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SEEING DOUBLE WITH *CANNABIS*: HETEROPLOID POPULATIONS IN *BIPOLARIS GIGANTEA*, CAUSAL AGENT OF BIPOLARIS LEAF SPOT

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Agriculture, Food and Environment
at the University of Kentucky

By

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and Dr. Christopher Schardl, Professor of Plant Pathology

Lexington, Kentucky

2021

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ABSTRACT OF THESIS

SEEING DOUBLE WITH *CANNABIS*: HETEROPLOID POPULATIONS IN *BIPOLARIS GIGANTEA*, CAUSAL AGENT OF HEMP LEAF SPOT

Bipolaris leaf spot (BLS) disease emerged with the reintroduction of hemp as a crop in the United States following more than 60 years of prohibition. The causal agent was identified as *Bipolaris gigantea* (=*Drechslera gigantea*), a known minor pathogen of monocots which causes devastating disease on hemp. BLS has been confirmed throughout Kentucky and reported in 15 states. Morphology and growth characteristics of isolates from eight counties across Kentucky were similar with the exception of some isolates producing protoperithecial-like structures. Phylogenetic and whole genome analysis indicated that some isolates were haploid, containing a single allele at each gene (*RPB2*, *TEF1*) and only one mating type idiomorph. Others were "heteroploid," having two alleles at each gene, both mating type idiomorphs, and an assembled genome approximately twice the size of haploid genomes. The phylogenies suggested that most heteroploids had a genome similar to most haploids, plus a related genome that was closely related but phylogenetically distinct. Haploids and heteroploids caused indistinguishable disease symptoms on field hemp and were both equally likely to be isolated from samples. The implications of the genetic diversity of populations causing BLS are unknown, as is the influence on the implementation and development of management strategies.

KEYWORDS: *Helminosporium giganteum*, hybridization, hybrid, ploidy, diploid, populations.

Desiree Szarka

May 7th, 2021

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CHAPTER 1. LITERATURE REVIEW

1.1 *Cannabis sativa*

1.1.1 Introduction

Cannabis sativa is one of the world's most controversial plant species. Its versatility allows for a popular fiber and grain crop, as well as an extensive history of medicinal, recreational, and spiritual usage (Russo 2007). Its psychoactive properties and a perceived potential for drug abuse have led to conflict with the plant's potential for reintroduction as an industrial crop. Many countries have laws that govern or restrict cultivation and use of *C. sativa*. To date, *C. sativa* is reported to have over 3,000 uses ranging from hempcrete for construction to biofuels as an alternative to fossil fuels. Additionally, *C. sativa* has potential as a source for drugs for a variety of medical conditions and recreational use. Recent interest in industrial hemp has emerged owing up to its wide potential, sustainability, and recent legalization.

Cannabis sativa is a dioecious flowering annual in the family Cannabaceae. The closest relatives are *Humulus* (hops) and *Celtis* (hackberry). Leaves are distinctive, palmately compound with 3–9 leaflets, and can be arranged in an opposite or alternative pattern. The majority of the plant is covered with trichomes, small hairs that can be either glandular or non-glandular (Raman et al. 2017). Cannabinoids are produced in glandular trichomes and are excreted in a resinous substance. Most *C. sativa* varieties are heliotropic, and flowering is triggered by shortening daylight length in late summer. Female plants are wind pollenated, with males dying shortly after anthesis (Small 2015). Females typically endure until frost, but under optimal conditions, can survive for years, though in decreasing vigor. Plants naturally reproduce by seed but can be vegetatively propagated to maintain desired traits.

There are over 100 informal, historical, or regional names commonly used in the English language for *C. sativa* or its derivative products (Small 2015). Most names relate to its usage as a psychoactive drug; several common names are pot, weed, bud, ganja, hashish, and marijuana (historically marihuana). *Cannabis sativa* is the accepted scientific

name, though "cannabis" may be used to refer to the plant in general. Although most experts agree that the genus *Cannabis* is monotypic, others argue that *C. indica* and *C. ruderalis* are separate species rather than subspecies of *C. sativa* (Long et al. 2017). Extensive hybridization and long-distance pollination, combined with a lengthy history of cultivation, complicate taxonomy and species identity (Small 2015). Breeding for a range of selected traits has resulted in substantial variation from wild ancestors and between cultivars. While most cultivars are dioecious, some are monoecious or autoflowering. Cultivars may vary greatly in appearance depending upon purpose. Cultivars are plant varieties that have been selected for a desired trait during cultivation and are propagated to maintain that trait. Consequently, tall and unbranched hemp is desirable for fiber cultivation, whereas increased branch development is advantageous to maximize floral or seed yields (Small 2015). The term 'strain' is popular within the marijuana industry and may be used in place of cultivar or variety within the literature.

Generally, *C. sativa* can be divided into two broad categories: hemp and marijuana. Hemp is grown for fiber, grain, or non-psychoactive cannabinoids such as cannabidiol (CBD), whereas marijuana is grown for the psychoactive properties of the cannabinoid Δ9-tetrahydrocannabinol (THC). While marijuana can be prescribed medically in some states, CBD from hemp is legal for therapeutic or health benefits (Agriculture Improvement Act of 2018 ; Mead 2019). Marijuana remains illegal on the federal level in the United States. Legality of *C. sativa* is dependent upon the level of THC present in the plant. The 2014 Farm Bill (Agricultural Act of 2014) defined hemp as *C. sativa* containing no more than 0.3% THC concentration at dry weight and the 2018 Farm Bill (Agriculture Improvement Act of 2018) separated hemp as defined from classification as a Schedule I controlled substance. Here, "marijuana" will refer to recreational or medicinal use of *C. sativa*, and "hemp" will refer to industrial uses including fiber or grain and therapeutic uses including CBD.

1.1.2 Origin and domestication

The precise origin and domestication of *C. sativa* is widely debated, though most agree that it originated in Eurasia, with domestication likely occurring in either China or Central Asia (Liu et al. 2017). Evidence also suggests the possibility of a multiregional

domestication, as centers of early utilization can be identified in both Europe and Asia (Long et al. 2017). The multipurpose use of *C. sativa* has led to selection pressures dependent upon human interests in fiber, grain, or resin during early cultivation, resulting in a variety of phenotypes (Clarke and Merlin 2013). Populations of *C. sativa* readily naturalized throughout both continents following human disturbance, thus complicating efforts to identify wild populations (Small 2015). Early evidence indicating human use in China (4,000 B.C) suggests that *C. sativa* was first cultivated for fiber (Li 1974; Liu et al. 2017). The Chinese also used *C. sativa* for grain, medicine, and, to a lesser extent, for its psychoactive properties (Liu et al. 2017; Zuardi 2006). References to *C. sativa* as a hallucinogen in China are rare, possibly due to its connection to ancient religions, which are also scarcely mentioned in historical texts. The use of *C. sativa* in India for medicine and recreation was much more prominent, and its role in religion was considered sacred. Northern India and the Himalayan foothills was one of the earliest regions to use the plant primarily for its psychoactive properties and likely assisted with the domestication of cultivars high in THC (Clarke and Merlin 2013). In Europe, pollen records indicated the presence of *C. sativa* as far back as the Bronze Age or Neolithic Age, but evidence suggests that cultivation began later, sometime between 8,000-6,000 BP (Long et al. 2017). Romans were the first Europeans to cultivate hemp on a large scale, likely following the introduction of hemp from China through the Middle East (Mercuri et al. 2002). However, evidence also suggests a presence of fiber hemp in Northern Europe centuries, if not millennia, prior to the Roman Empire (Clarke and Merlin 2013). These ancient Europeans likely made crude fiber nets for hunting or similarly styled products.

1.1.3 Cannabinoids

Cannabis sativa is known to produce numerous secondary metabolites, including cannabinoids, terpenes, and phenolic compounds (Flores-Sanchez and Verpoorte 2008). These compounds have drawn interest due to their pharmacological and medicinal potential. The possible interactions of the over 525 known compounds make its chemistry complex and attractive for research (Elsohly and Slade 2005; Radwan et al. 2009). Production of these compounds depends upon numerous environmental and genetic factors (Khan et al. 2014). Cultivar, plant maturity, and tissue type, combined with growth

conditions, determine the amount and concentration of compounds produced. Cannabinoids are produced throughout the plant, but the highest concentration of cannabinoids and other compounds is in mature flowers where glandular trichomes are most abundant. In comparison, seedlings and vegetative tissues contain relatively little.

The most well-known and studied metabolites in *C. sativa* are a unique group of C_{21} terpenophenolic compounds called cannabinoids, or phytocannabinoids (Andre et al. 2016). Although reported in other species within the genera *Radula* and *Helichrysum*, cannabinoids are primarily known as products of *C. sativa* (Appendino et al. 2011). Presently, scientific literature indicates that 141 cannabinoids have been identified, however there is a discrepancy of the exact number (Aizpurua-Olaizola et al. 2016; Elsohly and Gul 2014; Mudge et al. 2018). The most recognized cannabinoids are CBD and THC. The acidic forms of cannabinoids, THC-acid and CBD-acid, decarboxylate upon heating to become psychoactive (Mechoulam and Parker 2013). Cannabinoids are unique for their ability to bind to receptors in the human endocannabinoid system. This system influences anxiety, cognition, depression, neurogenesis, memory, and reward through endocannabinoids and receptors in the central neural system while also performing regulatory homeostatic functions in numerous organs (Mechoulam and Parker 2013; Russo 2016). Cannabinoids' ability to interact with this system has drawn interest for their potential use in medicine. The legal status of *C. sativa* has long restricted research, thus relatively little is known about the effects of cannabinoids on humans and specific interactions within the body (Williams and Williams 2019). Products derived from cannabinoids may be considered nutritional supplements and cannot be sold as medicine (Agriculture Improvement Act of 2018).

1.1.3.1 Δ9-tetrahydrocannabinol (THC)

THC is the primary psychoactive component of *C. sativa* and the most researched of the metabolites. Various initial attempts to isolate the pure form of THC were complicated by the similarities in structures and physical properties of cannabinoids (Mechoulam and Parker 2013). Thus, the isolation and elucidation of the structure in 1964 occurred relatively recently compared to other similar drugs (Gaoni and Mechoulam 1964).

The effects of THC are known to be biphasic and often result in opposing experiences at low and high doses. This presumably is partially responsible for the differences reported in historical and modern accounts of marijuana use, as well as between individual responses (Mechoulam and Parker 2013). Modern marijuana breeders have rapidly increased the amount of THC present in some cultivars in a relatively short time (Cascini et al. 2012; Mechoulam and Parker 2013). In the 1960s, THC-acid percentages averaged no higher than 3%, but some modern cultivars contain nearly 9 times that (Mechoulam and Parker 2013). By 2017, THC content ranged from 17–28% in the most popular cultivars found in Colorado dispensaries (Stuyt 2018). These cultivars also contained less CBD — one popular cultivar had only 0.09–0.2% CBD.

THC has both recreational and medicinal uses, but the former resulted in a damaging reputation, which eventually caused the illegalization of all forms of *C. sativa*, including hemp. Consumption of marijuana, typically smoked for recreation, produces a range of effects including euphoria, intoxication, relaxation, perceptual alterations, and intensification of senses (Appendino et al. 2011; Hall and Degenhardt 2009). Adverse effects include anxiety, depression, panic attacks, and psychosis, as well as functional impairments like memory or reaction time (Hall and Degenhardt 2009). Sustained use can result in the increased possibility of dependency, motor vehicle accidents, cardiovascular disease, and adverse effects on adolescent psychological development (Hall and Degenhardt 2009). These effects have contributed to the widely negative view of *C. sativa* despite its productive history.

Marijuana has been used for management of symptoms from cancer treatments such as nausea and anorexia, as well as for glaucoma, insomnia, post-traumatic stress disorder, and chronic pain (Appendino et al. 2011; Zuardi 2006). Research has shown support for the positive effects of *C. sativa* and these benefits have contributed to support of medical marijuana and legalization in some states as early as 1996, despite the negative connotation (Mead 2019). In addition to THC, consumption of marijuana for either purpose exposes the user to the full spectrum of compounds. For example, studies have shown that CBD moderates the effects of THC (Hudson et al. 2019; Schubart et al. 2011).

1.1.3.2 Cannabidiol (CBD)

Cannabidiol is the second major cannabinoid in *C. sativa* and is a compound which has generated much interest due to its potential therapeutic use against a variety of illnesses and ailments (Pisanti et al. 2017). Although not historically bred or harvested for CBD content, modern fiber hemp is typically high in CBD and low to moderate in THC, whereas the concentrations in marijuana are often the reverse (Giroud 2002; Small 2015; Small et al. 1975). Only in the last few decades that there has been increased interest in CBD, even though the compound was identified relatively early in research of *C. sativa* (Mechoulam and Parker 2013; Zuardi 2008). Cannabidiol is neither psychoactive nor does it bind to known cannabinoid receptors (Mechoulam et al. 2002). Rather, studies suggest that CBD affects the endocannabinoid system indirectly through other actions such as negative allosteric modulation on CB1 or activation of other receptors which activate CB1 (Corroon and Felice 2019; Costa et al. 2004; Laprairie et al. 2015). Cannabidiol research has identified potential for use in many areas of medicine, with possible therapeutic effects on seizure disorders, Alzheimer's disease, Parkinson's disease, nausea, and cancer (Devinsky et al. 2014; Pisanti et al. 2017; Zuardi 2008). In addition to possessing anxiolytic, anticonvulsive, and antipsychotic properties, CBD has potential for treating insomnia, inflammation, and some types of brain damage (Mechoulam et al. 2002; Zuardi 2006). The potential uses of CBD have generated much interest and have helped revive the hemp industry in the United States (Hamilton and Williams 2019) . However, CBD research is still in its infancy, and the full extent of its therapeutic properties and side effects are unknown.

1.1.4 Cultivation of *C. sativa*

1.1.4.1 Cultivation in the United States

Hemp was likely introduced to the American colonies in the early 1600s by the Puritans shortly after their arrival in New England where it quickly became an important crop (Dewey 1913). Early English colonists in some regions were required by law to cultivate hemp at the behest of the government (Clarke and Merlin 2013). Hemp was especially important for manufacturing rope for shipbuilding and in the fishing industry (Clarke and Merlin 2013). In 1699, restrictions placed by the British government on wool

made fiber hemp crucial for maintaining independence by manufacturing clothing locally. Hemp quickly became a staple throughout the lands and was used to produce a variety of household products (Clarke and Merlin 2013). Hemp shortages were common in the colonies and England as hemp processing was difficult and labor intensive (Clarke and Merlin 2013). Slaves and prisoners were sometimes responsible for stripping the outer fibers from the stalk by hand (Clarke and Merlin 2013).

Hemp continued to be widely grown throughout the Americas until the mid-1800s when the Civil War prevented southern hemp suppliers from selling to the North (Clarke and Merlin 2013). The American hemp industry never fully recovered as the demands were met by cheaper fibers and iron ties. Hemp production in the South thrived from 1840 to 1860, but by 1912 most hemp was produced only in Kentucky (Ehrensing 1998). The decline of hemp in the United States was caused by the difficulty in maintaining the labor force required for production. Additional causes of decline included the increase of profits from other crops, lack of specialized equipment, and competition of other fibers (Dewey 1913). Skills for processing fiber hemp, like retting and breaking, were labor intensive and required specialized knowledge, much of which was restricted to Kentucky (Dewey 1913). Although hemp acreage declined due to a multitude of reasons, it was hemp's relationship with marijuana that led to its end (Dewey 1913; Luginbuhl 2001; Mead 2019). Besides a brief allowance for fiber production in early 1940s for World War II, virtually no hemp had been legally cultivated since the initial restrictions until its eventual ban in 1970 (Ash 1948; Ehrensing 1998).

Following the first harvest of hemp in 1775 near Danville, Kentucky, hemp production increased. Kentucky went on to be the crop's leading industry center for nearly 100 years (Rogers 2011). Most hemp was produced in the Bluegrass region where the soil was generally moist and well-drained, conditions under which the crop flourished. During the peak period of fiber hemp production from 1840 until about 1901, Kentucky produced about 75% of the country's hemp and was one of the last regions to grow the crop. (Garland 1946). Kentucky was one of six states that grew the hemp for World War II during the "Hemp for Victory" campaign (Ash 1948; Hemp for Victory 1942; Luginbuhl 2001). Although Kentucky only grew 6,400 of the 250,100 acres planted between 1943-1944, they

provided nearly all the seed needed for production (Agricultural Statistics 1944; Agricultural Statistics 1945). In 1942, the commonwealth contributed 36,000 acres for seed production (Ash 1948). Kentucky's historical ties to hemp have contributed to reviving of the crop. Following the reintroduction of hemp in 2014 under the research pilot program launched in the 2014 Farm Bill (Agricultural Act of 2014), Kentucky was one of the first states to plant hemp (Hemp program overview) . Approximately 33 acres were planted the first year, and over 65,000 acres were planted in 2019 (Hemp program overview). Production was reduced in 2020, as 32,000 acres were approved but only 5,000 acres were planted, but markets are expected to rebound and stabilize.

1.1.4.2 Usage and cultivation

Cannabis sativa is a multipurpose crop that is cultivated for three distinct industries: fiber for textile and building materials, seed for grain and oil, and floral material for cannabinoid (e.g. CBD and THC) extraction. Hemp fiber and grain have been utilized by humans for millennia, and while cultivation was prohibited in most countries due to its association with marijuana, the hemp industry has persisted in other countries like China (Clarke and Merlin 2013; Ehrensing 1998). Despite its status, interest in the crop has caused some nations to reverse laws and permit cultivation of hemp (Clarke and Merlin 2013; Mead 2019). In contrast, marijuana has remained illegal in the majority of the world since the mid-20th century, and only recently have some countries allowed medicinal or recreational use (Mead 2017; 2019). Prior to state-specific legality of medical marijuana in some states, breeding and cultivation had been limited to illicit indoors production in order to hide from law enforcement (Small 2015). Thus, a variety of cultivation methods have been adapted for the different industries. While fiber and grain are cultivated using typical row-crop methods, hemp grown for cannabinoids is treated much like a horticultural crop (Hamilton and Williams 2019). Recent interest in the proposed health benefits of CBD has led an increased demand for cannabinoids, the cultivation of which currently dominates the American hemp industry (Hamilton and Williams 2019) . Like all cannabinoids, THC and CBD are produced primarily in the glandular trichomes on female inflorescences. Therefore, cultivation of hemp for this new purpose resembles marijuana.

Cultivation and production of the crop is determined by cultivar differences and the desired product. The phenotypic variation between fiber, grain, and floral cultivars was driven by selection during domestication for traits that would maximize yields for the desired products (Small 2015). Selection for fiber hemp favored traits that produced tall, unbranched plants with long internodes and a greater phloem or "bast" fiber percentage (Small 2015). In contrast, cultivars that are grown for grain or floral material tend to be shorter and have more branches. Smaller plants invest less energy in vegetative growth and therefore direct more towards inflorescence development where flowers and seeds are produced. While these traits are genetically fixed, planting density influences height and branching patterns. Other traits, such as variation in seed size, seed shattering, or sex ratio, have been retained from wild ancestors and can contribute to yield loss (Schluttenhofer and Yuan 2017). The variation of these traits among cultivars suggests that they can be improved through breeding to increase yields. The extended prohibition against *C. sativa* has delayed modern breeding and has led to the loss of locally adapted hemp cultivars. Combined with the need to maintain compliance with the THC legal limit, modern cultivars of both hemp and marijuana are vastly different than those cultivated historically.

1.1.4.3 Cultivation for fiber

Fiber is derived from the tall, unbranched stems of hemp plants. Seeds sown close together encourage these traits by promoting upward growth and by discouraging branching (Small 2015). Many years of production and research resulted in a wide range of suggested optimal densities, influenced by a variety of factors including cultivar and soil conditions (Amaducci et al. 2015; Cherney and Small 2016). Optimal seeding rates for fiber in Kentucky ranges from 18–27 kg (40–60 lbs.) acre^{-1} spaced by 20 cm (8 in) wide rows (Kostuik and Williams 2019). Fiber is harvested from both female and male plants, though male plants tend to produce a higher quality of fiber. Hemp should be harvested at the onset of reproduction: before male plant decline following anthesis and before blast fibers bind together in females (Fike 2019). Most fiber hemp cultivars are dioecious, as monoecious hemp tends to produce lower quality fiber. However, the later resolves the issue with sexual differences, maturation time, and quality (Small 2015). High density planting also prevents the establishment of weeds by shading and competition (Fortenbery

and Bennett 2004). Following harvest, fiber is removed from the stalk by retting the stems (Clarke and Merlin 2013). During this process, the outer valuable blast fibers are separated and removed from the inner core (hurd) by microorganism degradation of the largely pectin substances between strands. Historically, the hurd was a discarded byproduct, but new uses were devised that take advantage of its high absorbency and other properties (Small and Marcus 2002). There are several methods for retting hemp. Most common is field or dew retting, where harvested hemp is left on the ground and rotated repeatedly over the course of several weeks. Field retted hemp tends to result in inconsistent and poorer quality fiber; however, this method is the most economical. Water retting is a faster but more costly process that produces higher quality fiber with greater uniformity. The harvested stems are immersed in water and carefully monitored to ensure a steady temperature and even circulation. Proper retting determines the processability of the hemp; under-retting makes decortication difficult and over-retting causes deterioration of the fibers. Finer, high quality fiber is needed for textiles such as clothing, but cruder fiber is used for numerous industrial purposes (Small and Marcus 2002).

1.1.4.4 Cultivation for grain (hempseed)

Hempseeds have long been cultivated alongside fiber production and have numerous uses. Seeds can be utilized as food for humans and livestock (hemp for animal feed is not legal in the United States) or processed for oil (Industrial hemp in the United States: status and market potential 2000). Although referred to as seed or grain, the correct botanical term for the fruit is achene. Hempseeds are high in nutrients and proteins, containing approximately 25% protein and 30% oil (Callaway 2004). Hemp oil's low burning point prevents its use for cooking, but it can be used directly in products such as in salad dressings, cosmetics, and body care products (Industrial hemp in the United States: status and market potential 2000). Seeds do not contain THC; however, leaf material or resin may adhere to the surface of the seed and transfer cannabinoids to the hempseed product (Bosy and Cole 2000). Unlike fiber hemp, seed cultivars are sown further apart to encourage branching. As seeds develop in compact inflorescences on branches, more branches increase yield. Male plants are required for pollination; however excess numbers of male plants contribute to yield loss, as seeds only develop on female plants (Clarke and

Merlin 2013; Schluttenhofer and Yuan 2017). In Canada, China, Europe, and the United States, most hempseed is produced using dual-purpose cultivars, which are also harvested for fiber (Small and Marcus 2002). These tend to have less branching and are grown at a moderate density that falls somewhere between that of fiber and grain production standards. Recommended seeding rates for grain or dual purpose hemp in Kentucky is 14–18 kg (30–40 lbs.) acre^{-1} spaced in rows 20–41 cm (8–16 in) apart (Kostuik and Williams 2019). The fiber from dual purpose cultivars is of lower quality but is adequate for many purposes. Harvest should occur when 70% of seeds reach maturity. This prevents yield loss from shattering, a trait still retained from ancestral hemp in which seeds fall as they mature (Kostuik and Williams 2019). In addition to seed shattering, several traits can be improved upon to increase yield; research is ongoing.

