RUST AND LEAF SPOT DISEASES ON AMERICAN ELDERBERRY PLANTS

A Thesis

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By

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The undersigned, appointed by the Dean of the Graduate School,

have examined the Thesis entitled

RUST AND LEAF SPOT DISEASES ON AMERICAN ELDERBERRY PLANTS

Presented by Kaley Hensel

A candidate for the degree of

Master of Science

And hereby certify that, in their opinion, it is worthy of acceptance.

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ABSTRACT

Elderberry rust (*Puccinia sambuci* Schewin.) Arthur (=*P. bolleyana*) and leaf spot diseases are frequently found in commercial American elderberry (*Sambucus nigra* L. subsp. *canadensis* L.) plantings throughout the growing season in Missouri. Thus, studies were conducted to ascertain if rust infections affect plant growth, fruiting, or berry puree quality. Rust symptoms were observed in early April at 9 to 18° C, ≥ 3 h leaf wetness, and ≥ 85% relative humidity. When young, potted elderberry plants averaged 3 to 6 rust pustules/plant, vegetative growth was not adversely affected. However, field-grown elderberry plants heavily infected with rust (137 pustules/cane) lost nearly twice as many leaves as controls during the growing season, indicating rust-induced defoliation. Shoot dry weight of these heavily infected canes was also 32% less than that of controls. First and last harvest dates were advanced by the high level of rust infection on 'Wyldewood' elderberry canes, but not by low pustules numbers (< 6 pustules/plant) on 'Bob Gordon' or 'Ozark' plants. Similarly, berry yields were not significantly different at low infection levels, even though rust-infected 'Bob Gordon' plants had a 31% reduction in yield with an estimated $440/ha loss of income. Heavily-infected 'Wyldewood' canes had a significant loss in berry yield (47%) and potential income ($2,295/ha), assuming a conservative estimate of five canes/plant.

In another study, *Colletotrichum* was isolated from elderberry leaf spot lesions and identified before subsequent re-inoculation of elderberry plants with this pathogen. Three species of *Colletotrichum* (*C. salicis* Funkel, *C. kahawae* subsp. *ciggaro* Wollenw., and *C. aenigma* C.M. Tian & Z. Li) were putatively identified as being casual agents of leaf spot indicating the diversity of species within this genus on elderberry plants.

CHAPTER 1: ELDERBERRY LITERATURE REVIEW

American elderberry is a relatively new specialty crop grown in Missouri (Byers et al., 2014). Historically the bark, roots, stems, flowers, and fruit of elderberry have been foraged by native people with berries used as a folk medicine (Salamon and Grulova, 2015). Elderberries contain antioxidants and have immune system-stimulating, antibacterial, antiallergic, and antiviral properties (Inami et al., 1996; Schmitzer et al., 2012; Sidor and Gramza-Michalowska, 2015). Both flowers and berries are harvested for a wide variety of processed products such as wines, juices, teas, health supplements, jams, baked goods, and dyes (Stevens, 2001).

Elderberry plants are fruit-bearing, deciduous, multi-stemmed shrubs found native to eastern and central North America (Charlebois et al., 2010). Elderberries prefer sunny sites with moist well-drained soil (Byers et al., 2014). Throughout Missouri, wild elderberry plants are found growing along roadsides and in ditches and streams since birds consume the fruit and excrete seeds (Schmitzer et al., 2012). For commercial plantings, elderberry plants are clonally-propagated by hardwood or softwood cuttings (Charlebois et al., 2010). Underground rhizomes produce new elderberry canes throughout the growing season. At maturity, plants are ≈ 3 m in height with canes bearing multiple lateral shoots (Yatskievych, 1999). Leaves are opposite and odd-pinnately compound often having seven leaflets (Yatskievych, 1999) and bud burst occurs as early as late Feb. in Missouri (Byers et al., 2014).

