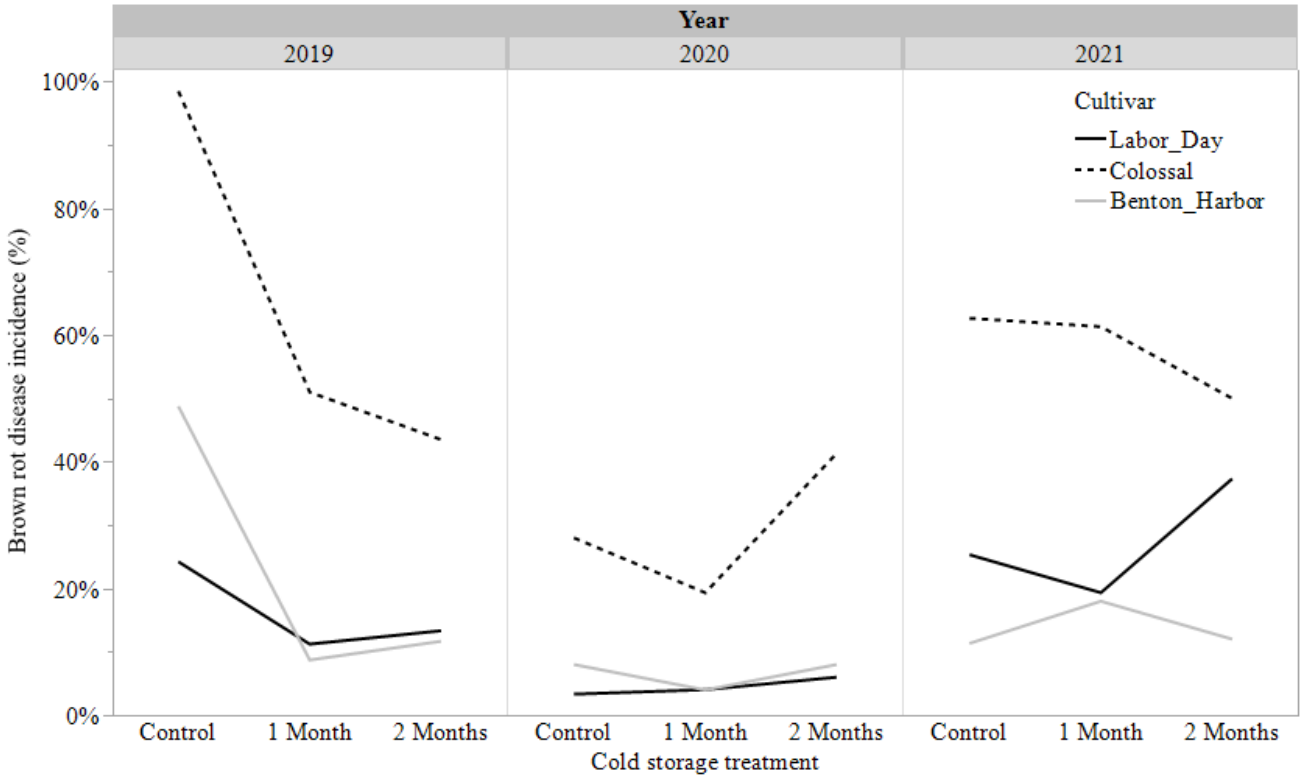

Figure 2.4. Incidence of brown rot disease for 3,873 naturally infected (disinfected yet unwounded nuts) nuts (~ 143 nuts/cultivar/year/cold storage treatment). Nuts were collected from three chestnut cultivars: 'Colossal', "Benton Harbor" and "Labor Day". The three storage conditions included: room temperature for two weeks (Control), 4 °C for 4 weeks and then room temperature for two weeks (1 Month), and 4 °C for 8 weeks and then room temperature for two weeks (2 Months).

Annual brown rot disease incidence after 1 month of cold treatment ranged from 9-33%, average of 22% (**Figure 2.4. and Figure 2.5.**). With 'Colossal' consistently exhibiting significantly higher annual and overall disease incidence (19-61%, average of 44%) than 'Benton Harbor' (4-18%, average of 10%) or 'Labor Day' (4-19%, average of 11%) (**Figure 2.4. and Figure 2.5.**). 'Benton Harbor' and 'Labor Day' disease incidence were not significantly different from each other.

Annual brown rot disease severity after 1 month of cold treatment ranged from 0-1 (**Figure 2.6.**). 'Colossal' consistently exhibited significantly higher annual and overall disease incidence (0-1) than 'Benton Harbor' (0-0) or 'Labor Day' (0-1) (**Figure 2.6.**). BH and LD disease severity were not significantly different from each other.

Annual brown rot disease incidence after 2 months of cold treatment ranged from 23-33%, average 25% (**Figure 2.4. and Figure 2.5.**). With 'Colossal' consistently exhibiting significantly higher annual and overall disease severity (19-61%, average 45%) than 'Benton Harbor' (4-18%, average of 11%) or 'Labor Day' (4-19%, average of 19%) (**Figure 2.4. and Figure 2.5.**). 'Benton Harbor' and 'Labor Day' disease incidence were not significantly different from each other.

Annual brown rot disease severity after 2 months of cold treatment ranged from 0-1 (**Figure 2.6.**). With 'Labor Day' consistently exhibiting significantly higher annual and overall disease severity (0-1) than 'Benton Harbor' (0) or 'Labor Day' (0-1) (**Figure 2.6.**). 'Benton Harbor' and 'Labor Day' disease severity were not significantly different from each other.