1.1.4.5 Cultivation for floral material

Cannabis sativa cultivars grown for THC (marijuana) and CBD (hemp) share a similar phenotype, as both are cultivated for floral material. The only distinguishing feature is their cannabinoid profiles. Aside from the legal requirement maintaining a THC concentration of less than 0.3%, CBD, hemp is bred to maximize cannabinoid percentages. Therefore, cultivation practices for CBD are nearly identical to marijuana production (Fike 2019). Cultivation can occur outdoors in fields or indoors in greenhouses and enclosed rooms. Plants are spaced at low density to promote branching which maximizes floral development (Clarke and Merlin 2013). As the majority of production for cannabinoids prior to 2012 was illicit or limited, no scientifically produced data existed defining conditions that maximize the yield of cannabinoids (Kostuik and Williams 2019). Research is ongoing and has yet to be published. A planting density of 10 plants m^{-2} was given as optimal for marijuana production, though one study found that 15 plants m^{-2} produced the greatest yield in inflorescences (Amaducci et al. 2015). In Kentucky, plant spacing is one plant per meter on rows one meter apart. In contrast to fiber and grain production, male plants are removed to prevent pollination, as seed development lowers yield. Some operations use plasticulture (plastic mulch over rows) or maintain plants in pots. Early CBD production methods were experimental and varied between growers, but as the industry develops, methods are being established (Williams 2019). As cannabinoids are

concentrated in the resin produced primarily from inflorescences, only female plants are desired. Plants can be cultivated from seeds with later rouging of male plants or by vegetative propagation to maintain high cannabinoid content. Cultivar appears to have the greatest influence on cannabinoid concentration (Toth et al. 2020). Additionally, the potential for CBD and THC concentrations are linked, therefore increases in the former can result in concentrations of the latter above the legal limit (Toth et al. 2020). The development of cultivars with higher CBD to THC rations could maximize CBD yield without violating the law. Currently, testing prior to harvest is required by United States law to ensure compliance.

1.1.5 Legality of *Cannabis sativa*

1.1.5.1 Legal history of *C. sativa* in the United States

In early American history, *C. sativa* was used for both medicinal and industrial purposes. Prior to the early 1900s, the plant was prescribed for a variety of ailments and used medicinally in the form of extracts combined with other ingredients (Mckenna 2014); no form of medicinal marijuana was smoked. Early recreational use was rare and limited primarily to Mexican American immigrants, with increased use by African Americans in larger cities after the 1920s (Bonnie and Whitebread 1970). Knowledge of marijuana became widespread in the 1960s and popularity increased among small groups of college students, hippies, and antiwar protesters (Mckenna 2014). Prior to this, the drug was relatively unknown. Rationale for illegalization varied, likely fueled by racial overtones, political ideologies, and inaccurate information (Bonnie and Whitebread 1970). Marijuana gained a reputation as a dangerous, addictive, crime-inducing drug that would replace alcohol (prohibition) and other restricted narcotics (opiates and cocaine). As its popularity rose among white middle-class Americans, misinformation was recognized and an interest in scientific research was generated (Bonnie and Whitebread 1970). Over the years, activist organizations and states attempted to legalize *C. sativa* in its many forms (recreational, medicinal, or industrial), both independently and jointly (Rawson 2005). The United States Federal Government and the Department of Drug and Alcohol Enforcement was resistant to changes and research continued to be restricted, particularly after its Schedule I

substance designation (Brady 2003; Kolosov 2009; Rogers 2011). International stances on marijuana were similar, though many countries separated marijuana from hemp and supported cultivation of the latter.

In the early 1900s, following an increased negative reputation, states began prohibiting medicinal use of *C. sativa*. In 1911, Massachusetts was the first state to ban the sale of derivative products without a prescription, and by 1931 twenty-two states had legislation prohibiting the drug (Bonnie and Whitebread 1970). The Marihuana Tax Act of 1938 was the first national law governing the cultivation of *C. sativa* (Mead 2017). Although not prohibiting marijuana, the Act increased taxation of all *C. sativa* and rendered handling the crop expensive and cumbersome. Anyone who cultivated, distributed, purchased, or handled *C. sativa* was required to pay a tax or risk punishment. Violators could be fined up to $2,000 or/and be imprisoned of up to five years. Despite the distinction made between marijuana and hemp (fiber stalk, oil, etc.), the Act still applied to cultivation for industrial purposes (West 1998). Follow up laws such as the Boggs Act of 1952 and the Narcotics Control Act of 1956 further restricted *C. sativa*.

The United States officially criminalized *C. sativa* with the passage of the 1970 Controlled Substance Act (CSA). The law classified *C. sativa* as a Schedule I substance, the most severe ranking defined by three factors: the potential for abuse, the potential for addiction, and lack of accepted medical use (CSA 21 USC 812). Schedule I substances, also including psilocybin, peyote, heroin, and D-Lysergic acid diethylamide (LSD), are defined as having no accepted medical use, a high potential for abuse, and considered unsafe even under medical supervision (Mead 2017). Classification as a Schedule I substance prohibited medical professionals from prescribing products derived from *C. sativa* and limited possession to only federally approved research programs. As the law made no distinction between hemp and marijuana, cultivation for fiber and grain were also prohibited.

1.1.5.2 Current legal status

Within the past few decades, opinions and attitudes concerning marijuana use have eased, and state laws have reflected this change. In 1996, California was the first state to

approve marijuana for medical use (Compassionate Use Act of 1996). By 2012, when Colorado and Washington legalized recreational marijuana, 19 states had laws permitting some form of medical marijuana. Despite individual states authorizing medical use, *C. sativa* remained a Schedule I substance under federal law. While medical professionals could recommend or suggest marijuana for a medical condition, they could not provide a prescription (Mead 2017). The situation was further complicated as states began to legalize recreational marijuana, as federal law remained unchanged.

Although focus and media attention were on marijuana, numerous attempts were made to separate hemp from its illicit counterpart and to legalize hemp (Rawson 2005). The 2014 Farm Bill (Agricultural Act of 2014, Sec. 7606) took the first step in reintroducing industrial hemp (<0.5 THC) as an agronomic crop to the United States. At the discretion of each state and in conjunction with institutions of higher learning, hemp could be grown under approved agricultural pilot programs for research purposes. Since research was not defined and hemp was not removed from its Schedule I listing, each participating state established distinct policies based on their interpretations of the Act (Mead 2017).

Despite the continuous legalization of medical and/or recreational marijuana under state laws and the approval for hemp cultivation, no changes were made to the federal status of *C. sativa* until the 2018 Farm Bill (Agriculture Improvement Act of 2018, Sec. 12619). The 2018 Farm Bill officially separated hemp from marijuana and, provided that the THC content was less than 0.3%, also removed it from the Controlled Substances Act. Regardless, as long as marijuana remains a Schedule I substance, hemp will require continued monitorization (Hamilton and Williams 2019).

1.1.6 Diseases of *Cannabis sativa*

Despite documentation of various diseases and pests of *C. sativa*, hemp had developed a reputation as a disease-free crop. General descriptions refer to hemp diseases as occurring sporadically or of low severity, with few serious diseases reported (Dewey 1913; Industrial hemp in the United States: status and market potential 2000; Mcpartland 1996). European growers report few to no pest or disease issues (Carus et al. 2013). Like

any other crop, however, hemp is susceptible to diseases caused by bacterial, fungal, nematode, and viral pathogens. Mcpartland et al. (2000) provides the most comprehensive information on pests and diseases of *C. sativa*, but the text was compiled prior to the 2014 legalization of the hemp in the United States. Most literature referenced in this and other texts is 60 years or older, generated before research on *C. sativa* became restricted. There is even less research available for marijuana diseases. Although many pathogens that infect one crop will likely infect the other, different methods of cultivation and growing environments also influence disease development (Punja et al. 2019). Already there has been an increase in disease reports and research following the recent changes in the legal status of *C. sativa*, the revival of the American hemp industry, and the growth of the CBD hemp industry. As legalization progresses, a strong understanding of hemp and marijuana diseases becomes crucial in anticipating issues that may arise from changes in growth environments, adaptation of new cultivation methods, and development of new cultivars.

1.2 *Bipolaris gigantea*

1.2.1 Genus *Bipolaris*

1.2.1.1 Taxonomy

The genus *Bipolaris* has a complex taxonomic record owed to frequent nomenclature changes and adjustments in classification. Presently, *Bipolaris* is placed within Ascomycota, Dothideomycetes, Pleosporales, Pleosporaceae (Manamgoda et al. 2014). Initial classification described *Bipolaris* species within the genus *Helmisporium*, later to be renamed as *Helminthosporium* (Persoon 1822). Historically, this genus included a large number of pathogenic species, many of which were associated with grasses and other monocots (referred to as graminicolous hosts). Fundamental differences in these specimens from the type specimen eventually led to the reclassification of these graminicolous *Helminthosporium* species into four new or existing genera with corresponding sexual states, *Bipolaris* (*Cochliobolus*), *Curvularia* (*Cochliobolus*), *Drechslera* (*Pyrenophora*), and *Exserohilum* (*Setosphaeria*).

Initially, morphological differences between the type specimen *Helminthosporium velutinum* Link (1809) and graminicolous members of the genus led to the creation of two subgroups, both of which were eventually elevated to genera status (Nisikado 1929). Species with cylindrical conidia capable of germinating from every cell and that often had a *Pyrenophora* type sexual state, "broad, fusiform, and muriformly-septated ascospores" (Nisikado 1929), were placed in the newly formed *Drechslera* (Ito 1930). The remaining subgroup, *Eu-Helminthosporium* became the genus *Bipolaris*, characterized by fusiform, straight, or curved conidia that germinated at both ends (Shoemaker 1959).

Morphological differences within *Bipolaris* influenced the creation of another new genus. While many *Bipolaris* species were associated with a *Cochliobolus* type sexual stage, some were noted to have *Trichometaphaeria* type sexual stages, identified by "lack of a clypeus, lysigenous development of the ostiole, occurrence of setae on the perithecial wall, absence of periphyses in the ostiole, and hyphomycetous conidial states" (Leonard and Suggs 1974). These species also differed by having conidia with a protuberant hilum. To accommodate these species, the asexual genus *Exserohilum* and the corresponding sexual genus *Setosphaeria* were formed (Leonard and Suggs 1974). The genus *Curvularia* was created to accommodate graminicolous *Helminthosporium* species (Boedijin, 1933). In addition to the morphological characteristics shared with *Bipolaris,* many *Curvularia* species were associated with *Cochliobolus* sexual stages. Consequently, morphology and sexual stage alone could not be utilized to fully delineate species between *Bipolaris* and *Curvularia* (Sivanesan 1987). Recent phylogenic studies have successfully separated the taxa into the two genera (Berbee et al. 1999; Manamgoda et al. 2012).

The genus *Bipolaris* was established in 1959 for graminicolous *Helminthosporium* species in the subgenus *Eu-Helminthosporium*, with *Bipolaris maydis* (*Cochliobolus heterostrophus*) designated as the lectotype specimen (Shoemaker 1959). Despite the teleomorph *Cochliobolus* being the oldest name associated with these species, *Bipolaris* was accepted as the sole name by the International Commission on the Taxonomy of Fungi (Manamgoda et al. 2014). The rationale for the conservation of *Bipolaris* over *Cochliobolus* was that the former was more commonly used in practice and in literature, thus minimizing confusion and sparing additional name changes (Rossman et al. 2013).

1.2.1.2 Morphology

Conidia of *Bipolaris* are melanized, single, branched, and sometimes arranged in small groups. Conidia are mostly curved, fusoid, straight or curved, and produce a single germ tube from each end. Conidia are 2–14 pseudoseptate/distoseptate (typically more than 6) and are hyaline to light brown. The hilum is inconspicuous or slightly protuberant. Bipolar conidial germination and hilum morphology are both considered distinguishing characteristics of *Bipolaris*.

In nature, *Bipolaris* species are observed in their asexual state, but the sexual morph can be induced under laboratory conditions. Sach's media amended with sterilized rice or wheat leaf material and incubated at 25 C is commonly used to induce ascomata development (Sinclair and Dhingra 1995). The sexual morph is similar to *Curvularia* and is therefore insufficient as an identifying characteristic of the genus. Pseudothecia are dark brown to black, mostly globose with a cylindrical long or short ostiolar neck. Ascomata can be immersed, erumpent, partially embedded or superficial, free, or on flat stroma. Asci are bitunicate and simple, containing 2–8 (mostly 8) ascospores. The ascus is cylindric to obclavate in form. Ascospores are fasciculate, filiform, or flageliform in shape; hyaline, pale yellow, or pale brown in color; septate; and helically coiled within the ascus with the degree of coiling variable.

Cochliobolus species were reported to produce protothecia, a structure superficially resembling ascomata, but containing no ascogenous material (Shoemaker 1955). Appearances and development are similar, but protothecia do not form asci or ascospores. *Bipolaris* species observed producing protothecia include *B. sorokiniana* (*C. sativus*) , *B. oryzae* (*C. miyabeanus*) (Nisikado and Miyake 1921), and *B. maydis* (*C. heterostrophus*) (Nelson 1957). Microconidia have also been observed in some *Bipolaris* species (Sivanesan 1987).

1.2.1.3 Ecology

Bipolaris includes numerous plant pathogens known to infect monocotyledons, primarily Poaceae. Several *Bipolaris* spp. are of economic importance due to their ability

to cause yield losses in cereals. *Bipolaris* species cause a range of diseases, including foliar and root diseases, damping off, crown rot, head blight, and black point (Manamgoda et al. 2014). *Bipolaris* pathogens have caused devastating disease on a number of important crops including corn, oat, rice, sugarcane, and wheat (Berbee et al. 1999). The Bengal Famine of 1943 in India was partially caused by yield losses from brown spot disease. The disease, caused by *Bipolaris oryzae* (syn *Helminthosporium oryzae*), led to the rice shortage that contributed to the death of over two million people (Padmanabhan 1973). *Bipolaris maydis* was responsible for the Southern corn leaf blight epidemic of 1970–1971 in the United States (Ullstrup 1972).

Several *Bipolaris* species are seedborne, including *B. sorokiniana* and *B. oryzae*. Infected seeds can reduce yields, lead to seed or seedling death, and provide major sources of inoculum for subsequent plant disease outbreaks (Neergaard 2017). Wheat and barley seeds infected with *B. sorokiniana* can transfer inoculum to seedlings and contribute to infections of the root and crown (Al-Sadi and Deadman 2010). Inoculum can arise from both infection and infestation of the seed. *Bipolaris sorokiniana* can colonize the entire seed (Acharya et al. 2011). Inoculum from *B. ory*zae can infect seed surfaces and sterile lemmas (Mew and Gonzales 2002). The resulting infection can cause necrotic spots on seeds, and heavy infections may prevent germination. *Bipolaris* species are also known to overwinter in debris and soil (Manamgoda et al. 2011).

The genus *Bipolaris* no longer includes clinically relevant human pathogens, although those pathogens were transferred to the closely related sister genus *Curvularia* (Manamgoda et al. 2012; Manamgoda et al. 2014). Members of *Bipolaris* produce several mycotoxins. Some of these toxins are host specific like HC toxin, HS toxin, and T toxin, whereas ophiobolins and carbotoxin are non-host specific toxins (Manamgoda et al. 2011; Sivanesan 1987).

1.2.2 *Bipolaris gigantea*

1.2.2.1 Description

Bipolaris gigantea was initially described in 1911 on Bermuda grass (*Cynodon dactylon*) in Texas (Heald and Wolf 1911). The description was abbreviated, with only brief details of symptoms and morphology. Conidiophores were characterized as dark brown with a slightly bulbus base, mulitseptate, average size 200–400 × 9–12 µm. Conidia were described as pale brown, cylindrical with slightly tapered ends, 5 septate, average size 300–315 × 15–22 µm, and with densely granular contents (Heald and Wolf 1911). The disease on grasses is called zonate eyespot (Drechsler 1928). Symptoms of the disease were described as numerous longitudinally elongated, yellowish spots with a narrow brown border and averaging 0.5−1 × 1–4 mm in size (Heald and Wolf 1911). Twelve years later, a series of three subsequent monographs by Charles Drechsler further detailed the fungus, expanding the host range and the know distribution. He introduced the term “eyespot” to describe the symptoms on grasses (Drechsler 1923). Drechsler further described the pathogen, expanded the host range to include an additional 34 species (Table 1.1), and described the symptom variation among these hosts (Drechsler 1923; 1928; 1929). The fungus was first renamed *Drechslera gigantea* during the restructuring of *Helminthosporium* species into newly created genera (Ito 1930) and then to *Bipolaris gigantea* based of molecular data (Lane et al. 2020).

Table 1.1 Known hosts of *Bipolaris gigantea.*

Host	**Scientific name**	**Common name**	**Reference**
Arecaceae	*Cocos nucifera*	Coconut	Meredith 1963a
Commelinaceae	*Commelina elegans*	White mouth dayflower	Meredith 1963a
Fabaceae	*Teramnus* sp.		Meredith 1963b
Musaceae	*Musa acuminata*	Banana	Meredith and Campbell 1962
Poaceae	*Agropyron elongatum*	Tall wheatgrass	Drechsler 1928
	Agropyron intermedium	Intermediate wheatgrass	Drechsler 1928

Table 1.1 (continued).

	Agropyron repens	Quackgrass	Drechsler 1923
	Agrostis canina	Velvety bentgrass	Drechsler 1929
	Agrostis stolonifera (= *Agrostis maritima*)	Creeping bentgrass	Drechsler 1929
	Agrostis tenuis	Colonial bentgrass	Jackson and Fenstermacher 1973
	Anthephora hermaphrodita	Oldfield grass	Meredith 1963a
	Andropogon pertusus		Meredith 1963b
	Argostis stolonifera	Creeping bentgrass	Drechsler 1928
	Bromus inermis	Smooth brome	Drechsler 1928
	Carex thunbergii		Honkura 2008
	Chaetochloa lutescens	Yellow foxtail	Drechsler 1928
	Cenchrus ciliaris (=*Pennisetum ciliare*)	Buffelgrass	Drechsler 1929
	Cenchrus brownii	Southern sandbur	Meredith 1963b
	Cenchrus echinatus	Burr grass, sandbur	Meredith 1963b
	Cenchrus purpureus (=*Pennisetum purpureum*)	Napier grass, elephant grass	Arnold 1986
	Cynodon dactylon	Bermuda grass	Heald and Wolf 1911
	Digitaria ciliari	Southern crabgrass	Honkura 2008
	Digitaria eriantha	Digitgrass	Camino-Vilaró et al. 2019
	Digitaria humifusa		Drechsler 1928
	Digitaria insularis	Sourgrass	Meredith 1963b
	Digitaria sanguinalis	Large crabgrass	Evidente 2006
	Echinochloa crus-galli	Cockspur grass	Drechsler 1928
	Eleusine indica	Indian goosegrass	Drechsler 1923
	Elymus virginicus	Virginia wildrye	Drechsler 1928
	Eragrostis caroliniana	Tuffed lovegrass	Drechsler 1929

Table 1.1 (continued).

	Eragrostis cilianensis	Stinkgrass, candy grass	Drechsler 1928
	Eragrostis pectinacea	Tuffed lovegrass	Sprague 1950
	Festuca hookeriana	Hooker's fescue	Drechsler 1929
	Glyceria depauperata	Mannagrass sp.	Sato et al. 1990
	Glyceria ischyroneura	Mannagrass sp.	Honkura 2008
	Hordeum secale	Barley	Isakeit et al. 2017
	Hordeum vulgare	Common barley	Gamba and Tekauz 2003
	Isachne globosa	Bloodgrass sp.	Honkura 2008
	Ixophorus unisetus	Mexican grass	Drechsler 1929
	Leersia oryzoides	Rice cutgrass	Greene 1955
	Leersia sayanuka		Honkura 2008
	Leersia virginica	Whitegrass	Drechsler 1928
	Microstegium vimineum	Japanese stiltgrass	Lane et al. 2020
	Miscanthus sp.	Silvergrass sp.	Miller 1997
	Muhlenbergia mexicana	Mexican muhly	Drechsler 1928
	Muhlenbergia schreberi	Nimblewill	Drechsler 1928
	Muhlenbergia sylvatica	Woodland muhly	Greene 1963
	Muhlenbergia uniflora	Bog muhly	Greene 1963
	Oryza sativa	Rice	Ahn 1980
	Panicum adspersum	Broadleaf panicum	Lenné 1990
	Panicum anceps	Beaked panicgrass	Drechsler 1928
	Panicum antidotale	Blue panicgrass	Arnold 1986
	Panicum capillare	Witchgrass	Greene 1952
	Panicum clandestinum	Deertongue, Kikuyu grass	Drechsler 1928
	Panicum coloratum	Kleingrass	Arnold 1986
	Panicum dichotomiflorum	Fall panicgrass	Drechsler 1928
	Panicum gattingeri	Gattinger's Panicgrass	Drechsler 1928
	Panicum maximum	Guinea grass	Meredith 1963b

Table 1.1 (continued).

	Pennisetum alopecuroides	Fountain grass	Drechsler 1928
	Phalaris arundinacea	Reed canarygrass	Drechsler 1928
	Phalaris aquatica (*Phalaris stenoptera*)	Bulbus canary grass	Drechsler 1929
	Phleum pratense	Timothy	Drechsler 1928
	Poa pratensis	Kentucky bluegrass	Drechsler 1928
	Saccharum officinarum	Sugarcane	Meredith 1963b
	Secale cereale	Rye	Sivanesan 1987
	Spodiopogon sibericus	Frost grass	Drechsler 1929
	Sporobolus neglectus	Small dropseed small rushgrass	Rogerson 1958
	Sporobolus sp.		Drechsler 1929
	Stipa splendens (*Lasiagrostis splendens*)	Chee grass	Drechsler 1928
	Triticum aestivum	Wheat	Chowdhury et al. 2005
	Tripsacum dactyloides	Gama grass	Drechsler 1929
	Zoysia japonica	Korean lawn grass	Sato et al. 1990
	Zizania aquatica	Wild rice	Kardin et al. 1982
	Zizania latifolia	Manchurian wild rice	Sato et al. 1990

1.2.2.2 Morphology and ecology

Symptoms of *B. gigantea* were noted to vary greatly between seasons, locations, and host (Drechsler 1928; 1929). The pathogen was reported to overwinter as dormant mycelia in infected material. Leaves of infected quack-grass (*Elymus repens*) exhibiting symptoms from the previous season were periodically collected during the spring of 1923. These samples were incubated in a moist chamber and observed to continuously produce conidia until the experiment was discontinued in early May (Drechsler 1928). Spore dispersal was described as localized, likely owing to the relatively few, short lived, and large-sized conidia produced by *B. gigantea*. Drechsler documented decreases in leaf

lesions observed the further away from an isolated, confirmed infected stand and found no symptoms at 20 m from the site.