Elderberry plants flower in mid-to-late June in Missouri with large cymes composed of many five-petaled, white flowers. Flowers occur on the terminal portion of 1-and 2-year old canes (Charlebois et al., 2010). Due to the late flowering habit, elderberry is not typically affected by late spring frost. Pollination of elderberry is not

well understood, however, it is recommended that two or more cultivars are present for optimal fruit set in commercial plantings (Byers et al., 2014). Flowers lack nectaries, but extrafloral nectaries are present on elderberry stems (Mizell, 2015; Yatskievych, 1999).

Fruit is purple-black and berries typically range from 5.0 to 6.5 mm in diameter (Schmitzer et al., 2012). Harvest is done by hand and is labor intensive due to the variability of ripening on plants and within individual cymes. In Missouri, elderberry harvest is from late July to September. Mature elderberry plants averaged 0.86 kg fruit/plant when dormant-pruned annually or averaged 1 kg fruit/plant if pruned every other year (Thomas et al., 2009). After harvest, fruit is de-stemmed, sanitized, and frozen for later processing. The selling price for de-stemmed berries is currently $4.40/kg (M. Warmund, personal communication).

The characteristic purple-black color of berries is derived from anthocyanins which are a subclass of primary polyphenols (Schmitzer et al., 2012; Sidor and Gramza-Michalowska, 2015). Other primary polyphenols found in berries are flavonols, phenolic acids, and proanythcynins (Sidor and Gramza-Michalowska, 2015). Fresh berries have the highest levels of primary and secondary metabolites and during processing and storage of elderberry products, the phenolic content often decreases (Johnson et al., 2015).

Berry puree and juice chemistry varies among elderberry cultivars (Perkins-Veazie et al., 2015; Thomas et al., 2013; Warmund et al., 2016). Soluble solid content of elderberry berry puree typically ranges from 8.9 to 12.5° Brix and pH varies from 4.3 to 5.2 (Perkins-Veazie et al., 2015; Thomas et al., 2013; Warmund et al., 2016). Titratable

acidity of berry puree is generally from 0.21 to 1.7 g/100 mL citric acid (Perkins-Veazie et al., 2015; Thomas et al., 2013; Warmund et al., 2016).

Several pests have been reported for elderberry in Missouri (Byers et al., 2014). Deer often feed on elderberry leaves and shoots and birds also consume fruit. Insect damage from Japanese beetle (*Popillia japonica* Newman), elderberry borer (*Desmocerus palliates* Forster), elder shoot borer (*Achatodes zeae* Harris) and sawfly (*Tenthrado grandis* Norton) can also restrict elderberry production (Byers et al., 2014; Charlebois et al., 2010). Spotted wing drosophila (*Drosophila suzukii* Matsumura) has recently become an increasingly important elderberry pest (Pinero, 2013). Adult *D. suzukii* females lay more than 300 eggs with larval feeding causing fruit loss (Pinero, 2013; Ryan, 2010). Two species of eriophyid mites (*Phyllocoptes*) are also commonly found on elderberry plants in Missouri, which cause tightly-rolled leaflet margins or crinkling of the foliage (Warmund and Amrine, 2015).

Various pathogens are also found on elderberry. *Pseudomonas viridiflava* Brunkholder (Byers et al., 2014), *Phyllosticta sambuci* Desm. (Horst, 2013), *Ramularia sambucina* Sacc. (Horst, 2013), and *Septoria sambucina* Pk. (Martin, 1887) have been reported as causal organisms of leaf spot diseases on elderberry. Elderberry plants also host tomato ring spot virus (Horst, 2013) and cherry leaf roll virus (Byers et al., 2014). Two claraviruses, EBCV153 and EBCV145, have been reported on elderberry in Missouri (Keller et al., 2015). A unique rust fungus of elderberry (*Puccinia sambuci*) causes bright orange pustules on leaves, stems, and petioles (Arthur, 1962). The life cycle of *P. sambuci* includes five spore types, including pycniospores, aeciospores, urediniospores, teliospores, and basidiospores (Arthur, 1962). Symptoms of elderberry

rust are first observed early in the growing season but a *Carex* species serves as an alternate host for this fungus in summer and through winter (Kellerman, 1904).