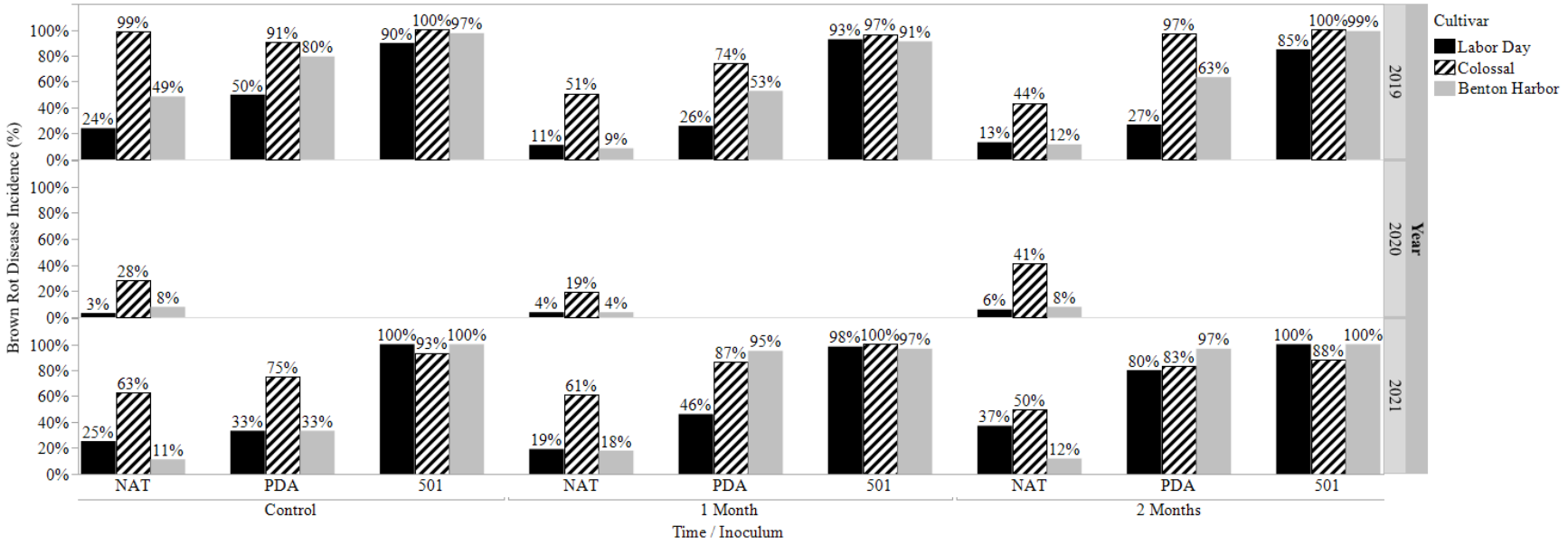

Figure 2.5. Incidence of brown rot disease for 7,180 nuts of cv. 'Colossal', "Benton Harbor" and "Labor Day" after three different storage treatments in: 2019, 2020, and 2021. Nuts were inoculated with PDA and *Gnomoniopsis smithogilvyi* isolate 501 (501), naturally infected (disinfected yet unwounded) nuts were also included.

PDA inoculated nuts

Annual brown rot disease incidence without cold treatment (control) ranged from 47-74%, average of 61% (**Figure 2.4. and Figure 2.5.**). With 'Colossal' consistently exhibiting significantly higher annual and overall disease incidence (75- 91%, average of 83 %) than 'Benton Harbor' (33-80%, average of 57%) or 'Labor Day' (33-50%, average of 42%) (**Figure 2.4. and Figure 2.5.**). 'Benton Harbor' and 'Labor Day' disease incidence was significantly different from each other.

Annual brown rot average disease severity (disease severity) without cold treatment (control) ranged from 1-3 (**Figure 2.6.**). With 'Colossal' consistently exhibiting significantly higher annual and overall disease severity (2-3) than 'Benton Harbor' (0-2) or 'Labor Day' (0-2) (**Figure 2.6.**). 'Benton Harbor' and 'Labor Day' disease severity was not significantly different from each other.

Annual brown rot disease incidence after 1 month of cold treatment ranged from 51-76%, average of 64% (**Figure 2.4. and Figure 2.5.**). With 'Colossal' consistently exhibiting significantly higher annual and overall disease incidence (74-87%, average of 81%) than 'Benton Harbor' (53-95%, average of 74%) or 'Labor Day' (26-46%, average of 36%) (**Figure 2.4. and Figure 2.5.**). 'Benton Harbor' and 'Labor Day' disease incidence was not significantly different from each other.

Annual brown rot disease severity after 1 month of cold treatment 1 (**Figure 2.6.**). With 'Colossal' consistently exhibiting significantly higher annual and overall disease severity (2) than 'Benton Harbor' (0-2) or 'Labor Day' (0-1) (**Figure 2.6.**). 'Benton Harbor' and 'Labor Day' disease severity was not significantly different from each other.

Annual brown rot disease incidence after 2 months of cold treatment ranged from 62-87%, average of 74% (**Figure 2.4. and Figure 2.5.**). With 'Colossal' consistently exhibiting significantly higher annual and overall disease incidence (83-97%, average of 90%) than 'Benton Harbor' (63-97%, average of 80%) or 'Labor Day' (27-80%, average of 54%) (**Figure 2.4. and Figure 2.5.**). 'Benton Harbor' and 'Labor Day' disease incidence were not significantly different from each other.

Annual brown rot disease severity after 2 months of cold treatment ranged from 1-2 (**Figure 2.6.**). With 'Colossal' consistently exhibiting significantly higher annual and overall disease severity (2) than 'Benton Harbor' (1-2) or 'Labor Day' (0-2) (**Figure 2.6.**). 'Benton Harbor' and 'Labor Day' disease severity was not significantly different from each other.