Morphological details were further expanded upon, including a description of microconidia (Drechsler 1928). Conidiophores were described as having septa at regularly spaced intervals, usually from 25–40 µm apart, and had an unpronounced scar marking the point of conidial attachment. Initial scars were measured at 140–250 µm from the base; successive scars were spaced at intervals of 40 µm (Drechsler 1923). Conidia were described as hyaline and filling homogeneous protoplasm, and the author proposed that the original conidia described by Heald and Wolf were of dead material. Conidia filled with "densely granular contents" were not observed to germinate (Drechsler 1923). Conidial ends were reported as rounded with the basal end exhibiting "a dark apicular projection" that fit seamlessly into the conidiophore scar (Drechsler 1923). Conidia germinated rapidly when exposed to water, typically producing groups of 3–4 germ tubes from both the basal and apical ends, sometimes from the middle segments, and occasionally produced single germ tubes. Germ tubes averaged 6–10 µm in width and grew "rapidly" (Drechsler 1923).

Pure cultures were cultivated on maize-meal agar, and optimal temperatures were documented to be between 25 C and 29 C (Drechsler 1928). Growth was noted as slow, with all but the aerial mycelia at the growing edge drying out and collapsing. Aerial mycelia were recognizable as large with a uniform width, a distinctive branching pattern, and having snarled and curved tips (Drechsler 1928). Older mycelia submerged within the medium were darker and had more septations than newer grower on the outer edges of colonies. Culture appearance was described as "somewhat granular or flaky white or grayish material, sprinkled irregularly over the surface of the culture" (Drechsler 1928). Sporulation in culture was sparse. Occasionally, conidia were observed to produce conidia instead of germ tubes. These secondary conidia also produced additional conidia, which in turn produced more conidia. These secondary and tertiary conidia were often smaller in size than conidia produced from leaf material. The resulting terminal conidia were produced in short branching chains, as little as 3.5–2.5 µm (Drechsler 1928). These branching chains of small conidia were not observed on collected material.

Further observations were made following the first report of *B. gigantea* in Jamaica causing eyespot disease on banana (Meredith and Campbell 1962). The disease was reported to be of little economic importance and that inoculum likely originated from Bermuda grass, a common weed in the area. In addition to descriptions matching those of Drechsler's, new observations were made regarding spore dispersal and release. Diurnal periodicity was established by the use of a Hirst spore trap, and spore release was concluded to be associated with decreasing vapor pressure. Air-borne conidia were captured in highest concentrations between 8:00 and 14:00 and after rain (Meredith 1963a). Under laboratory conditions, conidia were observed to discharge forcibly under drying conditions. Conidia likely matured at night and were released during the day as the temperature increased and humidity decreased, contributing to a decrease in conidiophore vapor pressure (Meredith 1963a). Laboratory studies showed changes in conidia and conidiophore turgidity when transferred from a moist environment. Meredith described a "gas bubble" that formed in conidiophores cells, caused movement, and contributed to spore release (Meredith 1963b). All dried conidia and conidiophores were reported to regain turgidity when submersed in water.

Additional reports of *B. gigantea* were scattered. The geographic distribution of the fungus was expanded to include regions in Central and South America, with the report of zonate eyespot disease on rice in Colombia, Panama, and Peru (Ahn 1980). *Bipolaris gigantea* was detected on wheat in Mexico during a study examining foliar pathogens of wheat in warm regions (Maraite et al. 1997). In Brazil, the pathogen was first reported causing zonate eyespot on specific rice cultivars in 2006 and has been observed seasonally since (Nunes 2008; Rivera et al. 2020). Similarly, the diseases has been continuously infecting wheat in India since it was first observed in 2005 (Chowdhury et al. 2005). The pathogen was also reported on rice in Japan (Sato et al. 1990) and on additional monocot hosts (Honkura 2008). Isolates from Japan were observed to form protothecia on V8 and leaf water agar (LWA) media. The structures were globose, black, and ranged from 200–450 µm in diameter. However, despite attempts to mate isolates from two regions in Japan, no asci or ascospores were produced (Honkura 2008). In Minnesota, wild rice (*Zizania aquatica*) was described as a new host of *B. gigantea* (Kardin et al. 1982). Barley (*Hordeum*

spp.) was also reported as a new host in both Texas and Uruguay (Gamba and Tekauz 2003; Isakeit et al. 2017).

1.2.2.3 Potential use of *B. gigantea*

Bipolaris gigantea is a well-known pathogen of many grasses. The fungus has also been investigated for its potential as a biological control agent against noxious weeds, both for direct infection and for the phytotoxic compounds it produces. Several studies assessed *B. gigantea* as a bioherbicide against invasive grasses. In Florida, studies examined *B. gigantea* independently and in a mixture with two other pathogens against seven grass species (Chandramohan and Charudattan 2001; Chandramohan et al. 2002). In laboratory experiments and under field conditions, *B. gigantea* was equally effective alone and as part of a mixture in reducing vegetation when applied to several grass species. The pathogen was also evaluated against 30 dicot crop species to ensure that no off-target species would be impacted. The studies concluded that *B. gigantea* has potential to control weedy grasses (Chandramohan and Charudattan 2001; Chandramohan et al. 2002). *Bipolaris gigantea* was also investigated as a bioherbicide against the invasive green foxtail (*Setaria virdis*) in the Canadian prairies (Green et al. 2004; Peng and Boyetchko 2006). The pathogen's ability to cause disease was evaluated under various moisture conditions and temperatures. Under optimal conditions, *B. gigantea* led to rapid symptom development, likely influenced by toxin production (Peng and Boyetchko 2006). Considering the semi-arid climate of the Canadian Prairies, the effectiveness of the pathogen may be limited by its moisture and temperature requirements (Peng and Boyetchko 2006).

1.2.2.4 Compounds produced by *B. gigantea*

Bipolaris gigantea produces a variety of bioactive compounds belonging to two classes of terpenoids: sesquiterpenes and sesterterpenoids. Many of these compounds have been isolated, characterized, and studied in *B. gigantea*. Investigations first identified 12 sesquiterpenes called eremophilanes, which were produced by *B. gigantea* in liquid culture (Kenfield et al. 1989). Eremophilanes are produced by both fungi and higher plants. A newly described eremophilane, gigantenone, was isolated from *B. gigantea* (Kenfield et al.

1989). When applied to leaves, gigantenone was observed to cause green islands, localized areas of chlorophyll retention, in monocotyledons, whereas application of gigantenone to dicotyledons resulted in necrosis. Similar results were documented with the eremophilane phaseolinone (Sugawara et al. 1993). Phomenone, phaseolinone, and petasol were isolated from *B. gigantea*, all of which have been previously isolated from other fungal species. Other eremophilanes isolated from *B. gigantea* were oxidized variants of known compounds. The majority of the isolated eremophilanes were phytotoxic and are assumed to play some role in symptom development (Sugawara et al. 1993).

Ophiobolins are sesterterpenoid phytotoxins produced as secondary metabolites predominantly by the genus *Bipolaris*. As phytotoxins, they are thought to have a role in disease development. Ophiobolin A, also known as cochliobolin, was independently discovered in *B. maydis* (*Helminthosporium oryzae)* by Canonica (1966) and Nozoe (1965). Since the discovery of the first ophiobolin, a total of 23 biogenic analogs have been identified (Au, 2000). Currently, eight ophiobolins, two of which were novel, have been isolated from *B. gigantea*, (Evidente et al. 2006a; Evidente et al. 2006b). Ophiobolin A, 6-epi-ophiobolin A, ophiobolins B and J were found to be toxic. While phytotoxicity varied between the compounds, monocots were more sensitive. Ophiobolin A was found to be highly toxic, even at the lowest concentrations, whereas ophiobolin I had no effects when applied at the highest concentrations (Evidente et al. 2006a).

1.3 Fungal diversity

1.3.1 Introduction

The phyla Ascomycota and Basidiomycota are both characterized by exhibiting a dikaryotic phase but are differentiated by the duration of that phase and the life stage in which it develops. Ascomycota exist primarily as haploids, whereas Basidiomycota exist predominantly as dikaryons. In Ascomycota, the dikaryotic state is restricted to ascogenous (sexual reproductive) hyphae. During the reproductive cycle, ascomycete fungi form a fruiting body known as an ascocarp. Ascocarp variation and other developmental characteristics define the six classes in which members of Ascomycota belong. *Bipolaris* belongs to the class Dothideomycetes, which is primarily characterized by bitunicate asci

and fissitunicate dehiscence (Hyde et al. 2013). Although ascomycetes typically exist primarily as haploids, there are several known exceptions in which a species may exhibit a prolonged alternative state including some yeasts, *Epichloë* spp., *Metarhizium* spp. and *Verticillium longisporum* (Ingram 1968; Kepler et al. 2016; Moon et al. 2004). These exceptions potentially arise from disruptions in the reproductive stage, during hybridization, or from whole genome duplication (Charron et al. 2019; Schardl and Craven 2003).

1.3.2 Hybridization

Hybridization is the union of individuals from different species or varieties. In fungi, intraspecific hybridization occurs between two individuals of the same species and interspecific hybridization occurs between individuals of two different species (i.e., same genus or family). Hybrids can differ from both of their parents in pathogenicity, host range, and other characteristics. Although hybridization often leads to decreased fitness, advantageous traits that the parents lack may appear in resulting hybrids (Stukenbrock 2016). Hybrids can arise through either sexual or parasexual processes and heteroploid offspring can sometimes result (Schardl and Craven 2003).

1.3.2.1 Sexual reproduction

In filamentous ascomycetes (subphylum Pezizomycotina), the sexual cycle generally begins when haploid hyphae of opposite mating types fuse together and undergo plasmogamy, the merging of the cytoplasm without nuclear fusion (Bennett and Turgeon 2016; Coppin et al. 1997). The resulting ascogenous hypha is dikaryotic (n + n) and the enclosed nuclei continue to divide independently as the ascocarp forms. Nuclei within ascogenous hyphae recognize non-self-individuals, pair with opposites, and migrate into a hook-like structure called a crozier, which forms from the hyphae. Following septum formation, the paired nuclei within the apical cell of the hook will undergo karyogamy to produce a diploid nucleus. This cell will develop into an ascus, and the diploid nucleus will rapidly undergo meiosis, followed by one or more mitotic divisions to form haploid ascospores. Germinated ascospores produces haploid hyphae until reproduction occurs.

Hybrids can result from either a complete or partial sexual cycle. Hybrids may persist as vegetative dikaryons following plasmogamy, karyogamy, or form following karyogamy and meiosis (Schardl and Craven 2003). A successful mating followed by a completed sexual cycle produces euploid hybrids, typically haploid in ascomycetes. In contrast, an incomplete sexual cycle can result in a heteroploid hybrid. Incompatible chromosomes from parents can disrupt normal mitotic or meiotic processes, resulting in unstable ploidy levels. These offspring are frequently sterile and can only propagate through asexual reproduction. Over time and development, some may regain their euploid state through partial or whole chromosome loss. In at least yeasts, the genome of such hybrids may double, thereby restoring fertility as seen in *Saccharomyces* spp. crosses (Charron et al. 2019). Successful hybridization can result in the formation of new species if the offspring contain advantageous traits or are adaptable to a new host range.

Genetic and other factors, such as environmental conditions or geological barriers, generally restrict mating of individuals to within a single species or to close relatives (Olson and Stenlid 2002). In heterothallic *Bipolaris* species, sexual development is dictated by a bipolar mating system comprising two mating types each determined by a distinct gene at a single locus designated *MAT1*. The different mating type genes are, therefore, referred to as *MAT1-1* and *MAT1-2* idiomorphs (Yoder et al. 1986). In *B. maydis* (=*Cochliobolus heterostrophus*), *MAT1-1* encodes an α-box protein and *MAT1-2* encodes an HMG-domain protein (Turgeon et al. 1995).

Homothallic species differ from heterothallic species in being capable of self-mating, such that a single strain can undergo sexual reproduction and produce ascospores. In homothallic fungi single individuals may contain both mating types whereas individuals of heterothallic species have one or the other. Such is the case for homothallic *Bipolaris* species described to date (Bennett and Turgeon 2016).

1.3.2.2 Parasexual reproduction

Parasexuality, also referred to as somatic recombination or vegetative fusion, is an asexual process of introducing variation through the transfer of genetic material between dissimilar individuals (Pontecorvo 1956; Tinline and Macneill 1969). The parasexual cycle

superficially resembles the sexual cycle with analogous steps, except there are clear differences governing compatibility and there is no meiotic stage (Parthiban et al. 2018). In asexual fungi, this process is an important alternative to sexual recombination, providing a substitute for evolution towards adaptive traits (Pontecorvo 1956). Beginning with anastomosis, the fusion of vegetative hypha forms a heterokaryon or a dikaryon (Schardl and Craven 2003). Heterokaryosis refers to the presence of genetically distinct nuclei occupying a single cell and is an integral part of the parasexual cycle. This stage may persist indefinitely as vegetative hyphae, or as more commonly observed, the unstable heterokaryons will result in homokaryons or follow with nuclear fusion (karyogamy) (Schardl and Craven 2003) resulting in a diploid nucleus. Mitotic crossing-over (recombination of linked genes) or haploidization at this stage can introduce variation (Day 1960). Repeated loss of chromosomes will reduce the ploidy of the cell-generating haploid, or potentially in aneuploid cells. However, in some cases (such as many *Epichloë* species) plasmogamy and subsequent karyogamy generates stable diploids or polyploids.

Allorecognition, distinguishing self from non-self, is a vital component of fungal biology and influences the parasexual cycle and heterokaryon formation (Glass and Kuldau 1992; Saupe 2000). As vegetative fusion results in the merging of cytoplasm and contents, this process would be detrimental if not strictly regulated. Fusion with unsuitable individuals can lead to decreased fitness, loss of self, and susceptibility to somatic parasites (Aanen et al. 2008; Czaran et al. 2014). Vegetative incompatibility (VI), or heterokaryon incompatibility (HI), regulates compatibility through alleles at *vic* or *het* loci (Glass and Kuldau 1992; Paoletti 2016). The occurrence of one or more incompatible alleles at either locus within a single cytoplasm triggers incompatibility and results in cell death, preventing further fusion. Although the same systems that regulate sexual or vegetative compatibility influence the potential for hybridization through either process, they can also function independently (Glass and Kuldau 1992; Tinline and Macneill 1969). Sexual incompatibility does not necessarily prevent vegetative fusion and heterokaryosis formation (Jacobson 1992; Mishra 1971), and not all filamentous ascomycetes have similarly restrictive VI systems (Chung and Schardl 1997a).

1.3.3 Examples of non-haploid ascomycetes

Although the majority of Ascomycetes exist as haploids for the majority of their life cycle, there are a few known exceptions.

1.3.3.1 *Epichloë*

Epichloë species (including anamorphs formerly classified as *Neotyphodium* species) constitute a well-known group of endophytic symbionts of grasses (Leuchtmann et al. 2014; Schardl 1996). Colonization of new hosts occur through both horizontal and vertical transmission. Sexual *Epichloë* species can produce stromata required for sexual reproduction and horizontal transmission via conidia and ascospores. Many sexual and all of the asexual *Epichloë* species can transmit vertically through the colonization of embryos in viable seeds. Germinating seedings are therefore already colonized. Horizontal transmission occurs when an *Epichloë* sp. completes its sexual cycle to produce ascospores, which infect developing seeds in the inflorescence (Chung and Schardl 1997b).

Molecular investigations have shown that sexual *Epichloë* species are predominantly haploid, whereas most asexual members are heteroploid, either aneuploid or polyploid (Moon et al. 2004). These heteroploid asexual members likely arose from interspecific hybridization between sexual ancestors through the parasexual process. As *Epichloë* lacks a vegetative incompatibility system and mating barriers are strong, hybridization likely occurs following anastomosis of two or more species colonizing a single host (Schardl and Craven 2003; Shoji et al. 2015). The host provides the opportunity for prolonged interaction between species.

Evidence for interspecific hybridization is demonstrated through the relationships of alleles in sexual and asexual species. Although sexual *Epichloë* species have consistently contained only a single allele of numerous genes examined, multiple alleles have been present at those same loci in asexual species (Moon et al. 2004; Schardl et al. 1994). Phylogenetic analysis demonstrates the alleles from asexual *Epichloë* species grouping with those from sexual species. In hybrid isolates with two or three alleles, this grouping indicates a close relationship to distinct species, thus multiple parental ancestors.

Additionally, asexual members have uninucleate spores that contain more genetic material then their sexual counterparts (Kuldau et al. 1999). The genome size of some asexual species is nearly the sum of its potential ancestors, indicating potential diploidy or (in the case of *Epichloë coenophiala*) triploidy.

1.3.3.2 *Metarhizium*

Metarhizium is a genus of soilborne entomopathogen fungi that parasitize a wide range of insects. Once considered to be entirely asexual, molecular phylogenetic studies link it to sexual species such as *Metacordyceps yongmunensis* (Liang et al. 1991; Liu et al. 2002; Sung et al. 2007). These species are heterothallic, requiring both mating types to complete sexual reproduction. Complex isozyme banding patterns in *Metarhizium majus* suggested that the species was diploid (Leger et al. 1992).

A study investigating the ploidy of isolates in the *M. majus* species complex has concluded that diploidy has arisen independently in two lineages (Kepler et al. 2016). The species investigated belong to the MGT clade (Bischoff et al. 2009), which includes two asexual species, *M. majus* and *M. guizhouense*, and two sexual species, *M. taii* and *M. indigoticum*. Sequencing results show that isolates of sexual species have a single allele at the eight microsatellite loci amplified and one mating type analyzed. These results are consistent with a haploid genome. Indicative of a diploid genome, the majority of asexual species have two alleles at a single locus and both mating types. Phylogenetic analysis groups diploid isolates into two clades within the *Metarhizium* majus species complex. Isolates were verified to have uninucleate conidia to indicate that diploid genotypes were derived from a single nucleus and not from binucleate heterokaryons (Kepler et al. 2016). Since diploid isolates consistently have both mating types, these lineages may have resulted from failed mating events.

1.3.3.3 *Verticillium*

Verticillium longisporum is a widespread hybrid plant pathogen that causes vascular wilt on a broad range of hosts. In the original description, *V. longisporum* was distinguished from *Verticillium dahliae* by having spores twice the size (Stark 1961).

Verticillium isolates producing large spores are also reported to contain a single nucleus (Hastie 1964). These characteristics have led to speculation that *V. longisporum* is a stable diploid or, alternatively, a large-spored haploid. Evidence to support stable diploidy was acquired by obtaining haploid spores from the suspected diploid and then re-uniting them (Ingram 1968). The derived haploids have small spores that, when recombined, have the typical characteristics of the parental diploid (i.e. stable large spored). Further studies have confirmed *V. longisporum* as a stable diploid (Clewes et al. 2008; Jackson and Heale 1985).

A study has provided evidence that *V. longisporum* is an allodiploid hybrid that arose from three independent hybridization events (Inderbitzin et al. 2011). Of the *Verticillium* spp. examined, all *V. longisporum* strains contain two alleles at eight different loci analyzed, whereas the three other *Verticillium* species analyzed contain only a single allele at those loci. Allele A1 is reported to be present in all *V. longisporum* strains, along with either alleles D1, D2, or D3. Although allele D2 and D3 are found in two different *V. dahliae* lineages, allele A1 and D1 are assumed to be from an unknown species. Thus, three hybridization events have apparently occurred between species A1 and D1, *V. dahliae* lineage D2, and *V. dahliae* lineage D3 to give rise to *V. longisporum*. Phylogenetic analysis of the mating type genes indicates that they correspond with the lineages determined by the allele pairs. *Verticillium longisporum* strains contained only one mating type idiomorph, *MAT1-1*, whereas *V. dahliae* strains had either a *MAT1-1* or a *MAT1-2*, although the latter was more common (Inderbitzin et al. 2011). These findings correspond with the results of previous studies investigating hybridization in *Verticillium* spp. (Clewes et al. 2008; Zeise and Von Tiedemann 2002).

1.4 Significance

The emergence of *Bipolaris gigantea* as a major pathogen on hemp resulted in challenges for understanding pathogen biology and for disease management. The pathogen is new to *C. sativa*, and all previously known hosts —with the exception of a *Teramnus* sp. —have been monocots. In addition, the crop itself is unique compared to most agronomic commodities. Hemp had been prohibited from modern cultivation until 2014. The controversy surrounding *C. sativa* had prevented any progress in modern hemp production and breeding. Further, there are limited pesticides approved for use on hemp. The

reintroduction of hemp as an agronomic crop in the United States coincided with the emergence of Bipolaris leaf spot. The new hemp industry had to refine cultivation methods for fiber and grain, as well as to incorporate the new CBD hemp while contending with disease. Concurrently, research into the pathogen revealed characteristics atypical in Ascomycetes, suggesting a need to understand how such a condition may arise. It is imperative to understand all aspects of the disease cycle to fully comprehend how the different aspects may interact and influence management.

CHAPTER 2. FIRST REPORT OF HEMP LEAF SPOT CAUSED BY A *BIPOLARIS* SPECIES ON HEMP (*CANNABIS SATIVA*) IN KENTUCKY

This chapter was previously published in Plant Health Progress and contains minor edits.

The passage of the 2014 Farm Bill (Agricultural Act of 2014) granted growers the opportunity to cultivate industrial hemp (*Cannabis sativa*) under university and state government research pilot programs. By 2019, over 1,000 growers representing approximately 9,700 ha (24,000 acres) participated in the program in Kentucky. Industrial hemp was grown outdoors in traditional fields for fiber and grain production. Hemp grown for cannabidiol (CBD) was raised in either fields or greenhouses. Cannabidiol cultivars continue to be the most widely grown type. In 2019, 92% of the acreage grown in Kentucky was intended for CBD extraction (https://www.kyagr.com/marketing/hemp- overview.html).