While several pathogens of elderberry have been noted in the literature, there is a paucity of information on fungi infecting commercial plantings in Missouri and the crop damage associated with these organisms. Thus, the purpose of this research was to document the effect of elderberry rust and leaf spot disease on commonly-grown cultivars.

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CHAPTER 2: ELDERBERRY RUST STUDIES

INTRODUCTION

Elderberry rust, *Puccinia sambuci* Schewin. Arthur (= *P. bolleyana*), is found on American elderberry in the eastern United States (Arthur, 1962; Kellerman, 1904). Symptoms of *P. sambuci* on elderberry occur as gall-like swellings on leaves, stems, and petioles (Arthur, 1962). *P. sambuci* is a heteroecious fungus which requires two alternate hosts, a sedge (*Carex* spp.) and American elderberry (Mims, 1981; Saccardo, 1891). Twelve species of sedge have been reported as the alternate host for *P. sambuci,* including *C. bebbii, C. bulata, C. comosa, C. crinite, C. crus-corvi, C. frankii, C. intumescens, C. lupulina, C. lupuliformis, C. lurida, C. tribuloides,* and *C. trichocarpa* (Arthur, 1962). The five spore stages of *P. sambuci* are pycniospores, aeciospores, urediniospores, teliospores, and basidiospores (Arthur, 1962).

Pycnia are the first symptoms observed on elderberry leaflets and petioles in early spring and appear as small yellow pustules. Pycnia are flask-shaped and contain receptive hyphae and pycniospores (Peterson, 1974; Littlefield and Heath, 1979). Pycniospores cannot infect elderberry tissue alone, instead, they must encounter receptive hyphae of a compatible mating type which undergo plasmogamy, resulting in the formation of dikaryotic mycelium (Mims, 1981).

After dikaryotization, mycelia grow intracellularly to the underside of the plant tissue using intracellular haustoria to gain nutrients and develop spore-producing aecia (Mims, 1981; Petersen, 1974). *P. bollyana* aecia are often observed on elderberry in May with large pustules causing deformed leaves, stems, and petioles. Aecia produce chains of

aeciospores which are wind blown to the alternate host, a *Carex* species (sedge) (Mims, 1981).

After germination on susceptible sedge leaf tissue in the summer, urediniospores formed within uredinia, continue to infect sedge plants (Bolley, 1889). In the late summer, sexual, two-celled teliospores are produced with thick cell walls to withstand colder winter temperatures (Arthur, 1962). Upon conducive conditions in March or April, each cell of the teliospore germinates and produces a basidium, the site of meiosis, resulting in four haploid basidiospores which are wind blown to elderberry plants (Petersen, 1974). Basidospore germination occurs laterally on elderberry tissue and a germ tube is produced, penetrating the host directly through the cuticle and epidermis with subsequent formation of haploid mycelium that forms pycnia (Agrios, 2005).

Although the disease cycle of *P. sambuci* has been described, the epidemiology of elderberry rust has not been investigated. Thus, studies were conducted in 2016 to ascertain environmental conditions associated with elderberry rust infection and determine the effect of this fungus on vegetative growth and fruiting. Additionally, the effect of *P. sambuci* on elderberry berry puree after harvest was evaluated.

MATERIALS AND METHODS

Collection of P. sambuci-infected plants. Twenty-five sedge plants (*Carex frankii* Kunth*)* infected with *P. sambuci* teilospores were obtained from a commercial elderberry planting near Hartsburg, MO in Oct. 2014, placed into 8.5 L polyethylene containers (A.M. Leonard, Piqua, OH) with native soil (Haymond silt loam) and transported to the MU Horticulture and Agroforestry Research Center (HARC) near New Franklin, MO.

Plants were maintained in a nursery area until they were covered with a polyethylene foam blanket (Hummerts, St. Louis, MO) and plastic sheeting for winter protection on 26 Nov. 2014. Two-year-old 'Bob Gordon' elderberry plants in 8.5 L polyethylene containers (A.M Leonard, Piqua, OH), that had been propagated from the same site were overwintered similarly. Sedge and elderberry plants were uncovered on 10 Mar. 2015 and were randomly interspersed in the nursery area to maintain *P. sambuci*. Plants were irrigated as needed throughout the growing season. By summer 2015, *P. sambuci* urediniospores from the elderberry plants had reinfected the sedge plants.