G. smithogilvyi inoculated nuts

Annual brown rot disease incidence without cold treatment (control) ranged from 96-98%, average of 97% (**Figure 2.4. and Figure 2.5.**). With 'Colossal' consistently exhibiting significantly higher annual and overall disease incidence (93-100%, average of 97%) than 'Benton Harbor' (97-100%, average of 99%) or 'Labor Day' (90-100%, average of 95%) (**Figure 2.4. and Figure 2.5.**). 'Benton Harbor' and 'Labor Day' disease incidence was significantly different from each other.

Annual brown rot average disease severity (disease severity) without cold treatment (control) ranged from 2-3 (**Figure 2.6.**). With 'Colossal' consistently exhibiting significantly higher annual and overall disease severity (3) than 'Benton Harbor' (2) or 'Labor Day' (1-3) (**Figure 2.6.**). 'Benton Harbor' and 'Labor Day' disease incidence was not significantly different from each other.

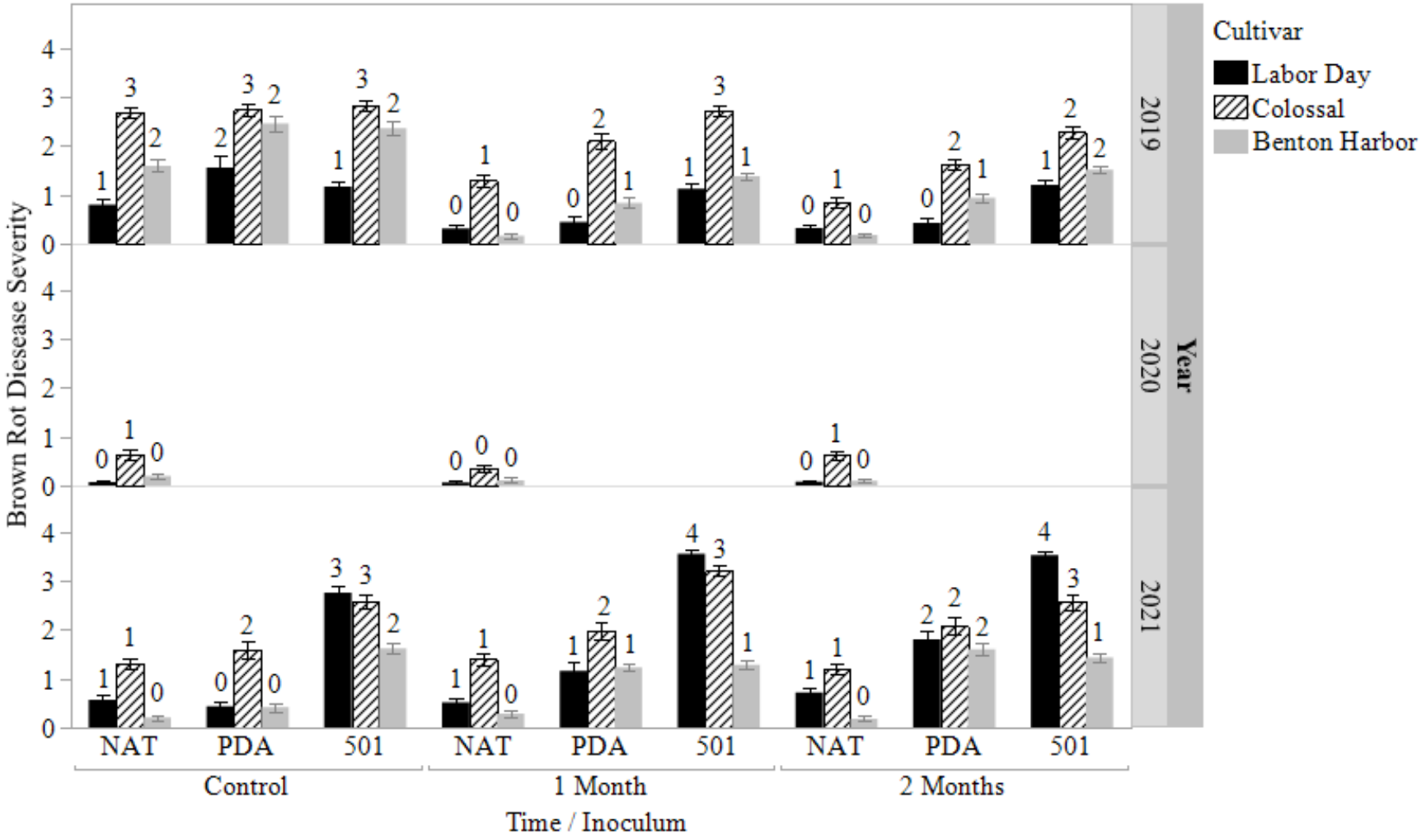

Figure 2.6. Average brown rot disease severity for 7,180 nuts of cv. 'Colossal', "Benton Harbor" and "Labor Day" after three different storage treatments in: 2019, 2020, and 2021. Nuts were inoculated with PDA and *Gnomoniopsis smithogilvyi* isolate 501 (501), naturally infected (disinfected yet unwounded) nuts were also included. Each error bar is constructed using 1 standard error from the mean.

Annual brown rot disease incidence after 1 month of cold treatment ranged from 94-98%, average of 96% (**Figure 2.4. and Figure 2.5.**). With 'Colossal' consistently exhibiting significantly higher annual and overall disease incidence (97-100%, average of 99%) than 'Benton Harbor' (91-97%, average of 94%) or 'Labor Day' (93-98%, average of 96%) (**Figure 2.4. and Figure 2.5.**). 'Benton Harbor' and 'Labor Day' disease incidence was not significantly different from each other.

Annual brown rot disease severity after 1 month of cold treatment ranged from 2-3 (**Figure 2.6.**). With 'Colossal' consistently exhibiting significantly higher annual and overall disease incidence (3) than 'Benton Harbor' (1) or 'Labor Day' (1-4) (**Figure 2.6.**). 'Benton Harbor' and 'Labor Day' disease incidence was not significantly different from each other.