In 2014, a grower in Jackson County, Kentucky, reported severe leaf spot symptoms on field-grown hemp cultivars. All plants in the field were affected. Yield losses reached 100% as severely infected plant material was discarded or diseased plants failed to produce sufficient quantities of CBD (> 4%) and were rejected by processors. No hemp had been planted in this area previously. In August 2015, samples were collected from the same fields that exhibited the same symptoms as previously reported. Cultivars included proprietary breeding lines with high CBD content and low tetrahydrocannabinol (THC) content (< 0.3% THC). Plants were approximately 3-mo-old and in the vegetative stages, and no flowers were present. Plants were spaced at 1.04 m (41 in.) apart, and rows were 1.04 m apart in a 2.63-ha (6.5-acre) plasticulture field. Similar leaf spots have since been reported in additional counties, including Marion County in 2017 and Nelson County in 2018 (Table 2.1). These fields were located 157.7 km (98 miles) and 141.6 km (88 miles) from the Jackson County field, respectively. Symptoms began as light green specks scattered on leaf surfaces (Figure 2.1). These specks expanded to round, 1–2-mm-diam spots within 2 wk and extended to the undersides of leaves. Symptoms were distributed evenly throughout each field. Spots appeared scattered on both mature and emerging leaves and ranged from dark brown with a darker margin to light tan with no border. Spots coalesced and formed irregularly shaped lesions (Figure 2.2). Dark conidiophores were visible in the centers of spots on both upper and lower sides of leaves with the aid of low-level or no

magnification. Coalesced lesions covered large areas on leaves, leaves became necrotic, and blighted leaves remained attached to plants. Symptoms were similar in all fields and for all cultivars. We refer to the disease as Bipolaris leaf spot, but growers commonly call it hemp leaf spot. Characteristic conidiophores and macroconidia were readily isolated from both dark- and light-colored lesions. Conidiophores were dark brown, (139.6–) 192.6–365.3 (–413.2) × (6.2–) 7.1–10.7 (–11.0) µm with 4–6 septa (n = 100). Conidiophores typically bore a single macroconidium, but occasionally two conidia developed on a single conidiophore (Figure 2.3). Conidiophores arose from leaf tissue singularly, but occasionally in pairs. Macroconidia were hyaline, cylindrical with rounded ends, measured (105.6–) 204.2–364.8 (–411.5) × (18.8–) 20.9–27.9 (–32.2) μm (n = 100) and contained a slightly protuberant hilum. Macroconidia were distoseptate with 3–6 septa and produced multiple germ tubes from both basal and apical ends and occasionally from an intercalary cell. Microconidia were hyaline to pale brown, formed in chains, contained 1–3 septa, and measured (9.0–) 12.9–28.2 (–32.8) × (4.0–) 4.5–6.0 (–6.5) µm (n = 100). Morphological characteristics, as well as culture descriptions, were consistent with the description of *Drechslera gigantea* (Drechsler 1923, 1928; Heald and Wolf 1911; Isakeit et al. 2017; Kardin et al. 1982).

To demonstrate pathogenicity, macroconidia were single-spore isolated and maintained on quarter-strength potato dextrose agar. Two different methods of inoculation were performed. Agar plugs from the edges of cultures were placed onto 6-wk-old greenhouse-grown hemp plants, and plugs were removed after 18 h. Drops (10 mm) of 104 conidia/mL suspensions were placed onto top sides of leaves, and plants were covered for 24 h with plastic bags. Plants were maintained under greenhouse conditions, and symptoms developed after 5 d. No symptoms were observed on uninoculated controls after 14 d.

Sequences from 28S and internal transcribed spacer (ITS) rDNA segments, *RPB2* (for RNA polymerase II subunit 2), and *TEF1* (for translation elongation factor 1-α) were used for molecular identification. A GenBank BLAST search for ITS resulted in 100% identity of all six hemp isolates to a *Bipolaris* species identified as *Drechslera gigantea* (GenBank AY004774.1, Zhang and Berbee 2001) and a 99% match to a published sequence from an isolate identified as *D. gigantea* (GenBank KY784633.1). All three 28S (domains D1 to D3)

sequences had 100% identity to a presumptive *D. gigantea* sequence (GenBank MH873929.1). Sequences from *RPB2* and *TEF1* failed to match GenBank accessions with >90% similarity.

All phylogenies (Figure 2.4) strongly grouped the pathogen as a species within the genus *Bipolaris*, as previously indicated by Zang and Berbee (2001). These results suggested that *D. gigantea* may be more accurately described as *Bipolaris gigantea*, though it is not the intention of this brief to reclassify the fungus. All sequences were uploaded to GenBank (Table 2.1).

Further field surveys and diagnostic laboratory samples were conducted between 2015 and 2019, and disease was confirmed on field hemp in at least 18 counties in Kentucky. Field surveys in Kentucky also indicated that grass and weed hosts near and within infected hemp fields were infected by *B. gigantea*, including *Acalypha ostryifolia* (hophornbeam copperleaf), *Eleusine indica* (Indian goosegrass), and *Microstegium vimineum* (Japanese stiltgrass) (Table 2.1, Figure 2.5). All isolates were identical to the original three isolates in morphological characteristics and DNA sequences.

Bipolaris leaf spot emerged in 2014 with the reintroduction of hemp in the United States and has become widespread. Disease can become severe and cause complete yield losses. In the past 3 y, hemp acreage in the United States increased by approximately 30-fold, and investment in processing and manufacturing has increased as well. Furthermore, the southeastern United States has become a major producer of hemp, with almost 100,000 acres (40,000 ha) planted in 2019 (data compiled from hemp crop reports at https://www.votehemp.com/u-s-hemp-crop-report/). Diseases of *C. sativa* are mostly unknown. Therefore, documentation of this and other diseases is critical for the emerging hemp industry.

Table 2.1 *Bipolaris gigantea* isolated from hemp leaves affected by Bipolaris leaf spot in Kentucky: isolate number, year of collection, Kentucky county origin, host, and GenBank accessions for 28S, ITS, RPB2, and *TEF1*.

Isolate information				**Amplified region (accessions)**			
Isolate	**Year**	**County**	**Host**	**28S**	**ITS**	***RPB2***	***TEF1***
15JK003	2015	Jackson	*Cannabis sativa*	MK947378	MK967573	MN868594	MN868608
17MA018	2017	Marion	*Acalypha ostryifolia*	MN915074	MN915068	MN868596	MN868610
18MA002	2018	Marion	*C. sativa*	MN915075	MN915069	MN868598	MN868612
18NL004	2018	Nelson	*C. sativa*	MK947385	MK967580	MN868601	MN868615
19FY001	2019	Fayette	*Microstegium vimineum*	MN915077	MN915071	MN868603	MN868617
19MA014	2014	Marion	*Eleusine indica*	MN915078	MN915072	MN868606	MN868620

Figure 2.1 Bipolaris leaf spot symptoms on field-grown *C. sativa*. A, B. Plants exhibiting severe spotting, leaf distortion, and necrosis.

Figure 2.2 Bipolaris leaf spot symptoms on leaves of field-grown *C. sativa*. A. Varied lesions caused by *B. gigantea*. B. Close-up with visible conidiophores in centers of spots. No magnification.

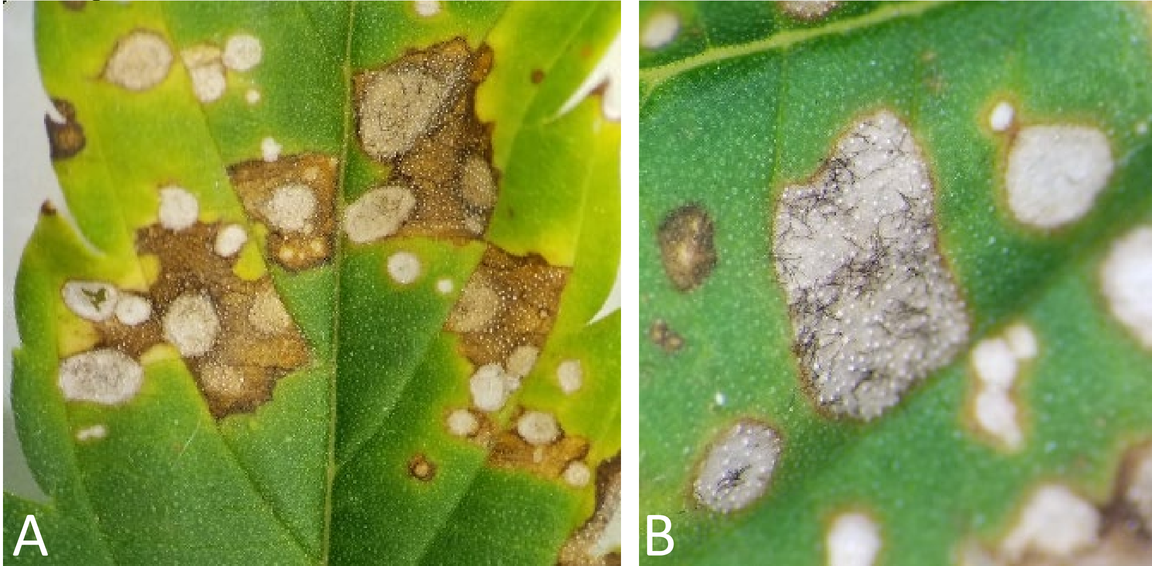


Figure 2.3 *Bipolaris gigantea* structures isolated from leaf spot on *C. sativa*. A. Germinating macroconidia. B. Ungerminated macroconidia. C. Macroconidia (left) and conidiophore (right) with contrasting size and color. Bars: 20 µm.

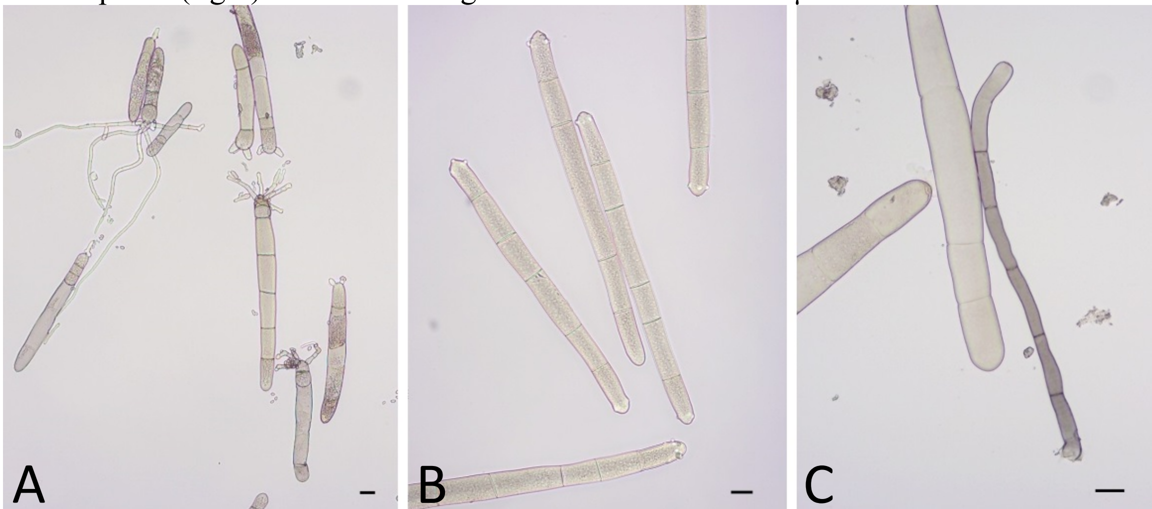


Figure 2.4 Maximum likelihood trees inferred using sequences of *B. gigantea* isolated from leaf spots of *C. sativa* in Kentucky. Phylogeny inferred from *TEF1*, *RPB2*, ITS and 28S. For each tree, all six Kentucky isolates (blue font, includes accession number and isolate number) were identical and grouped into a single clade.

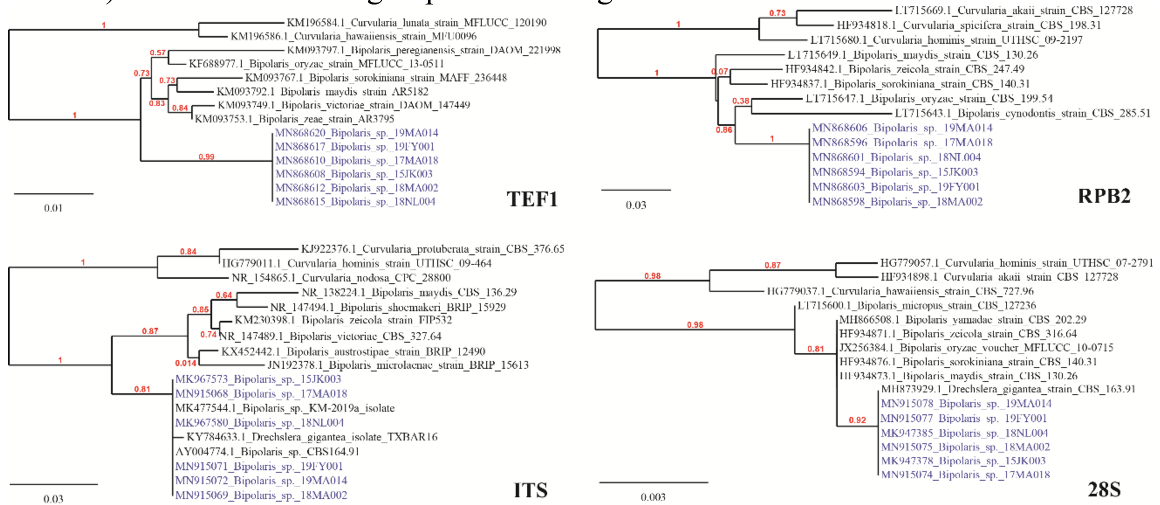
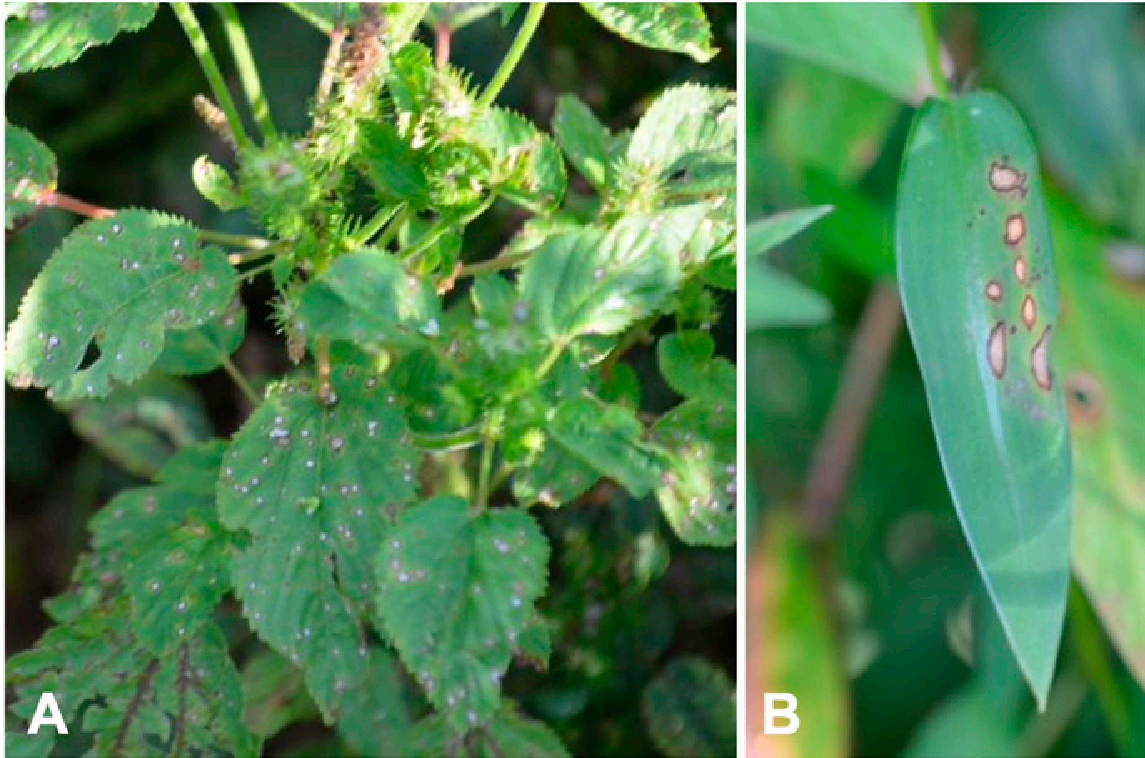


Figure 2.5 Leaf spot symptoms on alternative hosts caused by *B. gigantea*. A. Symptoms on *Acalypha ostryifolia* (hophornbeam copperleaf). B. Symptoms on *Microstegium vimineum* (Japanese stiltgrass).



CHAPTER 3. SEEING DOUBLE WITH *CANNABIS*: HETEROPLOID POPULATIONS IN *BIPOLARIS GIGANTEA*, CAUSAL AGENT OF BIPOLARIS LEAF SPOT

3.1 Abstract

Bipolaris gigantea (= *Drechslera gigantea*) was identified as the causal agent of Bipolaris leaf spot (BLS), a devastating new disease on *Cannabis sativa* that emerged with the reintroduction of widespread hemp production in the United States in 2014. The disease has been confirmed in 20 counties throughout Kentucky, and from 15 states in the eastern and midwestern United States. Isolates from BLS lesions in eight Kentucky counties were similar in morphology and growth characteristics, except that some produced structures resembling microsclerotia or protoperithecia. However, whole genome assembly sizes and sequence analysis of *RPB2* (RNA polymerase II subunit 2) genes, *TEF1* (translation elongation factor 1-α), and *MAT1* (mating type) idiomorphs indicated that some isolates were haploid whereas others were "heteroploid" in that they apparently contained two genomes; two alleles each of *RPB2* and *TEF1*, and both *MAT1* idiomorphs. Haploids (n = 13) all had identical *RPB2, TEF1* and (if present) *MAT1-2* alleles. Those alleles were also present in each heteroploid (n = 11) along with either of two related but distinct alleles for each gene. In contrast, haploids and heteroploids shared allelic variation of *MAT1-1*. In total, three haploid and two heteroploid genotypes were identified. The haploids and heteroploids were approximately equally common in the field and caused similar disease on hemp, but the heteroploids rapidly lost the ability to grow in subcultures. The possible implications of the genomic diversity of BLS for effective disease management strategies is discussed.

3.2 Introduction

In 2014, a widespread disease characterized by leaf lesions on hemp (*Cannabis sativa*) was first reported in Kentucky, coinciding with the reintroduction of industrial hemp production. The causal agent of this "Bipolaris leaf spot" (BLS) was identified as *Bipolaris gigantea* (Szarka et al. 2020), a minor pathogen of over 60 known host grasses (Poaceae) (Drechsler 1923; 1928; 1929). The fungus was originally described as *Helminthosporium giganteum*, and later reclassified as *Drechslera gigantea* (Heald and

Wolf 1911; Ito 1930) Based on molecular sequence analysis as well as reassessment of morphological relationships, the species has now been reclassified as *Bipolaris gigantea* (Lane et al. 2020). Since its description, the pathogen appears to be of little economic interest. Most recently in the United States, *B. gigantea* was reported on barley (Isakeit et al. 2017), and as a pathogen of the invasive grass *Microstegium vimineum,* and native grasses *Leersia virginica* and *Eragrostis* sp., where it is has been referred to as "*Bipolaris* taxon *megaspore*" (Stricker et al. 2016). Since *B. gigantea* was first observed as the causal agent of BLS, it has been reported in a total of 15 states.

To date, the genomic composition of *B. gigantea* has not been investigated, and limited phylogenetic analyses have been conducted on isolates from grasses (Lane et al. 2020; Stricker et al. 2016). The question, whether this species is genetically typical of other fungi in its genus or higher taxonomic levels, has not been addressed. In *Bipolaris* (teleomorph *Cochliobolus*), sexual development is controlled by a single mating type locus with two idiomorphs, *MAT1-1* and *MAT1-2* (Yoder et al. 1986). Members are predominantly heterothallic, requiring two strains containing opposite idiomorphs for successful reproduction. In homothallic species, the idiomorphs may be separate or fused together in a single open reading frame (Yun et al. 2000). Despite the presence of both mating types in a population, many *Bipolaris* spp. are not known to undergo sexual reproduction. Ascospores are produced in perithecia, but nonviable protoperithecia have been observed (Shoemaker 1955).

The sexual life cycles of Ascomycota and Basidiomycota both exhibit dikaryophases but largely differ in the duration and timing of the dikaryophases. Whereas most Basidiomycota grow primarily as dikaryons, members of Ascomycota subphylum Pezizomycotina propagate predominantly as haploids, and their dikaryophase is relatively brief and restricted to ascogenous (sexual reproductive) hyphae. Typically, in both groups the diplophase is transient because karyogamy leads directly to meiosis. However, exceptions have been identified among several Pezizomycotina. *Verticillium longisporum* is a naturally occurring stable diploid resulting from multiple preceding hybridization events (Inderbitzin et al. 2011; Ingram 1968). Similarly, persistent diploidy within the *Metarhizium* species complex is thought to have arisen from past failed mating events

(Kepler et al. 2016). Also notable are many *Epichloë* species that are asexual allopolyploids (diploids or triploids) each resulting from the fusion of a nucleus from one ancestor with a haploid nucleus of another ancestor, probably during vegetative growth (Kuldau et al. 1999; Moon et al. 2004; Schardl et al. 1994). Deviations from the haploid state can be detected by the presence of multiple alleles at a single locus, increased genome size, and occurrence of irregular mating type combinations. Variation of ploidy within Ascomycota is perhaps more diverse than generally appreciated, and additional exceptions are likely to be identified with the increased use of advanced molecular techniques.

In this study we investigate the phylogenetic and genomic variation of *B. gigantea* from infected hemp and other diseased plant species, finding that approximately half of the isolates were typical haploids with a single genome, but the others possessed two genomes ("heteroploids").

3.3 Methods and materials

3.3.1 Sampling and isolation

Leaves of hemp and other plants with lesions characteristic of BLS were collected from 11 counties in Kentucky between 2015 and 2019. Leaves were stored in the refrigerator (4 C) in individual plastic zipper bags for up to 2 d until fungal isolation. Whole leaves were surface disinfested with 10% bleach and then incubated in a moist chamber (clear plastic box lined with moist paper towels, 98% relative humidity) at room temperature (23–25 C) and ambient room light to induce sporulation. Single macroconidia were visualized with the aid of a dissecting microscope and isolated with a sterilized\ needle. Single-spored cultures were maintained on quarter-strength potato dextrose agar (1/4 PDA: 14 g/L Difco™ Agar Bacteriological, 10 g/L Difco™ Potato Dextrose Agar) on the benchtop under ambient light. Isolates 15JK003, 17CL005, and 17MA004 were deposited in multiple herbaria (Table 3.1).

Table 3.1 Voucher information for three *B. gigantea* isolates included in this study.

Isolate	Herbarium and voucher accessions			
	Cornell Plant Pathology Herbarium, Ithaca, New York, USA	Canadian Collection of Fungal Cultures, Ottawa, Ontario, Canada	U.S. National Fungus Collections Specimen Database, Beltsville, Maryland	University of Kentucky Herbarium, Lexington, Kentucky
15JK003	CUP-70688	DAOMC-252095	BPI 911225	Accession 50202
17CL005	CUP-70686	DAOMC- 252093	BPI 911223	Accession 50201
17MA004	CUP-70687	DAOMC-252093	BPI 911224	Accession 50204

3.3.2 Pathogenicity tests

To confirm pathogenicity, a combination of whole plants and detached leaves were used. Host plants included seed-grown cultivars 'Cherry Cherry' and a proprietary line. Leaves were inoculated with 5-mm mycelial plugs taken from the edges of 14-d-old colonies. In the greenhouse, plugs were placed onto fully developed leaves of 6-wk-old plants. Plugs were removed after 2 d, and inoculated plants were maintained under greenhouse conditions until symptoms developed (2–8 d). Detached leaves were prepared in 200-mm Petri dishes with petioles inserted into water agar. Plugs were placed onto adaxial sides of leaves and left in place until symptoms developed (2–8 d). Detached leaf assays were maintained on the benchtop at 23 C in ambient light. Water agar plugs were used as controls and were maintained for 21 d.