Rust infection of potted 'Bob Gordon' plants in 2016. 'Bob Gordon' and 'Wyldewood' elderberry plants were obtained from a commercial source (Botany Shop, Joplin, MO) on 11 Nov. 2015, transported to HARC, and then transplanted on 14 Nov. 2015 into 8.5 L polyethylene containers (A.M Leonard, Piqua, OH) using Pro-Mix BX (Premier Tech Horticulture, Quakertown, PA). On 20 Nov. 2015, elderberry plants were overwintered as previously described. Sedge plants maintained in the nursery area during the growing season were isolated from elderberry plants during overwintering to prevent an early rust infection.

Plants were uncovered on 11 Mar. 2016. Dormant oil (Damoil®; Drexel Chemical Company, Memphis, TN) was applied to elderberry plants for control of overwintering eriophyid mites (*Phyllocoptes wisconsinensis* Kiefer) at 7.5 mL a.i.·L^{-1} on 14 Mar. 2016. Elderberry plants were fertilized with 50 g 15N-9P-12K controlled-release fertilizer (Osmocote®; Scotts Company, Marysville, OH) on 21 Mar. 2016 and pruned to five nodes on 28 Mar. 2016.

To infect elderberry plants with *P. sambuci*, plants that were obtained in Nov. 2015 were arranged in six experimental blocks with four *C. frankii* sedge plants centrally located within each block. Each block contained 12 'Bob Gordon' elderberry plants with three in each north, south, east, and west orientation (Fig. 1). One block similarly arranged, but without the sedge was used for uninfected controls. Elderberry plants were arranged pot to pot, approximately 10 cm from each other. For pollination, one 'Wyldewood' elderberry plant was placed in the northwest, southwest, and southeast quadrant of each block. A clear polyethylene curtain (2.5-m-tall) was placed between blocks with and without sedge plants to prevent rust infection of control plants. Plants were sub-irrigated by filling 4 L saucers (Hummert; St. Louis) underneath pots as needed. After pycnia and aecia were observed on elderberry, all plants were moved to a nursery area with overhead irrigation and were arranged in a completely randomized design on 25 July 2016. From 22 Mar. 2016 to 25 July 2016, spirodiclofen (Envidor® 2 SC; Bayer, Research Triangle Park, NC) was applied at 240 g/L a.i. at 10 to 14 d intervals for mite control.

Micrometeorological data were recorded from 26 Feb. 2016 to 28 Sept. 2016 using a data logger remote monitoring system (U30; Onset, Bourne, MA) located 1 m east of the rust infected blocks. Temperature (S-TMB-M006; Onset, Bourne, MA), relative humidity (S-TMB-M006; Onset, Bourne, MA), and leaf wetness (S-LWA-M003 Onset; Bourne, MA) were recorded at 10 s intervals, averaged, and logged at 10 min intervals. Based upon the results of Beraha et al. (1960), temperatures > 8° C, relative humidity ≥ 85%, and leaf wetness periods ≥ 3 h were evaluated to determine infection periods.

After each fruit harvest, destemmed berries were stored at -22° C. For fruit compositional analyses, berries from each elderberry plant were pooled after thawing. A 50 g fruit sample from each plant was placed in a blender cup (Waring; Conair Corporation, Stamford, CT) with 50 mL double distilled water and processed for 30 s. Puree was then pressed through a mesh sieve to remove seeds. When harvested fruit was less than 50 g per plant, a 1 fruit: 1 double distilled water (w/v) sample was prepared. Soluble solids concentrations were determined with a digital refractometer (PAL-1; ATAGO USA, Bellevue, WA), using a 0.3 mL aliquot of puree. For pH measurements (HI222; Hanna Instruments, Woonsocket, RI) a 10 mL aliquot of puree was used. Another 2 mL aliquot of puree was mixed with 48 mL degassed deionized water and automatically titrated (G20 Compact Titrator; Mettler-Toledo, LLC, Columbus, OH) to an endpoint pH of 8.2 with 0.1 N sodium hydroxide. Titratable acidity (TA), expressed as citric acid, was then calculated.