Annual brown rot disease incidence after 2 months of cold treatment ranged from 95-96% (**Figure 2.4. and Figure 2.5.**). With 'Colossal' consistently exhibiting significantly higher annual and overall disease severity (88-100%) than 'Benton Harbor' (99-100%) or 'Labor Day' (85-100%) (**Figure 2.4. and Figure 2.5.**). 'Benton Harbor' and 'Labor Day' disease severity was not significantly different from each other.

Annual brown rot disease severity after 2 months of cold treatment ranged from 2-3 (**Figure 2.6.**). With 'Colossal' consistently exhibiting significantly higher annual and overall disease severity (2-3) than 'Benton Harbor' (1-2) or 'Labor Day' (1-4) (**Figure 2.6.**). 'Benton Harbor' and 'Labor Day' disease severity was not significantly different from each other.

Effect of temperature on G. smithogilvyi's growth in vitro

Cold storage treatments did not inhibit the radial mycelial growth of *G. smithogilvyi* isolate on PDA plates (**Figure 2.7.**). Post storage treatment each subsequent day the fungal colonies continued to grow (**Figure 2.7.**). The average radial growth rate was the highest in the

control (0.36 cm/day), followed by 1 month cold treatment (0.23 cm/day) and 2 month cold treatment, (0.24 cm/day) (**Figure 2.7.**). The control treatment was statistically different from the 1 month and 2 month treatments (**Figure 2.7.**). The 1 month and 2 month treatments were similar to each other (**Figure 2.7.**). The days post storage was not significantly different from each other for any of the treatments (**Figure 2.7.**).

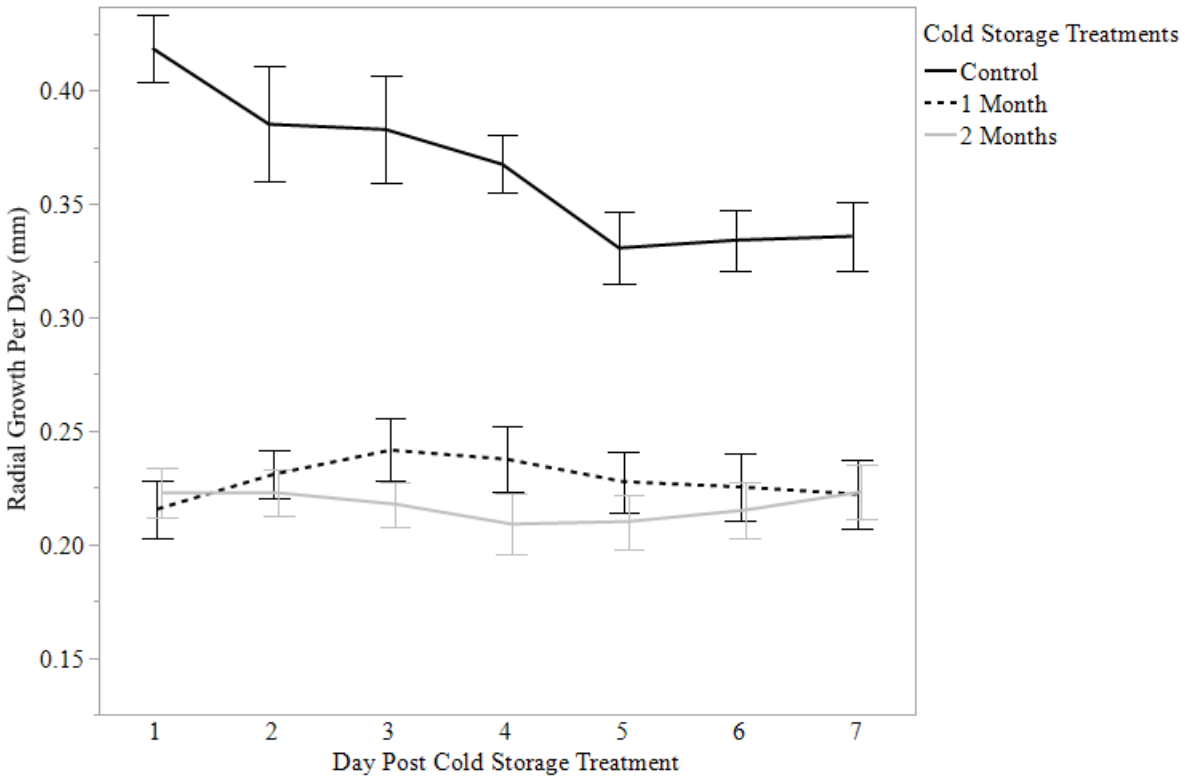

Figure 2.7. Summary of average radial growth per day in millimeters for *Gnomoniopsis smithogilvyi* isolate 501 incubated at 20°C after differing cold treatment (4°C) durations of: Control (no cold treatment),1 Month, or 2 Months. Each error bar is constructed using 1 standard error from the mean.

Fungi isolated from kernel tissues post-harvest

A total of 240 fungal cultures were isolated and sorted into 16 morphological groups corresponding to 12 fungal species and 4 unknown species (**Table 2.4.**). *Cytospora vinacea* (23 %), *Neofusicoccum* sp. (19 %), and *Sirococcus castaneae* (19 %) were the most frequently isolated species (**Table 2.4.**). Of these, 15 species were collected from symptomatic nuts and 12 from asymptomatic nuts, and 11 from both (**Figure 2.8.**). Unknown sp. 10, *Neofusicoccum* sp. (GenBank accession no. MT197527.1 and 100 % match), and *Sirococcus castaneae* (GenBank accession no. KX929760.1 and 81 % match) were most frequently isolated species from asymptomatic nuts (**Figure 2.8.**). *Cytospora vinacea*, (GenBank accession no. MT360050.1 and 100% match) *Sirococcus castaneae* and *Neofusicoccum* sp. were the most frequently isolated species from symptomatic nuts (**Figure 2.8.**).