3.3.3 Morphology

Morphological characteristics of eight isolates (Table 3.2; isolates 15JK003, 17CL005, 17LC008, 17MA004, 17RW001, 18BB004, and 18NL003) were compared using structures produced on inoculated hemp leaves. These isolates were selected from a range of geographic regions across Kentucky. Leaves from 3–5-wk-old hemp plants (proprietary cultivars) were collected from greenhouse-grown plants, surfaces were disinfected with 10% bleach for 30 s, and petioles were inserted into water agar (15 g/L agar slanted in 200-mm Petri dishes). Leaves were inoculated with 5-mm-diam mycelial

plugs taken from the edges of 7 d old colonies that were grown on PDA. Plugs were left in place for 2–8 d until symptoms were apparent, infection time varied between isolates. Leaves were pressed to dry at room temperature (23–25 C) for 1 wk and stored in paper envelopes until use. Leaves were rehydrated to induce sporulation by incubating for 2–5 d in moist chambers as described above and maintained under 12 h UV light and 12 h dark. Newly emerging conidia and conidiophores were examined using a DM1000 compound microscope (Leica Microsystems, Wetzlar, Germany), captured using Moticam 5.0 MP (Motic, Hong Kong), and measured using Motic Images plus 2.0 software (Motic Microscopy, Hong Kong).

Table 3.2 Sequence accessions for *B. gigantea* isolates used in this study.

				GenBank accession numbers					
Isolates	**County of origin (KY)**	**Year collected**	**Host**	**ITS**	***TEF1***	**28S**	***RPB2***	***MAT1-2***	***MAT1-1***
15JK003	Jackson	2015	*Cannabis sativa*	MK967573	MN868608	MK947378	MN868594	MT340235	–[a]
17CL005	Clark	2017	*C. sativa*	MK967574	MT318947 MT318948	MK947379	MT270155 MT270156	MT340236	MT318998
17CL014	Clark	2017	*Acalypha digyneia*	MN915067	MT318949 MT318950	MN915073	MT270157 MT270158	MT340237	MT318999
17LC003	Lincoln	2017	*C. sativa*	nd[b]	MT747176 MT747177	nd	MT747172 MT747173	MT340238	MT319000
17LC008	Lincoln	2017	*C. sativa*	MK967575	MT747178 MT747179	MK947380	MT747174 MK967580	MT340239	MT319001
17MA004	Marion	2017	*C. sativa*	MK967576	MN868609	MK947381	MN868595	MT340240	–
17MA018	Marion	2017	*A. ostryifolia*	MN915068	MN868610	MN915074	MN868596	MT340241	–
17RW001	Rowan	2017	*C. sativa*	MK967577	MT318951 MT318952	MK947382	MT270159 MT270160	MT340242	MT319002
17RW002	Rowan	2017	*C. sativa*	nd	MT318953 MT318954	nd	MT270161 MT270162	MT340243	MT319003
18BB004	Bourbon	2018	*C. sativa*	MK967578	MT318955 MT318956	MK947383	MT270163 MT270164	MT340244	MT319004
18CL011	Clark	2018	*C. sativa*	nd	nd	nd	nd	MT340245	MT319005
18FY001	Fayette	2018	*C. sativa*	MK967579	MT318957 MT318958	MK947384	MT270165 MT270166	MT340246	MT319006
18JK005	Jackson	2018	*C. sativa*	nd	MN868611	nd	MN868597	–	MT319008
18MA002	Marion	2018	*C. sativa*	MN915069	MN868612	MN915075	MN868598	MT340248	–
18MA031	Marion	2018	*C. sativa*	nd	MN868613		MN868599	MT340250	–
18NL001	Nelson	2018	*C. sativa*	nd	MN868614	nd	MN868600	–	MT319009
18NL004	Nelson	2018	*C. sativa*	MK967580	MN868615	MK947385	MN868601	–	MT319010
18NL007	Nelson	2018	*C. sativa*	nd	MN868616	nd	MN868602	–	MT319011
19AD003	Adair	2019	*C. sativa*	nd	MT340233 MT340234	nd	MT340230 MT340231	MT340252	MT319013
19FR009	Franklin	2019	*C. sativa*	MN915070	MT318959	MN915076	MT340232	–	MT319014
19FY001	Fayette	2019	*Microstegium vimineum*	MN915071	MN868617	MN915077	MN868603	MT340253	–
19HS002	Harrison	2019	*C. sativa*	nd	MT318960 MT318961	nd	MT270167 MT270168	MT340254	MT319015
19MA014	Marion	2019	*Eleusine indica*	MN915072	MN868620	MN915078	MN868606	–	MT319017
19MA018	Marion	2019	*C. sativa*	nd	MN868621	nd	MN868607	–	MT319018

[a]Not determined.
[b]No sequence present.

3.3.4 Nuclear condition

Macroconidia, microconidia, and mycelia were stained with DAPI (4'-6-diamidino-2-phenylindole, Sigma, USA). Isolate 17MA004 was examined as a representative of haploid isolates while 17CL005 was examined as a representative of heteroploid isolates. DAPI stock solution was made by heating 1 mg DAPI into 1 mL MilliQ water. Spores were stained using 0.7 µL/mL DAPI TBS working solution. TBS buffer consisted of 200 mM NaCl and 10 mM Tris-HCl buffer pH 7.5.

3.3.5 DNA extraction

Isolates were grown on cellophane overlaid on PDA at room temperature for 3 d under ambient light. DNA was extracted from 50–100 mg of mycelia using Quick-DNA Fungal/Bacterial miniprep kit (Zymo Research, Irvine, California) according to the manufacturer's protocol, except nuclease free water was used in place of the supplied elution buffer.

3.3.6 PCR amplification and sequencing

PCR amplification was preformed using Bio-Rad T100th Thermal Cycler in 25 µL volume reactions. Each reaction contained 1 µL of DNA template (10 ng/µL), and final concentrations of 0.1 µM of each primer (Table 3.3), 0.1 µM dNTP mix (Applied Biosystems™ GeneAmp™ dNTP Blend, Thermo Fisher Scientific, Waltham, Massachusetts), 2.5 µL 1× Titanium Taq PCR buffer (TaKaRa Bio USA, Inc, Mountain View, California, USA), 0.25 µL 0.5× Titanium Taq DNA Polymerase (TaKaRa Bio USA).

Regions amplified by PCR included ITS (internal transcribed spacer), 28S (D1–D2 domains of the 28S rDNA), and portions of *RPB2* (RNA polymerase II subunit 2) and *TEF1* (translation elongation factor 1-α) genes. The following thermal cycling program was used for the amplification of ITS and 28S, using primers ITS1/ITS4 (Glass and Donaldson 1995) and LROR/LR5 (Gruyter et al. 2009) respectively: an initial denaturation temperature at 95 C for 3 min, followed by 34 cycles of at 95 C for 30 s, 60 C for 30s, 72

C for 1 min, and a final extension at 72 C for 5 min adjusted from manufacturer's protocol (TaKaRa Bio USA). The *TEF1* region was amplified using primers EF983F/EF2218R (Gruyter et al. 2009), with the annealing temperature modified to 56 C and the extension time to 1 min 30 s. To amplify the *RPB2* regions, primer 7cR (O'donnell et al. 2007) and primer rpr2 (Table 3.3) were used respectively, with a modified annealing temperature of 65 C. Mating type was determined using the same modified PCR conditions and designed primers of MAT121F1/ MAT121R2 for *MAT1-2* and MAT111F1/ MAT111R2 for *MAT1-1* (Table 3.3). A total of 24 isolates were sequenced. The majority originated from *C. sativa* but four were isolated from alternative hosts; two from dicots and two from monocots (Table 3.2).

Table 3.3 List of targets and primers used in this study.

Target DNA	Primer	Primer sequence (5'→3')	Expected size (bp)	Reference
28S	LR0R	GTACCCGCTGAACTTAAGC	900	Gruyter et al. 2009
	LR5	ATCCTGAGGGAAACTTC		
TEF1	EF 983F	GCYCCYGGHCAYCGTGAYTTYAT	1200	Gruyter et al. 2009
	EF 2218R	ATGACACCRACRGCRACRGTYTG		
ITS	ITS1	TCCGTAGGTGAACCTGCGG	540	White et al. 1990
	ITS4	TCCTCCGCTTATTGATATGC		White et al. 1990
MAT1-1	MAT111F1	CTGCCACTCCAGAGAAAGCA	599	This study
	MAT111R2	TTGGAGGTACCTTGCGTGTC		
MAT2-1	MAT121F1	ATGGATGGCGGCAACAACTA	705	This study
	MAT121R2	ACGGATTGACTCAGCGTTGT		
RPB2	7cR	CCCATRGCTTGYTTRCCCAT	870	O'donnell et al. 2007
	Rpr2	CCCGTTGGACGTGATGGTAA		This study

Resulting PCR products were purified using ExoSap-it (USB, Cleveland, Ohio) according to the manufacturers protocol. Samples were submitted for Sanger sequencing (Eurofins Genomics, Louisville, Kentucky) with the same primers used for PCR amplification. Raw complementary sequences were aligned and edited in Geneious 6.0.6 (Biomatters Ltd., New Zealand). BLAST searches for consensus sequences for each isolate and target region were performed in NCBI against the GenBank nr database.

PCR-amplified fragments of *RPB2* and *TEF1* segments from 17LC008 were purified using ExoSap-it and cloned with pGEM-T EASY Vector System I (Promega, Madison, Wisconsin). Adjustments were made to the manufacturer's transformation instructions. Ligation reaction was diluted with 5 µL Tris-EDTA (pH 8) and 0.5 µL dilution was added to 40 µL EPI300 electrocompetent *Escherichia coli* cells in 0.5 µL

microcentrifuge tube on ice. Cells were transformed by electroporation using a Gene Pulser (Bio Rad, Hercules, California) with a 1-mm cuvette at 2 V. Cells were transferred into a 1.5 µL tube with 500 µL SOC medium (Invitrogen, Thermo Fisher Scientific) and incubated for 1 h at 37 C (200 rpm). Cells were plated onto Luria Broth (LB) plates each containing 5µL of 100 µg/mL ampicillin and 40 µL X-gal (100mg 5-bromo-4-chloro-3-indolyl-β-d-galactoside dissolved in 2 mL *N,N′*-dimethyl-formamide). Plates were incubated overnight at 37 C. Screened colonies were incubated overnight in liquid LB medium at 37 C and plasmids were extracted using Zippy Plasmid Miniprep Kit (Zymo Research). Product was submitted for sequencing and submitted to GenBank.

3.3.7 Whole genome sequencing

Genomic DNA for isolates 15JK003, 17CL005, 17CL014, 17MA018 and 17UKY004 was sequenced using Ilumina NextSeq 500 (Illumina, San Diego, California, USA) using mid-throughput configuration with paired-ends at 150 bp each. Isolates 17LC008, 17MA004, and 18NL004 were sequenced using Illumina MiSeq (Illumina, San Diego, California, USA) with paired-ends at 301 bp each. The genomes were assembled twice, using the MaSuRCA (Zimin et al. 2013) or SPAdes (Bankevich et al. 2012) genome assembler.

3.3.8 Phylogenetic analysis

Phylogenetic analysis was performed using phylogeny.fr (http://www.phylogeny.fr/) using "One Click" mode (Dereeper et al. 2008) with the default setting. Sequences were aligned using MUSCLE (Edgar, 2004) without GBlocks curation. PhyML (Anisimova, 2006) was used for building trees and TreeDyn (Chevenet, 2006) was used for rendering trees. Branch support values were set to display maximum likelihood and, estimated using the approximate likelihood ratio test (aLRT) with the SH-like option (Anisimova, 2006); all values under 50% were collapsed.

Multigene phylogenies were inferred using selected isolates from a range of geographic regions across Kentucky, and selected sequences were downloaded from GenBank during BLAST searches for ITS, 28S, *RPB2* and *TEF1* (Table 3.4). Phylogenies

for ITS and 28S were inferred using representative isolates (Table 3.2) of the counties sampled. Additional isolates (Table 3.2) were included in the phylogenetic analyses for *RPB2*, *TEF1*, and mating type genes.

Table 3.4 Accessions for sequences downloaded from GenBank for phylogenetic analysis.

		GenBank accession numbers					
Species	Isolate	28s	ITS	*RPB2*	*TEF1*	*MAT1-1*	*MAT1-2*
Bipolaris austrostipae	BRIP 12490	–	KX452442	–	–	–	–
Bipolaris cynodontis	CBS 285.51	–	–	LT715643	–	–	–
Bipolaris luttrellii	14643-1	–	–	–	–	AF129740	AF129740
Bipolaris maydis	CBS 130.26	HF934873	–	LT715649	–	–	–
B. maydis	CBS 136.29	–	NR 138224	–	–	–	–
B. maydis	AR5182	–	–	–	KM093792	–	–
B. maydis	C5	–	–	–	–	AF029913	–
B. maydis	C3	–	–	–	–	–	X68398
Bipolaris microlaenae	BRIP 15613	–	JN192378	–	–	–	–
Bipolaris micropus	CBS 127236	LT715600	–	–	–	–	–
Bipolaris oryzae	CBS 199.54	–	–	LT715647	–	–	–
B. oryzae	MFLUCC 13-0511	–	–	–	KF688977	–	–
B. oryzae	MFLUCC 10-0715	JX256384	–	–	–	–	–
Bipolaris peregianensis	DAOM 221998	–	–	–	KM093797	–	–
Bipolaris sacchari	764-1	–	–	–	–	–	X95814
Bipolaris shoemakeri	BRIP 15806	–	–	–	KX452469	–	–
B. shoemakeri	BRIP 15929	–	NR 147494	–	–	–	–
Bipolaris sorokiniana	CBS 140.31	HF934876	–	HF934837	–	–	–
B. sorokiniana	MAFF 236448	–	–	–	KM093767	–	–
B. sorokiniana	45130	–	–	–	–	–	AF275374
Bipolaris gigantea	642	–	KM507761	–	–	–	–
B. gigantea	D4-7F	–	MN902179	–	–	–	–
B. gigantea (=Bipolaris sp.)	KM-2019a	–	MK477544	–	–	–	–
B. gigantea (=Bipolaris sp.)	CBS164.91	–	AY004774	–	–	–	–
B. gigantea (=Drechslera gigantea)	CBS 163.91	MH873929	–	–	–	–	–
B. gigantea (=D. gigantea)	TXBAR16	–	KY784633	–	–	–	–
Bipolaris yamadae	M1320	–	–	–	KX774329	–	–
B. yamadae	CBS 202.29	MH866508	–	–	–	–	–
Bipolaris zeae	AR3795	–	–	–	KM093753	–	–
Bipolaris zeicola	CBS 247.49	–	–	HF934842	–	–	–
B. zeicola	CBS 316.64	HF934871	–	–	–	–	–
B. zeicola	26R13	–	–	–	–	AF032368	–
B. zeicola	FIP532	–	KM230398	–	–	–	–
Bipolaris victoriae	DAOM 147449	–	–	–	KM093749	–	–
B. victoriae	Hv033	–	–	–	–	–	AF032369
B. victoriae	CBS 327.64	–	NR 147489	–	–	–	–
Curvularia akaii	CBS 127728	HF934898	–	LT715669	–	–	–
Curvularia hawaiiensis	MFU0096	–	–	–	KM196586	–	–
C. hawaiiensis	CBS 727.96	HG779037	–	–	–	–	–
Curvularia hominis	UTHSC 09-2197	–	–	LT715680	–	–	–
C. hominis	UTHSC 09-464	–	HG779011	–	–	–	–
C. hominis	UTHSC 07-2791	HG779057	–	–	–	–	–
Curvularia homomorpha	ATCC 13409	–	–	–	–	–	AF129741
Curvularia lunata	MFLUCC 120190	–	–	–	KM196584	–	–
Curvularia nodosa	CPC 28812	–	–	–	MF490861	–	–
C. nodosa	CPC 28800	–	NR 154865	–	–	–	–
Curvularia protuberata	CBS 376.65	–	KJ922376	–	–	–	–
Curvularia spicifera	CBS 198.31	–	–	HF934818	–	–	–

3.3.9 Stability

DNA was extracted from cultures grown from single conidia isolated from inoculated leaves as previously described. Detached hemp leaves were inoculated as described above using isolates 17MA004 (*MAT1-2*), 18NL004 (*MAT1-1*), and 17CL005 and 18FY001 (heteroploids with both *MAT1-2* and *MAT1-1* idiomorphs). Sporulation was induced as described above. For each isolate, 10 single macroconidia were isolated from inoculated leaves and transferred to individual ¼ PDA plates. DNA was extracted using CTAB (Li et al. 2008) from colonies grown from each macroconidium (3-d-old spread plates [5 mL spore suspension spread on agar medium] from PDA plates overlaid with cellophane). PCR was preformed to amplify mating type sequences using mating type primers (Table 3.3), and gel electrophoresis was used to confirm mating type. The expected size for the PCR product from *MAT1-2* was 705 bp and the expected size for the product from *MAT1-1* was 599 bp.

3.3.10 Mating type experiment

Isolates representing each mating type, 17MA004 (*MAT1-2*) and 18NL004 (*MAT1-1*), were crossed in an attempt to induce the formation of protoperithecia-like structures. Isolate 17CL005 (*MAT1-1*/*MAT1-2*) has been observed to produce these structures previously and was used as a positive control. Mycelial plugs (5 mm) were taken from 1-wk-old plates and transferred to 10% PDA and Sach's media (Sinclair, 1995) plates; dry autoclaved hemp pieces were sprinkled onto the surfaces of both media. Two plugs were plated 2 cm apart from each other. Combinations were: two plugs from a single isolate or plugs from two different isolates. There were 20 plates for each combination, 10 per each media type. Plates were observed over a 3-wk period for the formation of structures. Crosses were also duplicated on hemp leaves inserted into WA slants. Plugs were removed after 1 wk and observed for 3 wk.

3.4 Results

3.4.1 Pathogenicity

Inoculated hemp plants developed symptoms within 5 d of inoculation, and characteristic macroconidia were isolated from resulting lesions. Time of symptom development varied among inoculated detached leaves, but all produced conidia within 21 d after inoculation. Controls with water agar plugs showed no symptoms after 27 d. All isolates used in this study were confirmed to infect hemp.

3.4.2 Morphology

All isolates produced morphologically similar conidiophores, macroconidia, and microconidia with no significant differences in size or shape (Table 3.5), and consistent with previous reports (Drechsler 1928; Isakeit et al. 2017; Lane et al. 2020) and the type specimen. Specifically, conidiophores were dark brown and multi-septate with a bulbus end (Figure 3.1A) and were produced on both the adaxial and abaxial sides of leaves. Conidiophores typically arose singularly but occasionally in pairs, with most producing a single macroconidium, and occasionally, two or three macroconidia developed from different attachment points on the same conidiophore (Figure 3.1B). Macroconidia were hyaline, distoseptate, and cylindrical with rounded ends and a slightly protuberant hilum (Figure 3.2A). Macroconidia frequently germinated while still attached to conidiophores. When exposed to water, macroconidia germinated within 20 min, typically producing multiple germ tubes from both the basal and apical ends and occasionally from any or multiple intercalary cells (Figure 3.2B, C). Microconidia were hyaline and formed in multiple chains, often from the tips of mature macroconidia or their conidiophores (Figure 3.3A-C). Microconidia germinated within 4–12 h to produce a single germ tube. Culture appearance varied among isolates, from a mix of white to dark brown and there were no distinct groupings (Figure 3.4).

A novel observation for this species was that only some isolates produced dark, spherical structures resembling microsclerotia or protoperithecia either immersed within leaf tissue or on the leaf surface (Figure 3.5A). These structures contained no identifiable

ascospores, and the contents resembled broken hyaline hyphae (Figure 3.5B). Isolates that produced these, and some other isolates, were less amenable to culture that others and after two or three subcultures would cease to grow.

Fungal structures from the holotype of *Helminthosporium giganteum* were examined on a sample of the type specimen acquired from the New York Botanical Garden (NY Barcode: 00946009). Type specimen was collected in Texas in 1909 and consisted of leaf lesions on blades of Bermuda grass (*Cynodon dactylon*). Whole and partial fungal structures were isolated from the sample. Conidia exhibited slightly protruding hila and septa similar to those of the isolates from hemp (Figure 3.6A, B). Conidiophores had a bulbus base and saucer shaped scars, also similar to those of the isolates (Figure 3.6C, D).

Table 3.5 Morphological characteristics of selected isolates comparing the range of length and width for macroconidia, macroconidiophores, and microconidia. These isolates represent a geographic and morphological distribution of *B. gigantea* in Kentucky at the time of this study. N > 50.