To evaluate the effect of four orientations and three placements of 'Bob Gordon' plants (1 = closest to sedge plants to 3 = furthest from sedge plants) on rust pustule development, data were analyzed as a 4 x 3 factorial experiment, using PROC GLMMIX procedure of SAS (Version 9.4; SAS Institute, Cary, N.C.). Means were separated by Fisher's protected least significant difference test (LSD), $P \leq 0.05$. Because of the relatively low incidence of rust pustules on 'Bob Gordon' elderberry plants, the T TEST procedure of SAS was used to statistically analyze the effect of *P. sambuci* on plant growth and fruiting. To test mean differences, a pooled test was used when variances were equal and the Satterthwaite test was used when variances were unequal, $P \leq 0.05$.

P. sambuci inoculation of potted 'Ozark' plants. Thirty-two 'Ozark' elderberry plants were obtained from a commercial source (Botany Shop, Joplin, MO) on 23 Mar. 2016, transported to HARC, and then transplanted to 8.5 L polyethylene containers (A.M Leonard, Piqua, OH) using Pro-Mix BX (Premier Tech Horticulture, Quakertown, PA. Elderberry plants were treated with dormant oil on 14 Mar. 2016 and fertilized on 21 Mar. 2016 as previously described before pruning to six nodes on 28 Mar. 2016.

On 8 Apr. 2016 potted sedge plants, maintained as previously described, were used as a source of *P. sambuci* inoculum. Sections (1 cm x 1 cm) of rust-infected sedge leaves were used to inoculate 'Ozark' elderberry leaflets at three levels. For low, medium, and high inoculation levels, either two, four, or eight elderberry leaflets, respectively, were smeared with a rust-infected foliar section of sedge after each elderberry plant was sprayed with 30 mL sterile water. Immediately after inoculation, potted elderberry plants were placed on an inverted 8.5 L polyethylene container and sealed in 208 L trash bags containing 10 L of water to maintain high humidity during incubation. Eight plants were inoculated for each rust treatment. Another eight plants were sprayed with 30 mL sterile water (i.e., controls) before bagging. All plants were incubated at 17° C for 48 h. After incubation, plants were arranged in a randomized complete block design (RCBD) in the nursery area at HARC and maintained as previously described. Plants were sub-irrigated by filling 4 L saucers (Hummert; St. Louis) underneath pots as needed.

Total leaf number, number of *P. sambuci*-infected leaves, and number of pustules per infected leaf were recorded on 29 Apr. 2016 and 19 May 2016. Date of flowering and fruit harvest (8 Aug. 2016 to 28 Sept. 2016), berry number, and berry weight were

also recorded throughout the season. On 19 Aug. 2016, the number of leaves per elderberry plant was recorded. Plants were pruned to 10 cm above soil surface and shoot dry weights were recorded on 10 Oct. 2016. Fruit compositional analyses were performed as described above.

Elderberry plant growth, fruiting, and fruit compositional data were subjected to ANOVA, using the PROC GLM procedure of SAS. Because significant differences between levels of rust infection were not found, infection data were pooled and the T TEST procedure of SAS was used to analyze effects of rust on infected versus uninfected elderberry canes. To test mean differences, a pooled test was used when variances were equal and the Satterthwaite test was used when variances were unequal, $P \leq 0.05$.

Natural rust infection of 'Wyldewood' elderberry canes at a commercial elderberry planting. Three-year-old 'Wyldewood' elderberry plants growing in a Weller silt loam soil in a commercial planting near New Bloomfield, MO were used for this study. Eleven elderberry canes of each of three *P. sambuci* rust infection levels (high, medium, and low) were visually assessed and flagged on 18 May 2016. Eleven individual canes of each infection level and uninfected controls without rust were also flagged in a completely randomized design. Total leaf number, number of *P. sambuci*-infected leaves, and number of pustules per leaf on each elderberry cane were recorded on 31 May 2016. The number of leaves on control canes were also recorded at this time.