Fungal Species associated with Cultivars

Nuts from 'Colossal' had the highest number of fungal isolates (67.9%), followed by 'Benton Harbor' (16.7%), and 'Labor Day' (15.4%) (**Table 3.4.**). Nuts from 'Colossal' had the highest number of fungal species isolated (13), followed by 'Benton Harbor' (7), and 'Labor Day' (7). *C. vinacea* was most commonly isolated from 'Colossal' nuts (**Table 3.4.**).

Of the species isolated from 'Colossal' nuts, 12 species were obtained from symptomatic and 7 from asymptomatic nuts, 6 from both. *Neofusicoccum* sp., *Sirococcus castaneae* and *Diplodia seriata* were most frequently isolated species from asymptomatic nuts (**Figure 2.8.**). *Cytospora vinacea*, *Sirococcus castaneae* and *Neofusicoccum* sp. were the most frequently isolated species from symptomatic nuts (**Figure 2.8.**). The most common fungal species isolated from 'Labor Day' nuts was *S. castaneae* (**Table 3.4.**). Of the species isolated from 'Labor Day' nuts, 5 species were collected each from symptomatic and asymptomatic nuts, 3 from both

(**Figure 2.8.**). Unknown sp. 10 and *Trichoderma atroviride* were most frequently isolated species from asymptomatic nuts. *Fusarium culmorum*, *S. castaneae*, *T. atroviride* and Unknown sp. 10 were the most frequently isolated species from symptomatic nuts (**Figure 2.8.**). The most common fungal specie isolated from 'Benton Harbor' nuts was *Neofusicoccum* sp. (**Table 3.4.**). Of these, 7 species were collected from symptomatic nuts and 5 from asymptomatic nuts, 5 from both (**Figure 2.8.**). *Neofusicoccum* sp., *S. castaneae* and *G. smithogilvyi* were most frequently isolated species from asymptomatic nuts. *Sirococcus castaneae*, *Neofusicoccum* sp., and *G. smithogilvyi* were the most frequently isolated species from symptomatic nuts (**Figure 2.8.**)

Table 2.3. Fungal species isolated from the nuts of chestnut cultivars 'Colossal', 'Benton Harbor' and 'Labor Day' harvested from at the MSU's AgBioResearch Center, Clarksville (Ionia county), Michigan in 2020.

		Number of isolations (%)			
Isolate	**Time Point**	**'Labor Day'**	**'Colossal'**	**'Benton Harbor'**	**Total**
Alternaria angustiovoidea	Control	-	-	-	-
	1 Month	-	-	-	-
	2 Months	-	-	1 (0.4 %)	1 (0.4 %)
Cytospora vinacea	Control	-	1 (0.4 %)	2 (0.8 %)	3 (1.3 %)
	1 Month	-	4 (1.6 %)	-	4 (1.6 %)
	2 Months	-	37 (15.4 %)	1 (0.4 %)	38 (15.8 %)
Diplodia seriata	Control	-	14 (5.8 %)	-	14 (5.8 %)
	1 Month	-	1 (0.4 %)	-	1 (0.4 %)
	2 Months	-	-	-	-
Fusarium culmorum	Control	1 (0.4 %)	-	-	1 (0.4 %)
	1 Month	1 (0.4 %)	-	-	1 (0.4 %)
	2 Months	1 (0.4 %)	2 (0.8 %)	-	3 (1.3 %)
Gnomoniopsis smithogilvyi	Control	-	-	1 (0.4 %)	1 (0.4 %)
	1 Month	-	1 (0.4 %)	1 (0.4 %)	2 (0.8 %)
	2 Months	-	-	3 (1.3 %)	3 (1.3 %)
Monochaetia ilexae	Control	-	1 (0.4 %)	-	1 (0.4 %)
	1 Month	-	1 (0.4 %)	-	1 (0.4 %)
	2 Months	-	-	-	-
Mucor circinelloides	Control	-	-	-	-

Table 2.3. (cont'd)

Isolate	Time Point	Number of isolations (%) 'Labor Day'	'Colossal'	'Benton Harbor'	Total
	1 Month	1 (0.4 %)	-	-	1 (0.4 %)
	2 Months	-	-	-	1 (0.4 %)
Mucor racemosus f. racemosus	Control	-	-	-	-
	1 Month	1 (0.4 %)	3 (1.3 %)	1 (0.4 %)	5 (2.1 %)
	2 Months	-	1 (0.4 %)	2 (0.8 %)	3 (1.3 %)
Neofusicoccum sp.	Control	-	31 (12.9 %)	14 (5.8 %)	45 (18.8 %)
	1 Month	1 (0.4 %)	-	-	1 (0.4 %)
	2 Months	-	-	-	-
Phacidium mollerianum	Control	-	-	-	-
	1 Month	-	-	-	-
	2 Months	-	8 (3.3 %)	-	8 (3.3 %)
Sirococcus castaneae	Control	-	6 (2.5 %)	2 (0.8 %)	8 (3.3 %)
	1 Month	-	11 (4.6 %)	3 (1.3 %)	14 (5.8 %)
	2 Months	2 (0.8 %)	14 (5.8 %)	8 (3.3 %)	24
Trichoderma atroviride	Control	3 (1.3 %)	-	-	3 (1.3 %)
	1 Month	-	-	-	-
	2 Months	1 (0.4 %)	-	-	1 (0.4 %)
Unknown sp. 1	Control	-	3 (1.3 %)	1 (0.4 %)	4 (1.7 %)
	1 Month	-	-	-	-
	2 Months	-	-	-	-
Unknown sp. 5	Control	-	1 (0.4 %)	-	1 (0.4 %)
	1 Month	-	9 (3.8 %)	-	9 (3.8 %)
	2 Months	-	-	-	-
Unknown sp. 6	Control	-	-	-	-
	1 Month	-	-	-	-
	2 Months	-	3 (1.3 %)	-	3 (1.3 %)
Unknown sp. 10	Control	-	-	-	-
	1 Month	25 (10.4 %)	2 (0.8 %)	-	27 (11.3 %)
	2 Months	-	-	-	-
	Total	37 (15.4 %)	163 (67.9 %)	40 (16.7 %)	240 (100 %)