Isolate	Macroconidia (µm)	Conidiophores (µm)	Microconidia (µm)
15JK003	Length (105.6–) 204.2–364.8 (–411.5) Width (18. 8–) 20.6–30.7 (–32.1) SD_{length} ±39.4, SD_{width} ±2.2 Septa 3–6	Length (139.6–) 192.6–367.8 (–413) Width (6.2–) 7.1–10.8 (–11.8) SD_{length} ±57.3, SD_{width} ±1.0 Septa 4–8	Length (9.1–) 13.1–26.6 (–32.8) Width (4.0–) 4.5–6 (–6.5) SD_{length} ±4.7, SD_{width} ±0.6 Septa 0–3
17MA004	Length (111.7–) 167.3–402.5 (–459.1) Width (14.9–) 17.4–30.9 (–33.4) SD_{length} ±81.3, SD_{width} ±3.4 Septa 3–6	Length (156.9–) 207.5–360.1 (–481.9) Width (6.6–) 7.6–10.8 (–11.7) SD_{length} ±60.2, SD_{width} ±0.9 Septa 4–8	Length (8.08) 25.04–21.08 (–23.7) Width (5.1–) 5.81–5.1 (–5.81) SD_{length} ±4.1, SD_{width} ±0.9 Septa 0–2
17CL005	Length (110.5–) 210.8–388.7 (–442.9) Width (14.5–) 16.2–23.6 (–25.3) SD_{length} ±42.6, SD_{width} ±1.9 Septa 4–6	Length (143.9–) 204.0–424.2 (–504) Width (7.8–) 8.7–12.1 (–13.2) SD_{length} ±70.0, SD_{width} ±1.2 Septa 4–8	Length (7.8–) 10.3–20.7 (–23.7) Width (2.7–) 3.5–5.3 (–6.2) SD_{length} ±3.1, SD_{width} ±0.5 Septa 0–2
17RW001	Length (98.2–) 167.5–432.9 (–512.4) Width (13.0–) 15.3–22.9 (–25.6) SD_{length} ±87.3, SD_{width} ±2.4 Septa 3–5	Length (201.9–) 270.5–499.3 (–556.6) Width (6.6–) 7.2–8.7 (–9.2) SD_{length} ± 96.0, SD_{width} ± 0.7 Septa 5–10	Length (7.6–) 10.3–20.9 (–24.0) Width (2.7–) 3.3–5.6 (–6.3) SD_{length} ±3.6, SD_{width} ±0.5 Septa 0–2
17LC008	Length (139.2–) 183.6–385.5 (–430.0) Width (14.3–) 16.6–25.8 (–29.1) SD_{length} ±84.2, SD_{width} ±3.2 Septa 3–5	Length (185.5–) 255.4–493.3 (–609.7) Width (7.8–) 8.5–10.8 (–11.8) SD_{length} ±73.7, SD_{width} ±1.0 Septa 6–9	Length (8.0–) 11.2–25.0 (–28.4) Width (3.7–) 4.4–6.8 (–8.0) SD_{length} ±3.8, SD_{width} ±0.7 Septa 0–3
18FY001	Length (18.1–) 115.5–330.6 (–432.2) Width (15.3–) 17.5–26.3 (–29.5) SD_{length} ±76.2, SD_{width} ±2.9 Septa 3–4	Length (204.2–) 274.3–478.28 (–586.0) Width (7.1–) 8.3–11.5 (–12.1) SD_{length} ± 80.9, SD_{width} ± 0.97 Septa 6–13	Length (8.1–) 11.3–26.4 (–28.9) Width (3.6–) 4.0–5.6 (–6.1) SD_{length} ±4.3, SD_{width} ±0.5 Septa 0–1
18BB004	Length (99.1–) 135.7–309.9 (–338.6) Width (10.5–) 15.0–27.6 (–31.2) SD_{length} ±42.7, SD_{width} ±3.2 Septa 2–5	Length (108.3–) 175.8–415.8 (–478.4) Width (7.1–) 7.9–11.1 (–12.1) SD_{length} ±64.7, SD_{width} ±1.1 Septa 3–8	Length (8.9–) 12.2–26.9 (–30.3) Width (3.7–) 4.2–6.0 (–6.6) SD_{length} ±4.7, SD_{width} ±0.6 Septa 0–2
18NL004	Length (108.4–) 176.3–354.4 (–394.6) Width (15.6–) 18.1–27.1 (–30.4) SD_{length} ±62.7, SD_{width} ±2.6 Septa 3–5	Length (146.4–) 187.7–345.1 (–412.2) Width (7.5–) 8.4–8.4 (–12.0) SD_{length} 63.6±, SD_{width} ±0.8 Septa 3–7	Length (7.3–) 11.1–24.5 (–32.5) Width (3.8–) 4.3–5.7 (–6.36 SD_{length} ±4.6, SD_{width} ±0.5 Septa 0–2

Figure 3.1 Characteristics of conidiophores of *B. gigantea*. A. Conidiophore with bulbus end (arrow). B. Second conidiophore (arrow) arising from single cell. Bars: 20 μm.

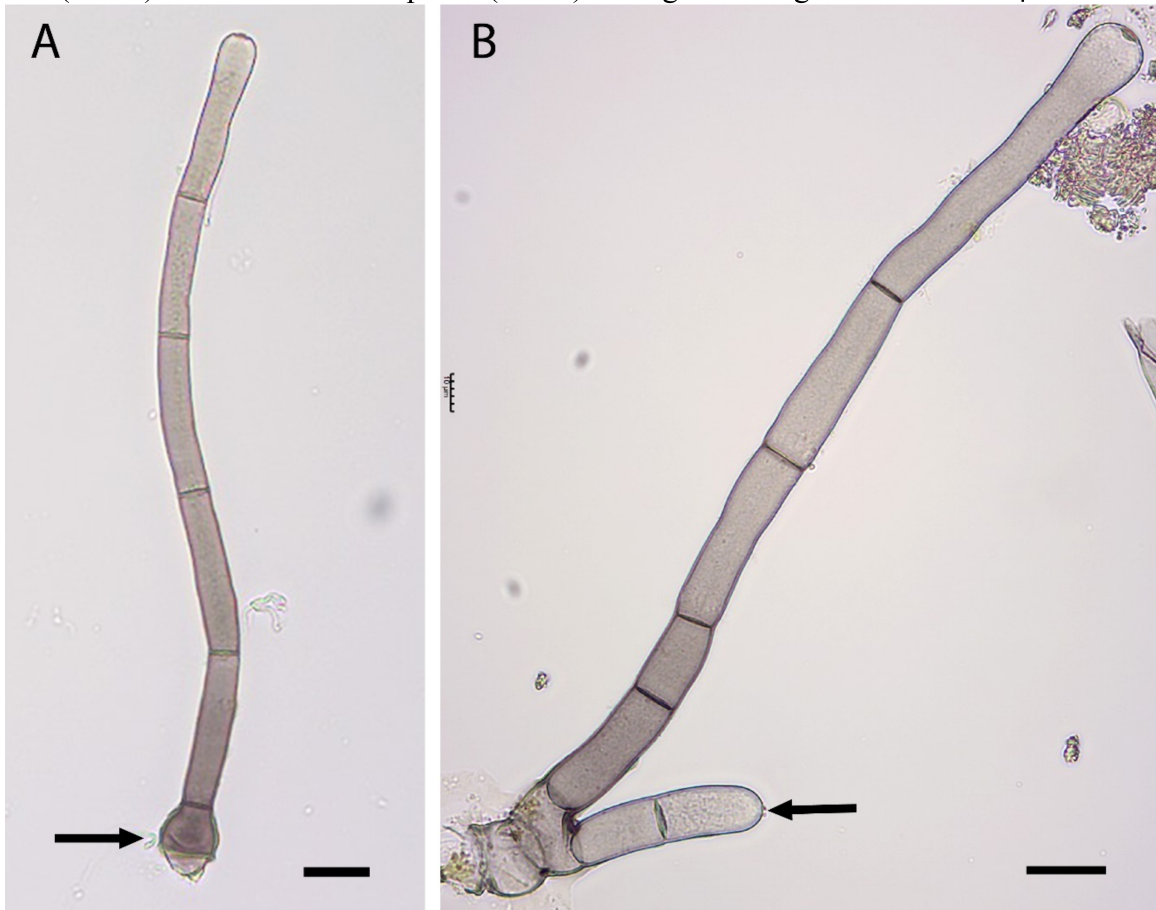


Figure 3.2 Characteristics of macroconidia for *B. gigantea*. A. Macroconidium with distosepta (black arrow) and a slightly protrudent hilum (white arrow). B. Macroconidium with bipolar germination (indicated by arrow at each end). C. Macroconidium germinating from each cell (arrows). Bars: 20 µm.

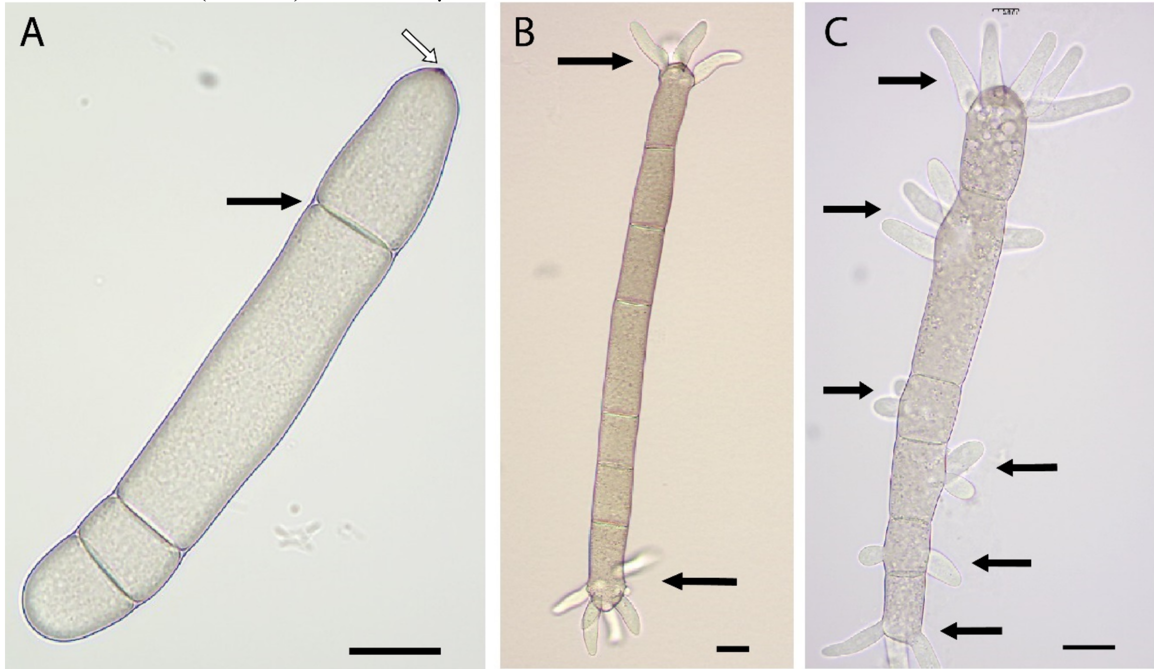


Figure 3.3 Characteristics of microconidia of *B. gigantea*. A. Microconidia developing at the tip of a macroconidium. B. Size comparisons of microconidium (arrow) to macroconidium. C. Chains of microconidia with septa and branching pattern visible. Bars: 20 μm.

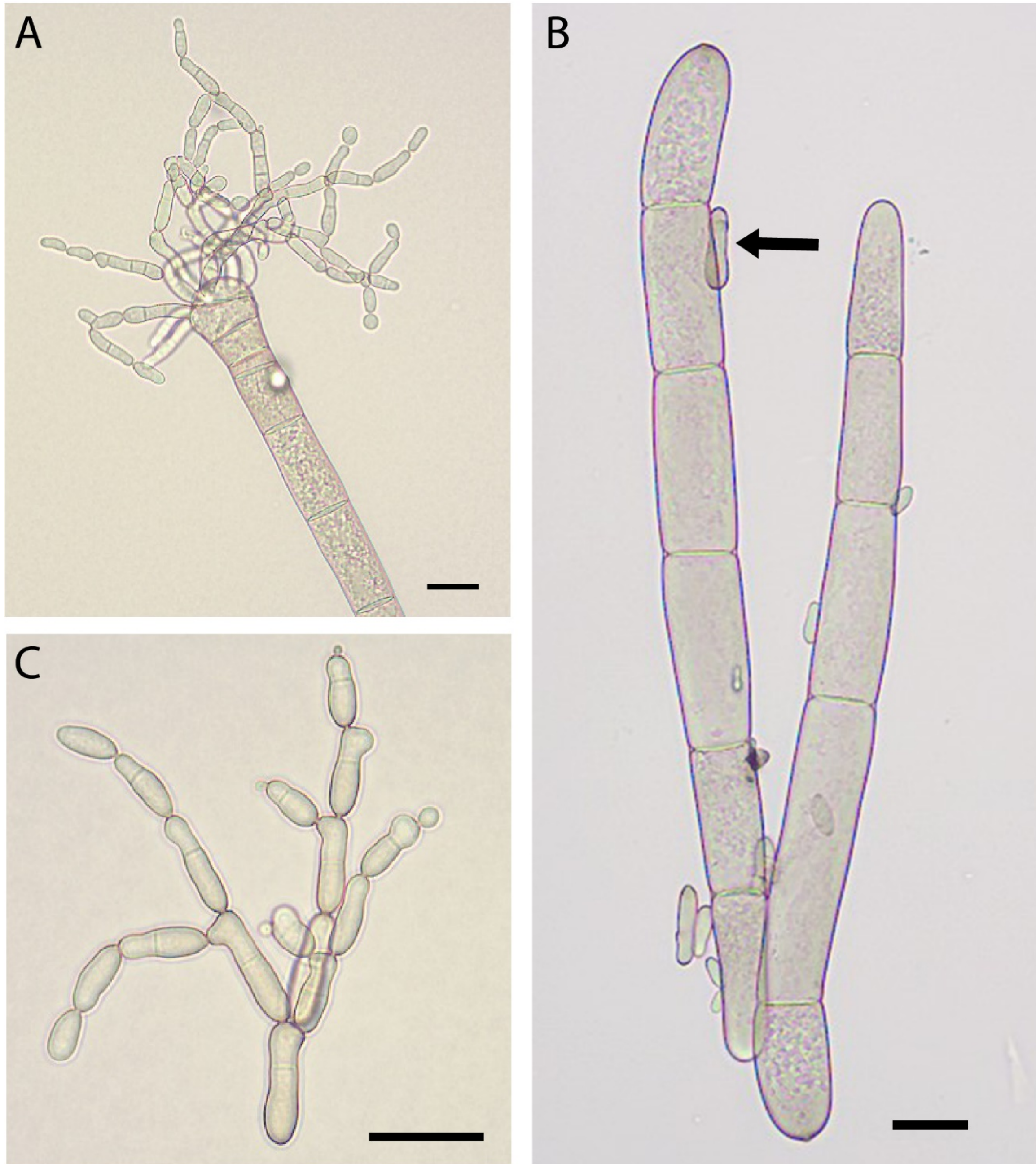


Figure 3.4 Colonies each grown from a single macroconidium from four different *B. gigantea* isolates on ¼ PDA at 14-d-old. A, B. Haploid isolates 17MA004 and 18NL004. C, D. Heteroploid isolate 17CL005 and 18FY001.

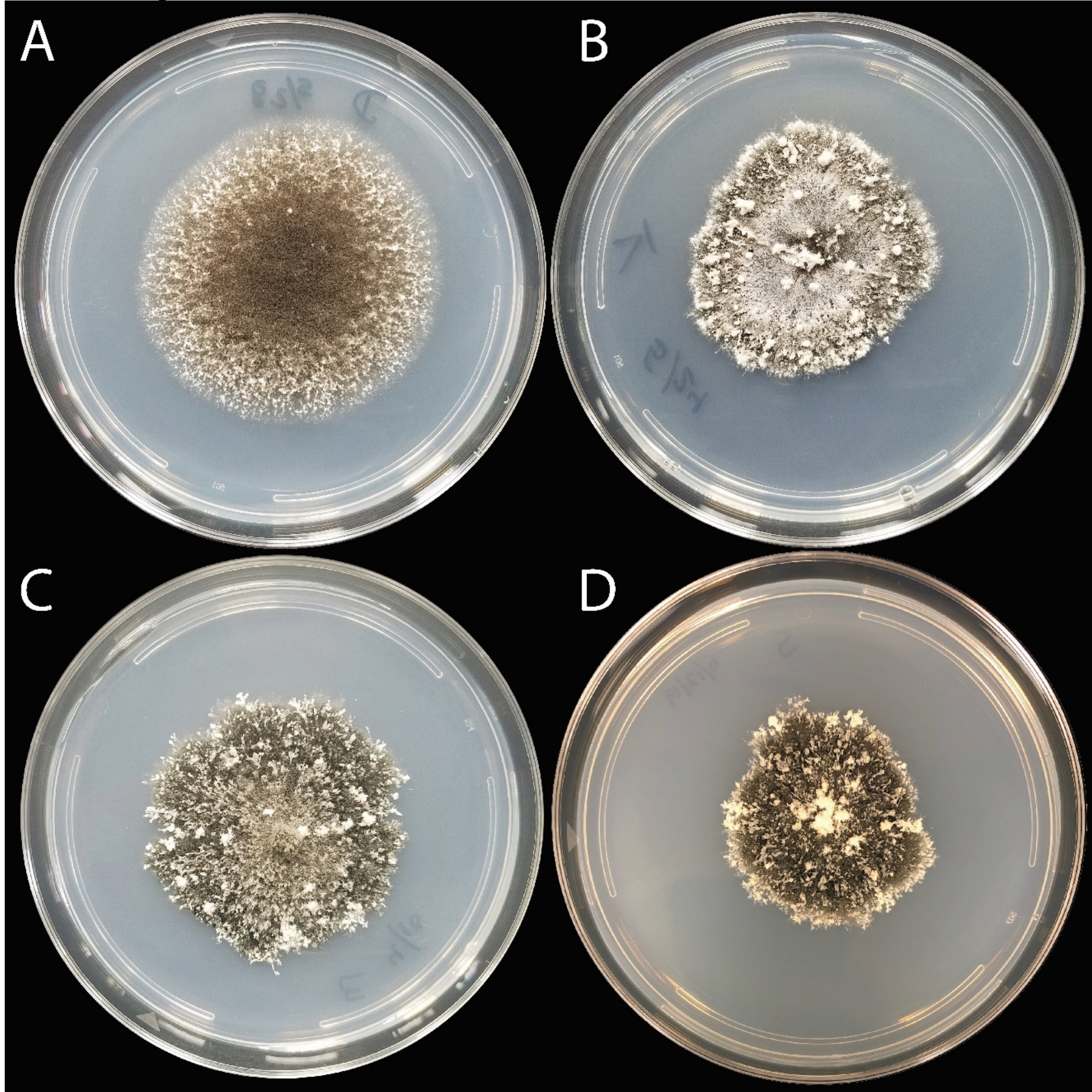


Figure 3.5 Structures produced by some *B. gigantea* isolates. A. Structures forms on leaf tissue. B. Inner contents of a structure contained no ascospores.

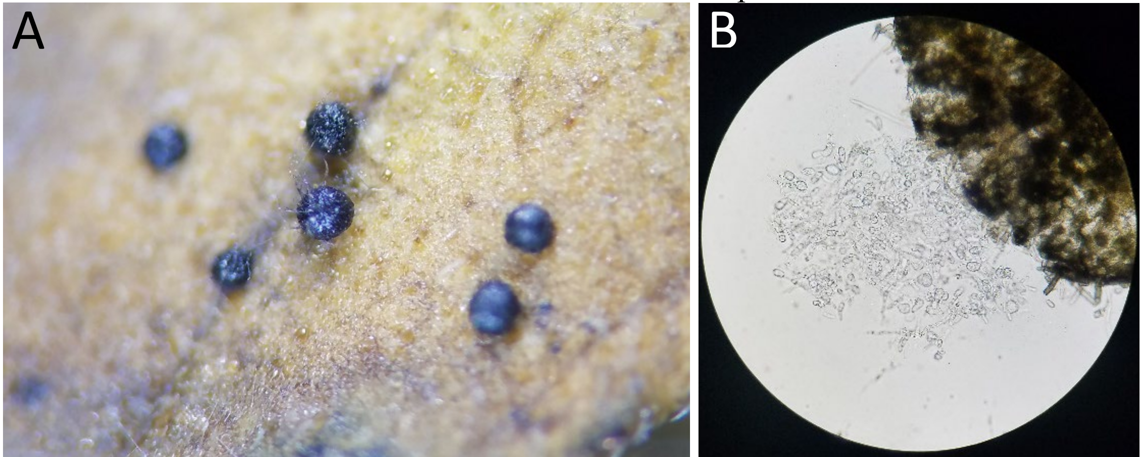
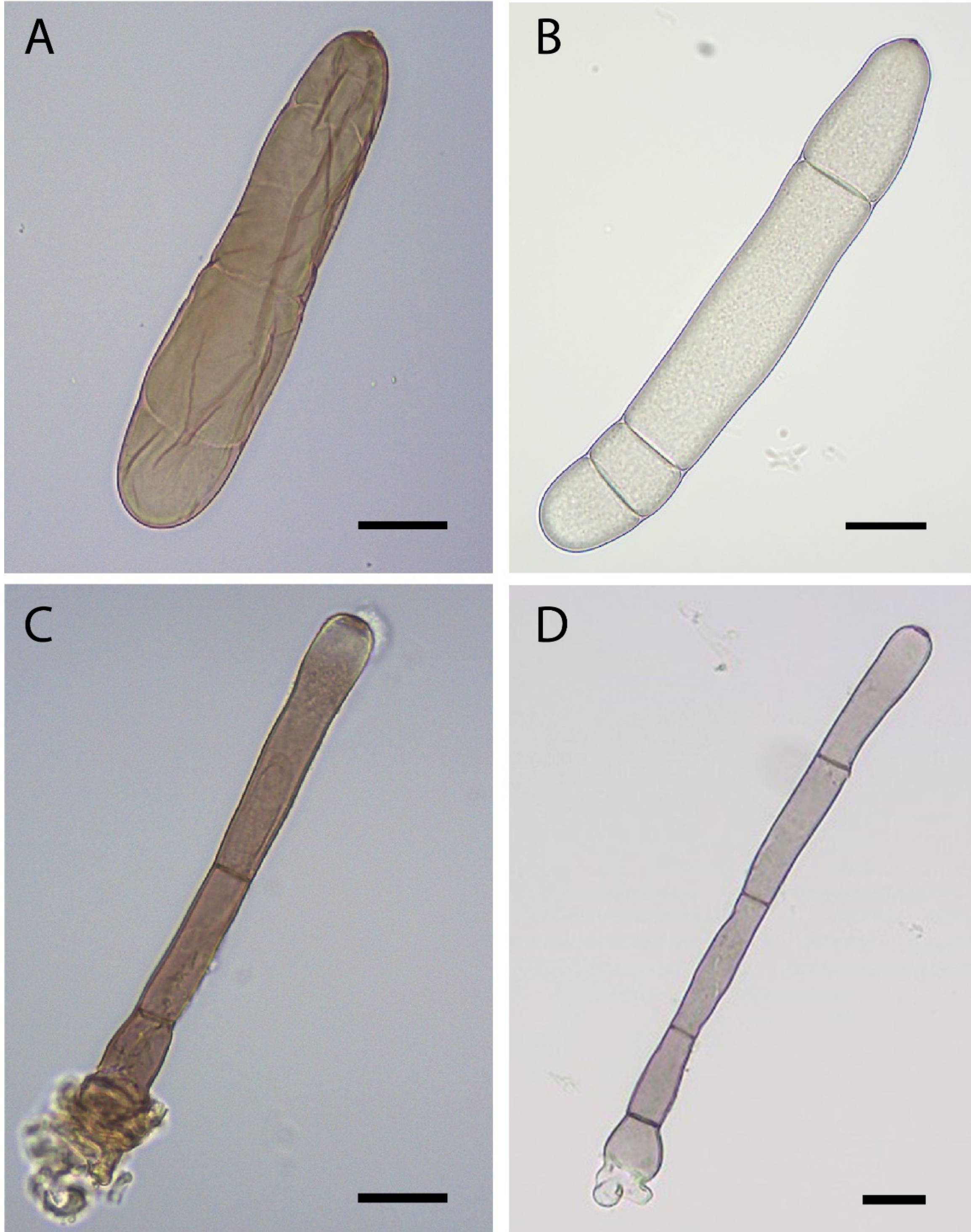


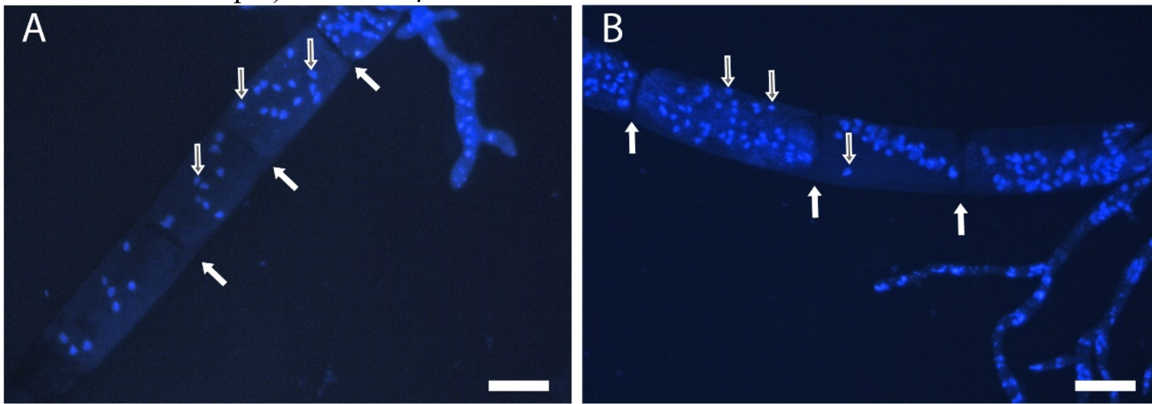
Figure 3.6 Type culture. A. Macroconidium from type specimen of *Helminthosporium giganteum* (= *B. gigantea*). B. Macroconidium from hemp isolate C. Conidiophore from type specimen D. Conidiophore from hemp isolate. Bars: 20 µm.



3.4.3 Nuclear condition

Macroconidia, microconidia, and mycelia for isolate 17MA004 and isolate 17CL005 showed multiple nuclei per cell (Figure 3.7) when stained with DAPI. Nuclei appeared to demonstrate a strong tendency to aggregate in cells thus relative sizes or staining intensities could not be measured to indicate if all heteroploids were heterokaryotic or diploid.

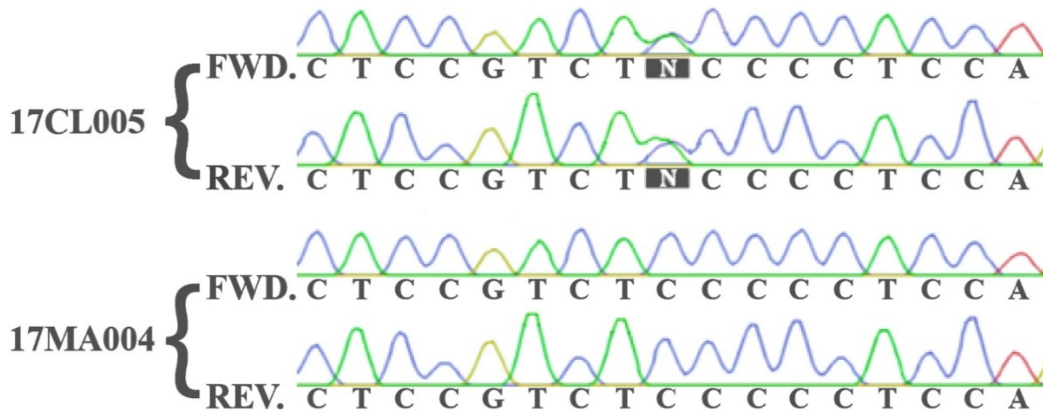
Figure 3.7 *B. gigantea* macroconidia stained with DAPI. A. Heteroploid isolate 17CL005 macroconidium with multiple nuclei (gray arrows) per cell (white arrows indicate septa). B. Haploid 17MA004 macroconidium with multiple nuclei (gray arrows) per cell (white arrows indicate septa). Bars: 20 µm.



3.4.4 Sequences

Single gene/region sequences for ITS, 28S, *RPB2*, and *TEF1* were determined from PCR products and submitted to GenBank (Table 3.2). Alignment of complementary sequences of some isolates for *RPB2* and *TEF1* showed otherwise clean traces with conflicting peaks in both directions (Figure 3.8). Such polymorphisms were observed at several positions within the sequences of both genes and were indistinguishable between two possible bases. Of the isolates sequenced, 11 contained these nucleotide variations and were therefore inferred to possess two alleles each of *RPB2* and *TEF1*. In the remaining 13 isolates there was no apparent allelic variation for these genes.

Figure 3.8 Excerpt of aligned sequences of an isolate (17CL005) containing polymorphisms compared to an isolate (17MA004) that has no polymorphisms.



Whole genome sequencing resulted in assemblies for eight isolates (Table 3.6) and were submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The genome assemblies revealed two sets of alleles for four isolates (17CL005, 17CL014, 17LC008, 17UKY004) and a single allele for four isolates (15JK003, 17MA004, 17MA018, 18NL004) for both *RPB2* and *TEF1*. Each of the former isolates contained both *MAT1-1* and *MAT1-2*, whereas each of the latter isolates contained one mating type idiomorph, either *MAT1-1* or *MAT1-2*. Alignment of sequences for both *RPB2* and *TEF1* from the whole genomes showed that all eight isolates shared an identical allele for each gene, which was designated allele-a. The remaining allele in 17CL005 and 17UKY004 was designated allele-b. Isolate 17LC008 had allele-a and another allele designated allele-c. Genome assembly results indicated that four isolates (15JK003, 17MA004, 17MA018, 18NL004) had genomes averaging approximately 30 Mb and the remaining four isolates had genome assemblies, up to nearly double that value depending on the assembler software used (Table 3.5).

RPB2 and *TEF1* sequences extracted from the genomes of isolates 17CL005 and 17LC008 were used to separate the PCR-product sequences with conflicting base calls into three sequences representing different alleles within the isolates. Isolates with PCR-product sequences identical to those extracted from the genomes maintained the same designation for each allele identified, allele-a, allele-b or allele-c. Isolates inferred to be

haploids contained only allele-a, whereas those inferred to be heteroploids contained either allele-a and allele-b or allele-a and allele-c.

Table 3.6 Whole genome sequence and assembly data.

SPAdes assembly					
Isolate	Number of contigs	Largest contig	Total length	N50	Number of reads
15JK003	680	773,279	30,059,364	127,381	33,073,486
17CL005	18,954	147,007	49,025,550	5,062	33,408,032
17CL014	16,819	158,365	48456,778	6,346	31,016,376
17MA018	1,006	286,181	29,692,755	69,302	32,556,082
17UKY004	16,499	206,426	50,223,234	6,943	37,345,442
MaSuRCA assembly					
15JK003	1,308	468,354	30,993,781	120,514	33,073,486
17CL005	4,708	144,384	41,349,018	5,062	33,408,032
17CL014	4,183	195,956	41,286,854	14,270	31,016,376
17LC008	2,268	117,522	31,525,264	26,161	34,693,374
17MA018	1,381	234,794	30,077,013	58,044	32,556,082
17MA004	2,268	117,522	31,509,268	26,161	34,333,578
17UKY004	7,786	200,618	52,853,584	13,548	37,345,442
18NL004	1,789	168,126	31,596,652	32,877	28,442,090
Velvet assembly					
15JK003	1,219	311,952	29,793,655	53,299	33,073,486
17MA004	25,379	97,855	24,280,052	906	34,333,578

3.4.5 Phylogenetic analysis

ITS and 28S sequences were identical or nearly identical for all isolates from which they were determined, and in each case the sequences grouped into a single distinct clade within the genus *Bipolaris* (Figure 3.9). Phylogenetic analysis of ITS grouped sequences with four GenBank accessions KM507761.1, MN902179, MK477544.1 and AY004774.1 annotated as *Bipolaris* sp., and accession KY784633.1 (Isakeit et al. 2017) annotated as being from *D. gigantea* (Figure 3.9A). Accessions KM507761 and MN902179.1 were determined to be *D. gigantea* and concluded to belong to *Bipolaris* (Lane et al. 2020). Attempts to amplify the ITS region from the type specimen using multiple methods and primer sets were unsuccessful. The 28S sequences were all identical except for that of isolate 17MA004 which had a single nucleotide difference from the rest (Figure 3.9B). Included within this clade was sequence accession MH873929.1 annotated as *Drechslera*

gigantea. Phylogenetic analyses for each of *RPB2* and *TEF1* grouped the three alleles from the *B. gigantea* isolates into three related clades with high aLRT support (Figure 3.10A, B).

Figure 3.9 Maximum likelihood trees inferred independently using partial gene data sets for ITS and 28S indicated our isolates group together as a distinct clade within *Bipolaris*. A. Phylogeny inferred from ITS. All our isolates are identical and with several confirmed or suspected *D. gigantea* isolates. B. Phylogeny inferred from 28S. All our isolates are identical except 17MA004 which has a single nucleotide difference. Blue indicates heteroploid isolates with allele-b and red indicated haploid isolates. Green indicates heteroploid isolates with allele-c. Internal node values give aLRT support.

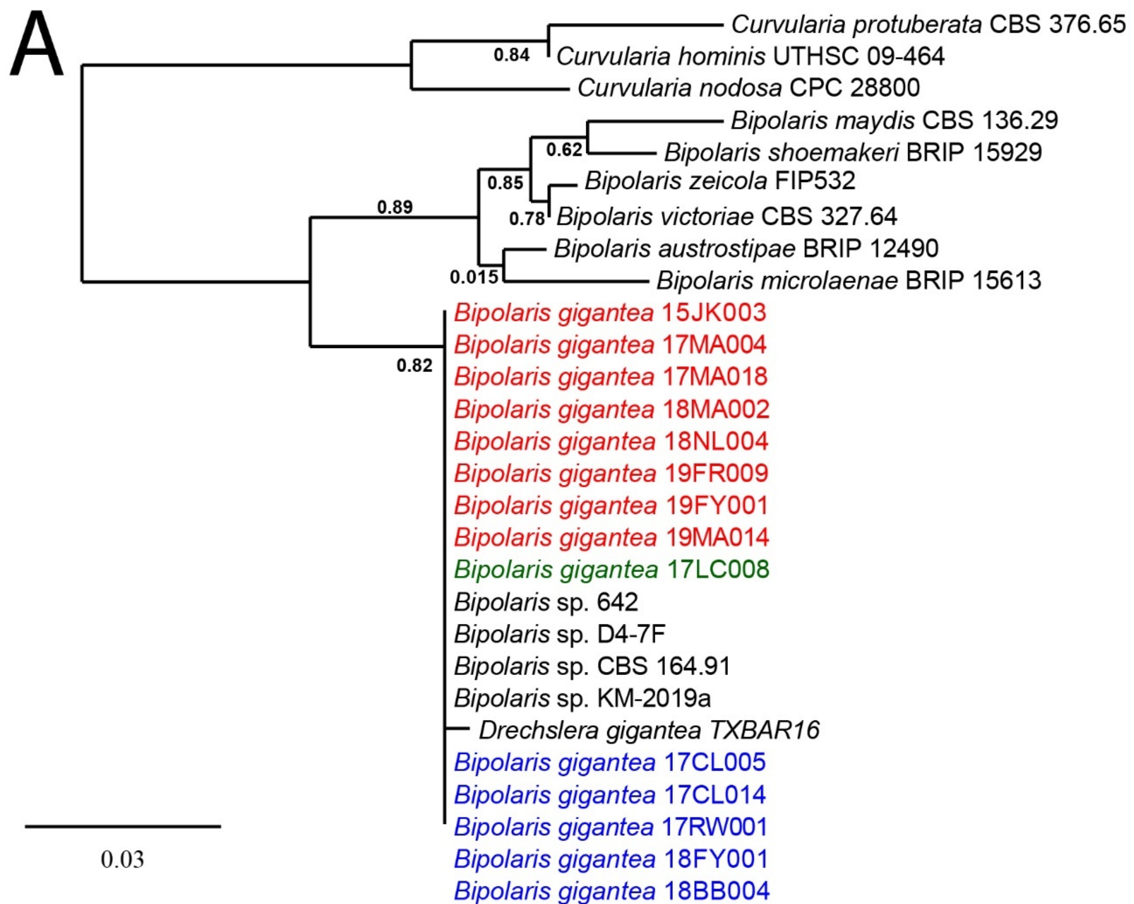


Figure 3.9 (continued).

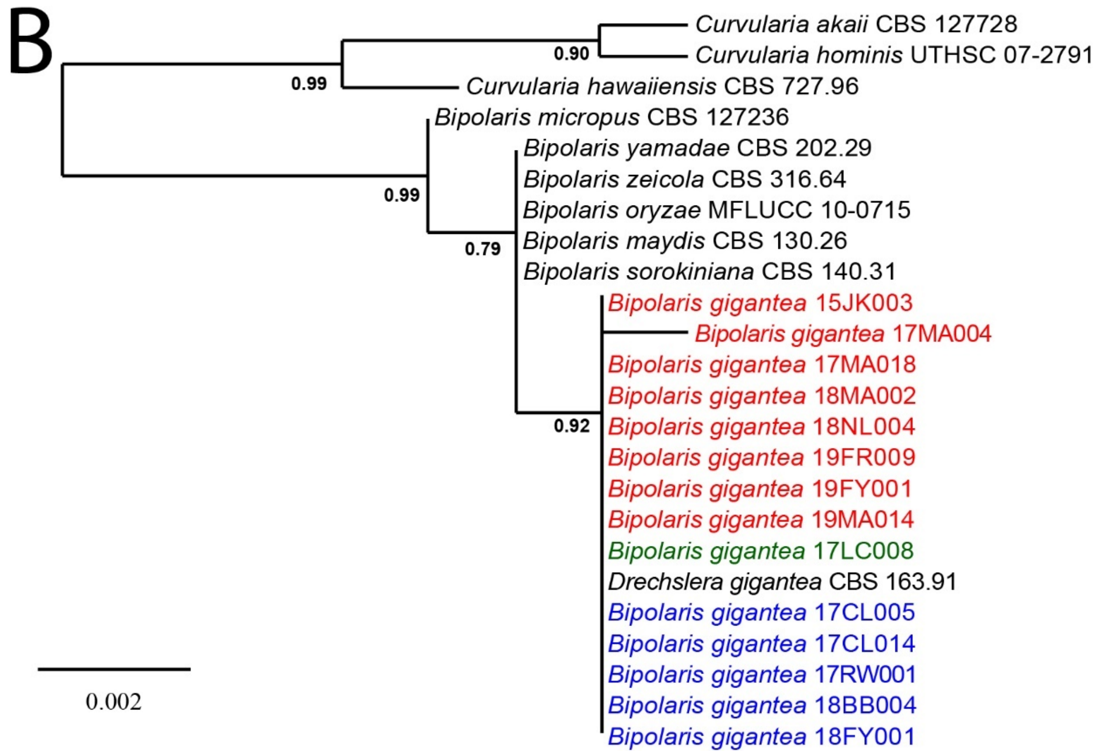
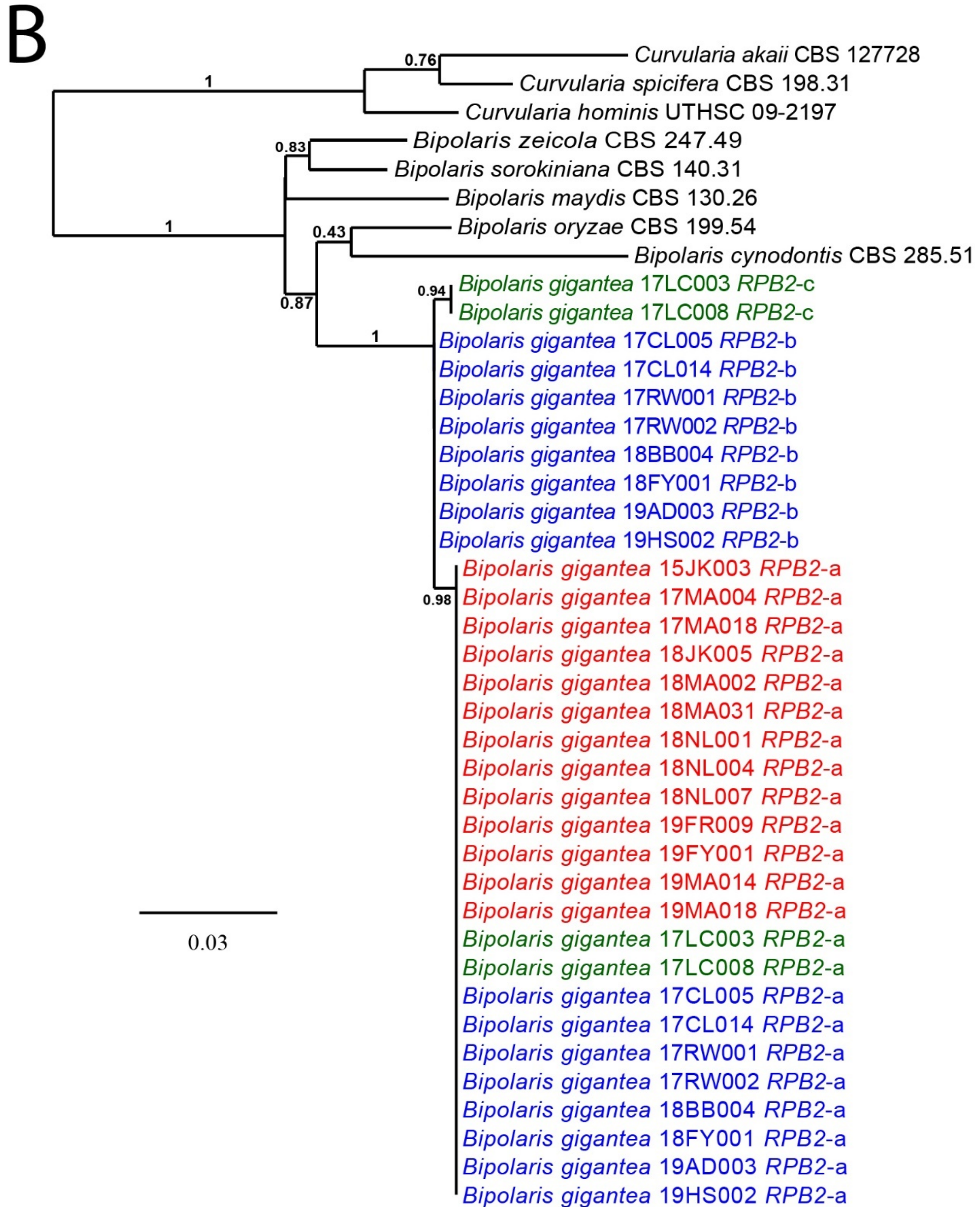


Figure 3.10 Maximum likelihood phylogenies inferred independently from two data sets grouped isolates into three distinct but closely related clades within *Bipolaris*. Haploid alleles group together with allele-a from heteroploids. Remaining alleles from heteroploids grouped into two clades. A. Tree inferred from *TEF1* sequences. B. Tree inferred from *RPB2* sequences. Blue denotes alleles from heteroploid isolates with allele-b. Green represents isolates containing allele-c. Red represents haploid isolates. Branch support values at nodes are given by aLRT.

Figure 3.10 (continued).

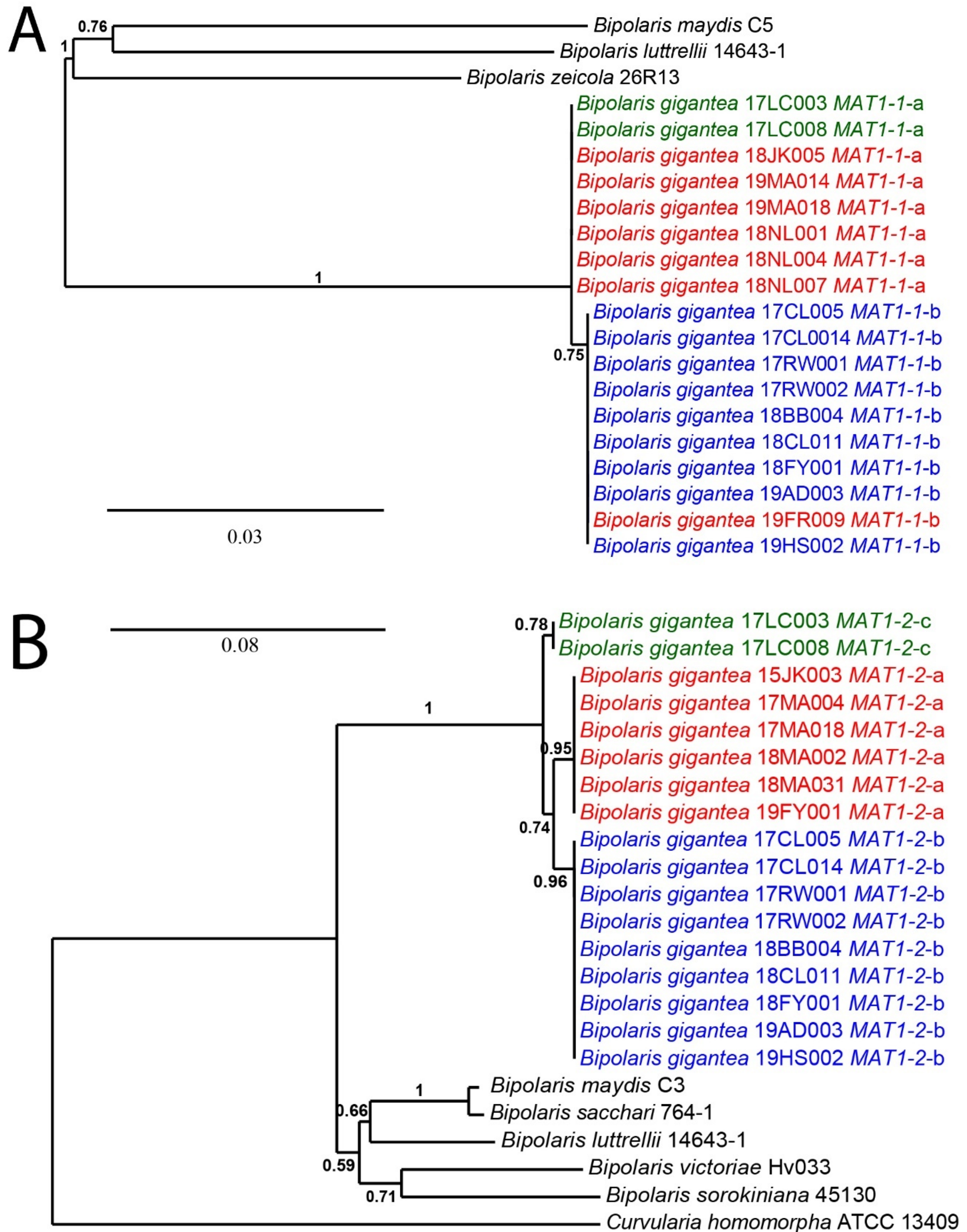


3.4.6 Stability

Gel electrophoresis of DNA extracted from cultures grown from 10 macroconidia from each of the four isolates examined revealed the same mating types as the inoculum.

Mating type sequence amplification separated the 24 isolates into three groups: seven isolates with only *MAT1-1*, six isolates with only *MAT1-2*, and 11 isolates with both mating type idiomorphs (Table 3.1). Inferred phylogenetic trees grouped *MAT1-1* sequences into two separate but related clades (Figure 3.11A). *MAT1-2* phylogeny grouped the isolates into three related clades (Figure 3.11B). In both trees, most mating-type sequences grouped together according to heteroploid or haploid genotype. Isolates with a single allele for *RPB2* and *TEF1* grouped into one clade, and those with two alleles grouped in another clade. Of the isolates from alternative hosts, three were haploid and one was heteroploid, and they grouped as expected. There were two sets of exceptions. Heteroploid isolates 17LC003 and 17LC008 group instead with the haploids/heterokaryons in the *MAT1-1* phylogeny but in a separate clade in the *MAT1-2* phylogeny. (Figure 3.11B). Isolate 19FR009, which contained only one mating type, group with the heteroploid isolates in the *MAT1-1* phylogeny.