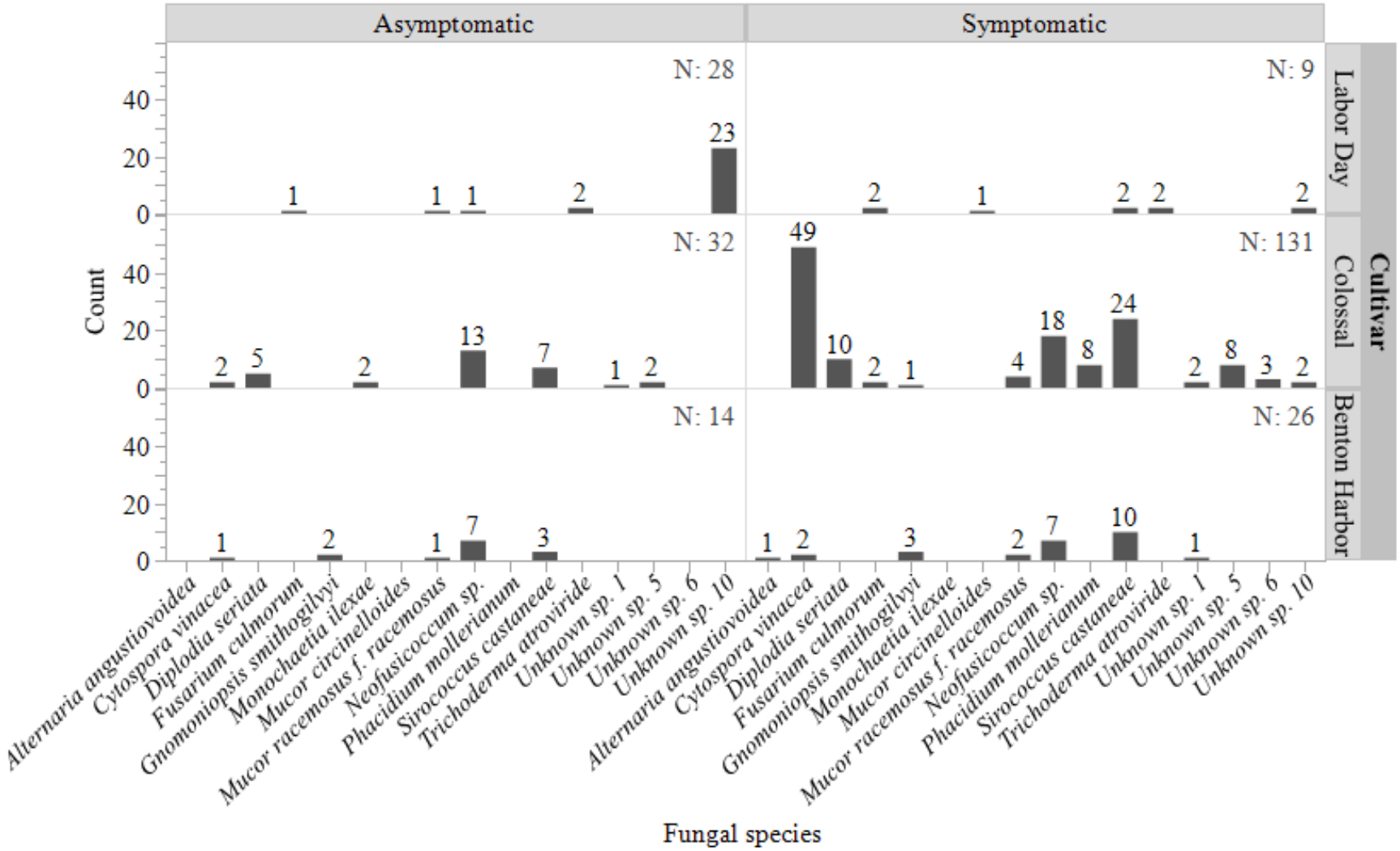

Figure 2.8. Fungal species from the nuts of chestnut cultivars ‘Colossal’, ‘Benton Harbor’ and ‘Labor Day’ isolated from brown rot asymptomatic or symptomatic nuts harvested at the MSU’s AgBioResearch Center, Clarksville (Ionia county), Michigan in 2020.

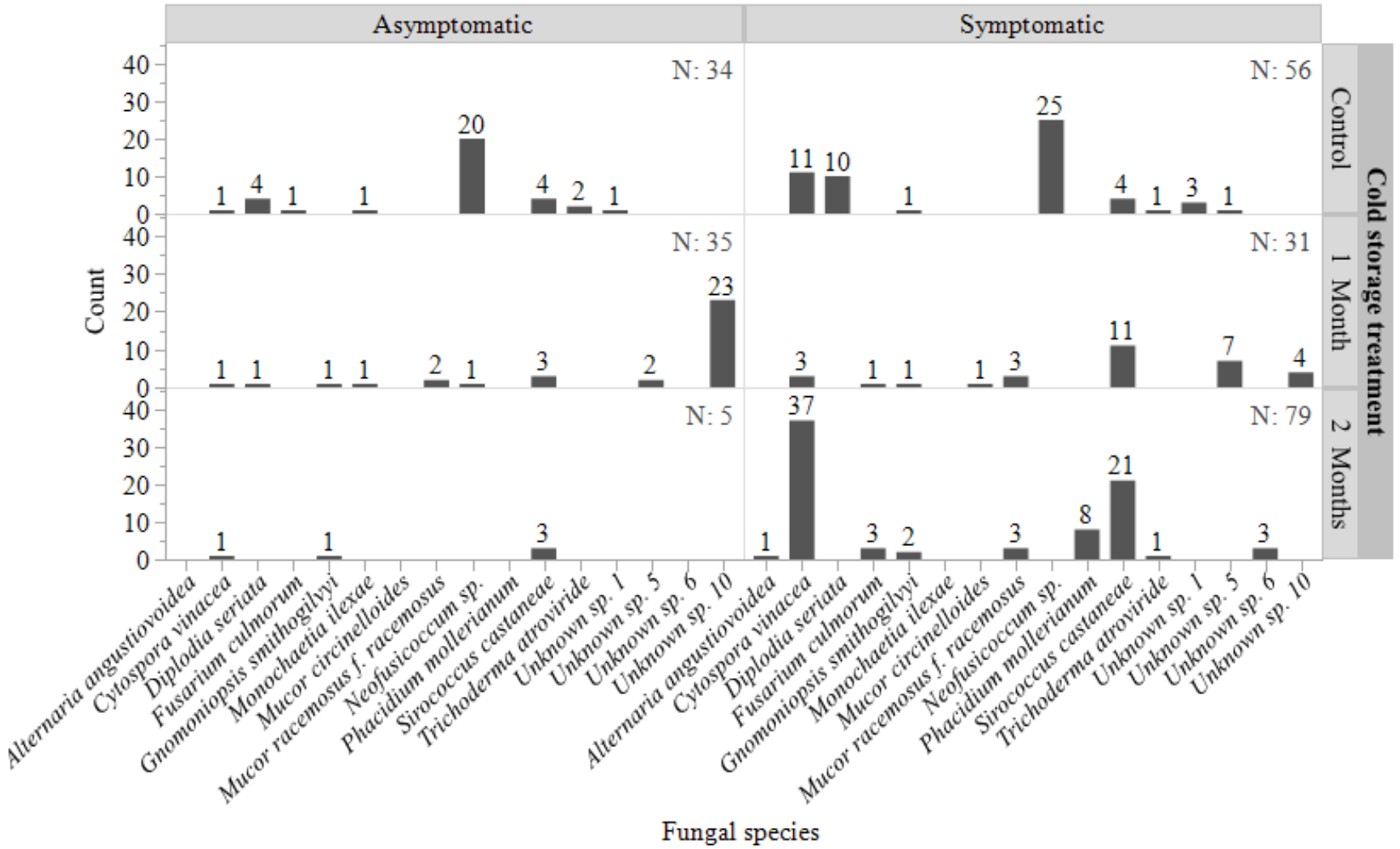

Figure 2.9. Fungal species from the nuts treated with no cold storage (control), 1 month of cold storage (1 month) or 2 months of cold storage (2 months isolated from brown rot asymptomatic or symptomatic nuts harvested at the MSU's AgBio Research Center, Clarksville (Ionia county), Michigan in 2020.

DISCUSSION

This is the first study to determine the susceptibility of three cultivars of *Castanea* to brown rot under different storage conditions. This study supports cold storage in harvested nuts to suppress brown rot disease. ‘Colossal’ was most susceptible to brown rot followed by ‘Benton Harbor’ and then ‘Labor Day’. ‘Colossal’ was most susceptible to both brown rot and general rot symptoms in both naturally infected and inoculated nuts, across all years sampled and in all storage conditions evaluated. Michigan’s nut producers use ‘Colossal’ or a cultivar of a similar genotypic background due to the large nuts produced (*C. sativa* x *C. crenata* hybrid). Some Michigan growers grow ‘Benton Harbor’ (*C. mollissima*) and ‘Labor Day’ (*C. crenata*) which are favored in the midwestern and eastern U.S. As chestnut growers consider the appropriate cultivars in new orchards or replacing trees in established orchards, cultivar resistance to postharvest rot will be important.

For all cultivars, brown rot incidence and severity increased with time in storage. However, both parameters were reduced when nuts were placed directly into cold storage compared to room temperature. The greatest effect of cold storage was observed in 2019 under high disease pressure. In 2020 and 2021, there was a reduced effort of the cold storage on disease incidence and severity. Cold storage is important after harvest and during transportation, marketing, and at consumers homes, and necessitates educating stores and consumers on how to properly store chestnuts (Sommer, 1985).

In our studies, cold storage did not completely suppress *G. smithogilvyi* growth in culture. *G. smithogilvyi* maintained reduced growth at 4°C during the 1 and 2 month cold storage compared to the room temperature control indicating that cold storage effects last for at least seven days post storage. Visentin et al. (2012) observed that *G. smithogilvyi* can grow at

temperatures ranging from 5°C to 35°C. Disease incidence was lowest at harvest (the baseline of disease), highest at room temperature, and limited with cold storage indicating consistency between the in vivo an invitro studies. Cold storage suppresses *G. smithogilvyi* and the potential inoculation of other nuts post-harvest (Wills and Golding, 2016).

The isolation frequency of fungal species varied by cultivar and the time of sampling. *C. vinacea* and *S. castaneae* were similar to *G. smithogilvyi* because their frequency of isolation increased the longer nuts were kept in storage indicating that cold temperatures do not completely arrest development. However, *Neofusicoccum* sp. was primarily isolated from nuts in the control treatments indicating that that this genus or particular specie could not grow and/or compete with other microorganisms at 4°C. *C. vinacea* was typically isolated from 'Colossal' nuts and Unknown sp. 10 was recovered from 'Labor Day'. This may indicate an increased susceptibility of these cultivars to these fungi, but controlled studies are needed for verification.