Figure 3.11 Maximum likelihood phylogenies inferred for mating-type idiomorphs of *Bipolaris gigantea* isolates and representatives of related species. A. Phylogenetic tree for *MAT1-1*. B. *MAT1-2* phylogeny. Names of isolates are color coded as in Figure 3.10.



3.5 Discussion

This investigation of the fungus causing Bipolaris leaf spot (BLS) disease in Kentucky reveals haploid and heteroploid genotypes of *Bipolaris gigantea*. The 24 isolates from the sampled Kentucky population group into five genotypes, three of which are haploid and two heteroploid. The heteroploid genotypes suggest a hybrid origin, and their possession of both mating type idiomorphs presents the possibility that they arose via an incomplete sexual cycle. It is conceivable that the sexual cycle is interrupted in such hybrids because the parents are sufficiently divergent to limit their sexual compatibility. Such a scenario is consistent with the observation that all of the sampled haploids share alleles for *RPB2* and *TEF1*, and that each heteroploids share those same alleles plus another allele that is closely related but distinguishable by sequence. Furthermore, although both mating types were represented in the common haploid genotypes, none of the observed heteroploids can be explained as hybrids between them. Such a hybrid should be homozygous for *RPB2* and *TEF1* (allele-a of each), but no isolate with both *MAT1-1* and *MAT1-2* had that genotype. Instead, all exhibited heterozygosity for *RPB1* and *TEF1*, as well as one of the two *MAT1-2* alleles that were not found in the haploids. Apparently, each heteroploid is the product of hybridization between a common haploid genotype and one or the other of genotypes that are either rare or reside in habitats (perhaps other hosts) that are yet to be identified.

To our knowledge the widespread occurrence of hybrids on hemp and other dicots has never been reported. A single report made mention of *B. gigantea* on a dicot—a *Teramnus* sp. (Fabaceae)—, however, little to no sporulation was observed on that host (Meredith 1963b). No genetics were given. It may be that this is an unusual situation associated with colonization of a group of hosts that is atypical for the fungal genus, considering that *Bipolaris* spp. are primarily pathogens of monocotyledonous plants. However, such an explanation would be incomplete at best considering that haploids were approximately as abundant as heteroploids in our survey. A survey of *B. gigantea* on the grasses *Microstegium vimenium* and *Elymus virginicus* resulted in five isolates (based on *TEF1* sequences) that were all similar to the haploid BLS pathogens (Lane et al. 2020), but such published surveys should be considered cautiously in light of our observation that the

heteroploids were refractory to maintenance in culture. Another difficulty with the heteroploids is that their genome assemblies based on Illumina (short-read shotgun) sequences were consistently poor, and it seems likely that researchers who are similarly faced with PCR-sequence ambiguities may be frustrated should they follow up with such a genomics approach in an effort to resolve them. For these reasons, it is possible that heteroploids, such as the ones identified in this study, are more common in *Bipolaris* spp. or other filamentous ascomycetes than realized.

The simplest explanation of the heteroploid genotypes is two hybridization events with subsequent propagation as clones. One may be between genotype *RPB2*-a *TEF1*-a *MAT1*-1-b (similar to haploid isolate 19FR009) and the as-yet unsampled genotype *RPB2*-b *TEF1*-b *MAT1*-2-b, and the other may be between genotype *RPB2*-a *TEF1*-a *MAT1*-1-a (similar to the other sampled haploids) and *RPB2*-c *TEF1*-c *MAT1*-2-c. Considering that our results suggest that at least one parent genotype in each hybridization event is uncommon, these hybridizations may have been rare events. If so, then the abundance of hybrids would suggest a selective advantage, although the basis for that advantage is not obvious from our observations to date.

Haploid and heteroploid isolates are distinguished by the number of alleles present for each gene, the presence of one or both mating type idiomorphs, and apparent genome size based on whole genome shotgun sequencing. Remarkably, only a single allele was identified in the haploids, and in each case the same allele is one of the two alleles in each heteroploid. Three haploid genotypes were identified, as well as three heteroploid genotypes. The three haploid genotypes differed only in possessing either *MAT1-1* or *MAT1-2*, and in a polymorphism in the *MAT1-1* idiomorph – seen in a single isolate that groups instead with one heteroploid genotype. In addition to hemp, the pathogen was isolated from four additional dicot hosts (Szarka et al. 2020), a shift in observed hosts, identified as all previously known hosts, have primarily been monocots.

This study demonstrated that both haploid and heteroploid populations of *B. gigantea* cause Bipolaris leaf spot disease on hemp in Kentucky. The causal pathogen was identified both by molecular and morphological methods. Heteroploids were distinguished from haploids by having a genome sequence assembly totaling approximately twice the size of

those of the haploid isolates, containing two alleles each of *RPB2* and *TEF1*, and containing both mating-type idiomorphs.

We have confirmed identification of the BLS causal pathogen by examining both morphological and molecular characteristics. Morphologically, the organism is consistent with the original description of *H. giganteum* (Heald and Wolf 1911), and macroconidia, microconidia, and conidiophores are also consistent with those described in other reports (Drechsler 1928; Kardin et al. 1982; Lane et al. 2020; Meredith 1963a; Sato et al. 1990). However, only Sato et al. (1990) made mention of protothecia, and attempts to induces ascospore development were unsuccessful. Although there has been no DNA sequence comparison with the 1909 type specimen (and we were unable to accomplish it), the sequences of the BLS isolates we report from rDNA ITS and 28S, as well as the *TEF1* gene, are identical or nearly identical to published sequences attributed to *B. gigantea* (Lane et al. 2020; Stricker et al. 2016; Zhang and Berbee 2001).

Bipolaris gigantea has been reported in monocots throughout much of the southern United States and in many parts of the world (Ahn 1980; Drechsler 1929; Lane et al. 2020; Meredith 1963a; Sato et al. 1990). Potentially, haploids containing allele-b or allele-c may only occur on monocot hosts. It is also possible that haploids with allele-b or allele-c occur in populations that have yet to be sampled or exist outside of the sample area. Alternatively, any haploids with the genotype attached to the allele-b or allele-c may have a deleterious element that caused them to be short-lived or to die prematurely, considering that the heteroploids are observed with weaker growth and more rapid decline than haploids in culture.

Morphology of conidia, conidiophores, and microconidia from isolates representing each sampling location had similar characteristics, which indicates a single species, *B. gigantea*, as the causal agent. Although, culture appearance alone could not be used to distinguish heteroploids from haploids because both groups exhibited variation in characteristics such as color and growth rate, protoperithecial-like structures were sometimes observed on inoculated leaves or in cultures of the heteroploids, but never in cultures of the haploids. This suggests that the species designation is not as well defined as otherwise indicated.

Symptoms and disease progression observed within infected fields were consistent across sample locations and within isolate types. Although severity varied between fields and location, there was no indication that this was due to different ploidy types of the pathogen. Severity was potentially influenced by weather, host cultivar, microclimate, or disease history. No observations suggested that either heteroploids or haploids were more aggressive.

The presence of both *MAT1-1* and *MAT1-2* in the haploid populations in approximately equal frequencies in our sample of isolates suggests that *B. gigantea* is sexually reproducing. Conceivably, the heteroploids may be an intermediate stage in the sexual cycle, but if so, it is difficult to explain the phylogenetic patterns we observed in the housekeeping gene sequences and the *MAT1* idiomorph sequences for *MAT1-2*. In the phylogenetic trees for *RPB2*, *TEF1*, and *MAT1-2*, haploid sequences grouped together in one clade separate from the two clades formed of the heteroploid allele-b and allele-c. Only *MAT1-1* sequences grouped haploids and heteroploids together. Allele-c heteroploids grouped together with the majority of haploids, while allele-b heteroploids grouped with a single haploid isolate (19FR009). This phylogenetic result indicates that the genome that characterizes the haploids does not readily recombine with the genomes that carry allele-b or allele-c of the genes that we sequenced (*RPB2, TEF1,* and *MAT1*). The pattern of protoperithecia production by natural isolates may be regarded as consistent with this conclusion, except that pairings of opposite mating types of the haploids also failed to progress beyond protoperithecium development, indicating that conditions to complete the sexual cycle were not reproduced in our experiments. However, consistent phylogenetic distinctiveness of the haploids and heteroploids at our level of sampling (13 and 11 of each respectively), and that both occurred throughout our sampling range, suggest that contributors of allele-b and allele-c lack sexual compatibility with contributors of genome-a.

The absence in our sample of any haploids identified with allele-b or allele-c may have any of several intriguing explanations. Perhaps allele-b or allele-c haploids are rare, but somehow when they hybridized with allele-a haploids the result was highly competitive heteroploids. However, heteroploids seemed no more competitive than the haploids on

hemp, which is the major crop host in Kentucky. A second possibility is that allele-b or allele-c haploids have gone extinct, and their genomes only persist now in heteroploids. However, considering the occurrence and approximately equal proportions of alleles-b of both *MAT1-1* and *MAT1-2* in the heteroploids, it seems likely that at least allele-b undergoes sexual recombination that should result in allele-b haploids in nature. A third possibility, which seems the most plausible, is that allele-b and allele-c haploids are present and abundant in nature but not on the plants sampled in ours or prior surveys (Lane et al. 2020; Stricker et al. 2016). Such an explanation would imply that although allele-a haploids and the heteroploids are both broad host-range pathogens, allele-b or allele-c haploids may be much more restricted in host range. There are over 50 known hosts of *B. gigantea*, yet only five species have been confirmed in Kentucky (Drechsler 1928; 1929; Gamba and Tekauz 2003; Isakeit et al. 2017; Meredith 1963a; Meredith 1963b; Sato et al. 1990; Szarka et al. 2020). Of the small sample of isolates from alternative hosts, none were haploids with allele-b or allele-c. Nevertheless, there so far appears to be no significant crop pathogen with those genomes since no representative sequences were available in the databases before our study.

The genomic variation in *B. gigantea* isolates may be significant in its host range, which is very broad compared to its congeners. *Bipolaris* species are most often identified as pathogens of grasses (Ahn 1980; Drechsler 1929; Lane et al. 2020; Sato et al. 1990). *Bipolaris gigantea* has reported on monocots such as grasses, wheat and banana (Meredith 1963a), but the identification of *B. gigantea* on hemp and other dicots is exceptional. Although our evidence suggests a lack of sexual recombination between allele-a, allele-b, and allele-c haploids, it is possible that genes or whole chromosomes are exchanged by parasexual means. In fact, horizontal chromosome transfer is known in *Fusarium oxysporum* strains (Ma et al. 2010) and suspected in *Alternaria arborescens* (Manamgoda et al. 2012), with effects on their host ranges. The latter is related to *Bipolaris* species, and the two genera share the characteristic that their host-selective toxins play important roles in host range. Therefore, a reasonable question for future inquiry is whether hybridization between *B. gigantea* strains may facilitate its broad host range by formation of stable heteroploids, exchange of chromosomes or genes between haploids, or both. Genome

sequence analysis of an extensive collection of *B. gigantea* isolates, and perhaps particular attention to genes for specialized metabolites (potential pathotoxins) may address this possibility.

In order to effectively manage Bipolaris leaf spot, it is important to better understand the pathogen and its lifecycle. Studies are needed to determine the potential for sexual reproduction, hybridization, and parasexual gene and chromosome transfers, as indicated by this study. Additionally, *B. gigantea* has numerous reported monocot hosts and at least four dicot hosts that potentially serve as sources for inoculum in the BLS disease cycle. Investigations into disease incidence and the distributions of these alternative hosts, as well as possible overwintering and sporulation of *B. gigantea* on plant debris, may help determine the main sources of inoculum and inform the best management strategies.

CHAPTER 4. CONCLUSIONS AND REFLECTIONS

4.1 Reflections

The reinstatement of hemp as a legal crop in the United States resulted in numerous challenges and opportunities. As with any crop, management of diseases is an integral part of production, particularly to protect yield. Bipolaris leaf spot (BLS) was first observed in 2014, the same year that hemp was reintroduced to Kentucky. Unfortunately, the >60-year gap in production led to the loss of locally adapted cultivars and stymied the growth of knowledge. It is unknown if *Bipolaris gigantea* had historically infected hemp or if the introduced cultivars were susceptible to a local pathogen. Regardless, Bipolaris leaf spot and its causal agent were unfamiliar to researchers and to growers. Initial attempts to identify the pathogen were challenging. Sequences and morphological characteristics of our Kentucky isolates matched *Drechslera gigantea*, a somewhat obscure pathogen of grasses that was first described in Texas, 1911 (Heald and Wolf 1911).

Bipolaris leaf spot was the primary focus of my research, especially as reports increased and concern of yield loss became more severe. As hemp acreage increased each year, the disease was reported in more hemp fields and in more counties across Kentucky.

The ambiguous legal status of *C. sativa* before 2018 meant there were no fungicides labeled for hemp, and the pesticide industry was hesitant to initiate such processes. The passage of the 2018 Farm Bill legally separated hemp from marijuana (>0.3% THC) allowing for new opportunities not restrained by classification as a Schedule I substance (Agriculture Improvement Act of 2018). Growers had few options for disease management, no resistant cultivars were available, and management options were limited to cultural practices. It became crucial for us to elucidate and understand the life cycle of *B. gigantea*. The lack of basic knowledge proved to be an obstacle for preforming advanced experiments. For example, it was first necessary to determine optimal media and growth conditions for the pathogen, as well as short- and long-term storage conditions for our isolates, in order to conduct routine laboratory experiments.

The causal agent of BLS was confirmed nearly five years after it was first observed. Prior to identification, we worked under the assumption that the pathogen was a new

species within *Bipolaris,* or a new genus closely related to *Bipolaris*. Thus, I initially used existing knowledge of other *Bipolaris* spp. as a guide for working with this pathogen. Through gene sequencing and comparison against similar organisms, the pathogen identity most closely matched *Drechslera gigantea.* However, phylogenetic analysis clearly placed the organism within the genus *Bipolaris*, which was confirmed by the recent reclassification (Lane et al. 2020). As *B. gigantea* was relatively obscure with minimal investigation, data was limited. Thus, identification did not provide sufficient insight into the pathogen, aside from an extensive list of confirmed monocot hosts.

4.2 Review of major findings

In addition to identifying the pathogen and determining the need for reclassification, I discovered several interesting findings about *Bipolaris gigantea.* Since its description, *B. gigantea* has been reported on at least 50 monocot hosts including several economically important species such as rice and corn. Hemp was not only the first verified dicot host observed, but also one of the first in which *B. gigantea* caused major disease.

In the years following the reintroduction of hemp and the expansion in cultivation, BLS has been reported in several states throughout the eastern United States. Reports of BLS coincide with geographical region of historical reports of *B. gigantea* on other hosts. Literature also reveals an international distribution of the pathogen on other hosts. Reports indicate its presence in South America, India, and islands like Japan and Jamaica. Currently, only American hemp has been reported infected by *B. gigantea*.

The most interesting discovery is the ploidy of *B. gigantea*. While some isolates were haploid and contained a single genome, as expected for an ascomycete, other isolates were heteroploid and contained two genomes. Additionally, the isolates sampled in this study resulted in the discovery of at least five genotypes comprising both haploid and heteroploid populations.

Bipolaris spp. are common on a wide range of grass and weed hosts, so we surveyed vegetation in and around infected fields for potential alternative hosts. In 2017, four dicots were identified as hosts: *Abutilon theophrasti*, (velvetleaf), *Acalypha ostryifolia* (hophornbeam copperleaf), *Acalypha virginica* (Virginia copperleaf), and *Boehmeria*

cylindrica (false nettle). In 2018, two monocots were identified as alternative hosts: *Microstegium vimineum* (Japanese stiltgrass) and *Eleusine indica* (Indian goosegrass). The pathogen was confirmed on these weed hosts on multiple occasions and at different field locations, suggesting that they are all common hosts of *B. gigantea*. Until now, all previously known hosts (with the exception of a *Teramnus* sp.) of *B. gigantea* were monocots, but my work identified five dicot hosts. A more comprehensive investigation is necessary to determine whether other potential hosts are present in and around hemp fields or in the absence of hemp.

While working with the pathogen, several signs indicated that there was some unknown factor influencing differences among isolates. As previously mentioned, some isolates did not subculture well beyond three or four transfers; growth eventually stopped, and isolates appeared dead. I soon realized that these same isolates were also the isolates that produced a protoperithecia-like structure (primarily on inoculated hemp leaves but occasionally in culture). Genetic analysis further supported a divergence among isolates. Many isolates were as expected for haploid ascomycetes; they contained a single allele at the genes *RPB2* and *TEF1* and had either mating type *MAT1-1* or *MAT1-2*. Other isolates, those that sub-cultured poorly and produced protoperithecia, had two alleles at *RPB2* and *TEF1,* and both *MAT1-1* and *MAT1-2* mating types (designated as heteroploids). Phylogenetic analysis has revealed interesting data and patterns in the alleles. Haploids each with a different mating type and heteroploids were isolated from multiple populations and in different locations across Kentucky. However, isolates examined only represent a small sample of the population infecting hemp. Bipolaris leaf spot has been reported from most hemp growing states in the eastern United States, but only a few isolates examined originated outside of Kentucky. Further studies are required to understand the population makeup and the influences it may have on disease management.

4.3 Minor studies

My research included several minor studies, most of which were necessary to determine some basic information about the pathogen and resulting disease. Some of that information is outlined here in hopes that it may be useful for anyone who works with *B. gigantea* or BLS.

Macroconidia develop in lesions after 48 h of incubation in moist chambers, but optimal sporulation occurs if field samples or inoculated leaves are air dried or pressed for at least seven days and then rehydrated in a moist chamber. Macroconidia typically appear within 2–3 days of incubation. A sterile needle can be used to isolate and transfer conidia from conidiophores under a dissecting scope. Conidia may be lost to static or movement, so dipping the needle into agar or water can help conidia adhere to the tip. A faster method of isolating conidia is to gently press the sporulating side of the leaf onto a water agar (WA) plate. Conidia will adhere to the media and are easy to isolate. This also greatly improves the potential for conidia to persist during transfer. Bottom lighting of the WA plate improves visibility for selecting individual conidia. To prevent transfer of contaminants, I occasionally transfer spores on another WA plate and allow them the germinate overnight. Individual spores can then be examined under a stereoscope to check for contamination before subsequent transfers. Conidia were typically transferred and maintained on quarter-strength PDA (¼ PDA), which assures reduced mycelial mass and increased conidial production. Colonies were maintained for longer periods on a low-nutrient medium such as ¼ PDA. Disinfestation of leaves using 10% bleach for 30 s before incubation in the moist chamber also helps prevent contamination or mixed cultures upon conidial transfer.

I initially ran several experiments to determine the optimal conditions for sporulation but made several discoveries which either delayed pending projects or resulted in adjustments. Cultures reacted differently when plates were not sealed compared to when they were sealed with parafilm (parafilm is standard in the lab). Cultures exhibited different coloration when grown enclosed with parafilm or unsealed, therefore, I questioned whether sporulation was also impacted by the presence of parafilm. Further in-depth investigation is needed to determine the effects of parafilm, but I maintained all cultures in unsealed plates for the majority of my tenure in the lab.

Also, I noted early on that some isolates behaved differently than others. For example, upon removal from -20 C storage, some isolates grew normally while others failed to acquire radial growth. Isolates varied in vigor, with some declining after three or four transfers or subcultures. These declining isolates were recovered by infecting hemp leaves and then reisolating them. Upon reisolation, these isolates regained their original

vigor and viability. This cycle had to be repeated for certain isolates to maintain vigor. *Bipolaris gigantea* sporulated best in lower nutrient media, such as Sach's agar, 10% PDA, or 10% V8 agar, with increased sporulation when dried hemp leaf pieces were added to the surface of molten agar. Ideal light conditions were alternating 12 h UV light and 12 h. dark conditions. Continuous light promoted conidiophore elongation and led to production of fewer conidia. Continuous darkness appeared to promote the production of microconidia. Different isolates also sporulated differently on the same media; some isolates sporulated well while others produced only a few conidia. No media was found to be optimal for sporulation of all isolates. Currently, Sach's agar appears best for sporulation. We used ¼ PDA for growth and maintenance, as Sach's agar resulted in little to no vegetative growth. Temperature optimization still needs to be explored; currently cultures are grown at room temperature (25–26 C), but the disease prospers in fields during hot summer months so warmer temperatures may be optimal. Drechsler (1923) suggests 25-29 C, ranges which I did not investigate.

4.4 Implication of this work

While *B. gigantea* has long been known as a minor pathogen of over 60 monocot hosts, including several economic crops, it has only become a severe disease on hemp, a dicot. Molecular investigations have revealed both haploid and heteroploid isolates, yet disease severity caused by these populations appears similar and is identified with similar frequency. Sequencing of some *B. gigantea* isolates from other states have thus far indicated similar patterns of haploid and heteroploid populations compared to those found in Kentucky. However, molecular data is limited outside of our study area. Therefore, the true population makeup is unknown, especially considering additional hosts and the worldwide distribution of this pathogen. Currently, at least five genotypes have been identified among the populations sampled in Kentucky. How the genetics of the pathogen will influence management is unknown. Further research is needed.

4.5 Future directions

There is still much unknown about Bipolaris leaf spot and the causal agent. My studies have only explored a few aspects of the disease cycle. Disease management is limited to cultural practices and biological control products until fungicides can be labeled.

While some products are currently being tested, trials will need to be repeated. There is much work to be done to determine which products are effective and then to acquire Environmental Protection Agency (EPA) registration. Several cultivars are also being evaluated for susceptibility to BLS, but new hemp cultivars are constantly being introduced and bred. A larger selection of cultivars will need to be tested.

Aspects of the pathogen life cycle, particularly within field conditions is still unknown. For example, knowing sources of primary inoculum, means for overwintering, inoculum disseminated, and the role of microconidia are important for developing management plans. The extensive list of known hosts of *B. gigantea* suggest that additional host species may provide a reservoir for inoculum. Alternative hosts have included warm season plants, but no cool season host species have been detected. Investigation of the role of debris in overwintering warrants inquiry, particularly since some *Bipolaris* spp. are known to overwinter in field debris.

Several aspects of fungal biology also warrant investigation. Isolates are currently stored on filter paper disk at -20 C and have remained viable for at least two years. However, it is unknown how long they will be viable in these conditions. Long term storage conditions will need to be established to maintain the isolate collection. Attempts to store *B. gigantea* in 15% glycerol were unsuccessful at -20 C, but other methods have not been tested. Storage methods for other *Bipolaris*, *Cochliobolus*, and related species may be worth investigating.

Grower recommendations and outreach are needed. All observations and data will also need to be translated and presented in a form that is usable to growers to needing to implement disease management.

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- 2019 University of Kentucky Field Day
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