Cold storage may select for microorganisms able to infect and grow at low temperatures requiring additional strategies such as surface disinfection (Sommer, 1985). The infection of nuts while in the orchard indicates that management practices including applications of biological or chemical controls may be needed (Prusky et al., 2013; Lione et al., 2019). The comparison of *Castaneae* cultivars for their susceptibility to brown rot provides important information to Michigan growers facing increasing disease pressure. Growers may want to plant 'Benton Harbor' or 'Labor Day' which are less susceptible to brown rot than 'Colossal'. The mechanisms for disease susceptibility among the cultivars is unknown. Results indicate from the storage study that nuts from 'Colossal' inoculated with *G. smithogilvyi* isolate 501 were the most susceptible, followed by 'Labor Day'; 'Benton Harbor' was the least susceptible. The data from natural infection show that 'Labor Day' and 'Benton Harbor' have similar susceptibility to *G.*

smithogilvyi. Taken together these results may indicate that ‘Labor Day’ and ‘Benton Harbor’ demonstrate differing mechanisms of resistance to *G. smithogilvyi*. The reduced susceptibility of ‘Labor Day’ may be due to it genetic background. ‘Benton Harbor’ is susceptible to *G. smithogilvyi* when the fungus is introduced to the kernel tissue. There may be a host tolerance and floral age component contributing to host resistance. ‘Colossal’ was most susceptible and may be due, in part, to its flowering time coinciding with the production and release of *G. smithogilvyi* spores as observed by Shuttleworth and Guest (2017).

Future work could include a positive control of nuts inoculated with *G. smithogilvyi* to reduce the reliance on natural incidence which is variable among years. Noting the timing of each chestnut cultivars’ bloom period and its relationship to the production of *G. smithogilvyi’s* primary inoculum is needed. It is theorized that that the chestnut flower is the point for primary infection for *G. smithogilvyi* (Shuttleworth and Guest, 2017). The environmental factors associated with bloom, post bloom, and the production and infection of *G. smithogilvyi* ascospores will support efforts to advance control measures. Increasing nut sample size could determine whether *G. smithogilvyi* is the causal agent of the symptoms classified as brown rot on the kernel or if other fungi cause similar symptoms.

This study fills a gap regarding the susceptibility of three chestnut cultivars representing three genotypes. Results may be applicable to cultivars with similar germplasm. The storage study emphasizes the requirement for cold storage of nuts post-harvest to limit brown rot in chestnuts. Continued education and outreach to markets and consumers on the proper storage in order to maintain the highest quality of nuts prior to consumption will be needed. This study also demonstrates the abundance of fungal species living in the chestnut kernel tissues and their

importance in the environmental ecosystem framework. Future work is needed to understand the interactions between the chestnut host, *G. smithogilvyi*, and their environments.

FUTURE WORK

Future work on the elucidation of brown rots disease cycle should include the route of infection of *G. smithogilvyi* leading to brown rot at harvest. While observational studies have been undertaken to learn the timing of spore production and release, the period of infection of the has been theorized (Shuttleworth and Guest, 2017) however the exact location and infection court of *G. smithogilvyi* and its host (*Castanea*) not yet been determined (Pasche et al., 2016). Determination of the infection court by *G. smithogilvyi* on chestnuts should include light and electron microscopy studies to observe the area of infection (proposed to be the chestnut flower) that *G. smithogilvyi* is targeting for infection (Hartill and Everett, 2002; Ngugi and Scherm, 2006; Salinas and Verhoeff, 1995; Sergeeva et al., 2008; Shinners and Olson, 1996; Shuttleworth and Guest, 2017; Viret et al., 2004). Determination of the location of where *G. smithogilvyi* begins its' pathogenic phase is critical to the development of strategies to disrupt the disease cycle of brown rot (Ngugi and Scherm, 2006).

During this study it was observed that multiple species of the genus *Gnomoniopsis* were observed causing symptoms similar to BR on nuts in Michigan. It has been proposed that the causal organisims of the disease, brown rot of chestnuts, should be updated to include other *Gnomoniopsis* species, similar to brown rot of *Prunus* caused by multiple species of *Sclerotinia* (Jiang and Tian, 2019, Westwood, 1993). Future work could look at characterizing the diversity of *Gnomoniopsis* species associated with asymptomatic and symptomatic nuts. Further identification and classification of the fungal species causing post-harvest decay in chestnuts in Michigan will allow for management strategies to be developed to manage those fungi prior to harvest instead of post-harvest. Management strategies could include evaluation of biocontrol (the competition of other fungi to outcompete *G. smithogilvyi* on chestnut tissues), cultural management (such as removal of burs and debris from the orchard floor), chemical management

(implementation of fungicides during the timing of infection by *G. smithogilvyi* of chestnut flowers) in Michigan orchards (Lione et al., 2019, Pasche et al., 2016; Silva-Campos et al. 2022). It is also critical for the continued monitoring and evaluation of fungal populations associated with nut decay in Michigan to continue to support chestnut growers in their work and to maintain sustainable orchards and businesses.

Other fungal species outside of the *Gnomoniopsis* genus were found associated with fungal decay in nuts post-harvest in this study. Neofusicoccum sp. and *Diplodia seriata* were isolated frequently in nuts (17% and 13% respectively) while also having disease severities higher than the negative controls in the pathogenicity trial conducted. This may indicate that these pathogens should be of note to Michigan chestnut researchers and producers. While these species or their genera have been reported as pathogens on chestnut species before (as fungi causing cankers or nut decay), further research on their effect on Michigan chestnut production is needed (Dar and Rai, 2017; Seddaiu et al., 2021). It would be prudent to not only study *G. smithogilvyi* but also continue to explore and learn what other fungi are affecting the health of nuts and chestnut trees post-harvest to understand their biology and to create possible management strategies.

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