Fruit harvest, berry number, and berry weight were recorded from 20 July 2016 to 23 Aug. 2016. Leaves of each cane were counted on 26 Aug. 2016. Canes were pruned to 10 cm and the plant material was harvested for dry weight measurements on 13 Oct. 2016. Fruit compositional analysis was performed as described above.

Elderberry plant growth, fruiting, and fruit compositional data were subjected to ANOVA, using the PROC GLM procedure of SAS. Because significant differences between levels of rust infection were not found, infection data were pooled and the T TEST procedure of SAS was used to analyze effects of rust on infected versus uninfected elderberry canes. To test mean differences, a pooled test was used when variances were equal and the Satterthwaite test was used when variances were unequal, $P \leq 0.05$.

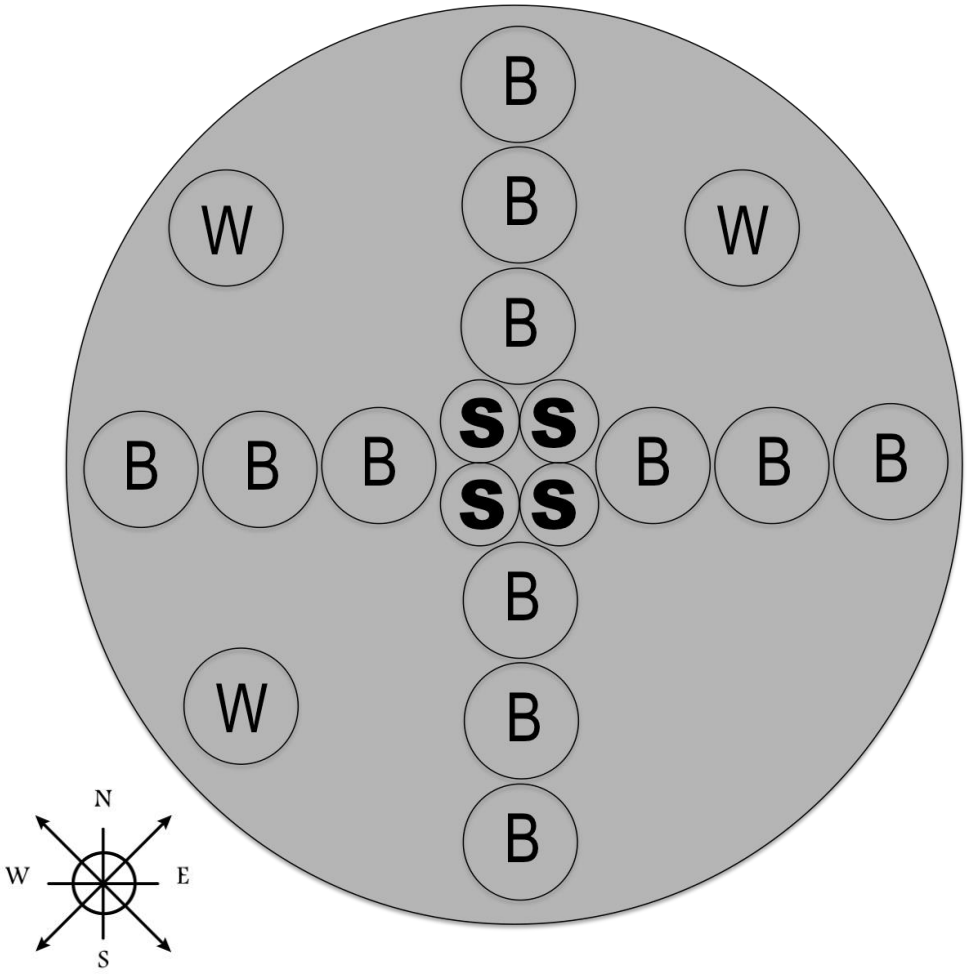

Fig. 1. Experimental design for potted *Sambucus nigra* subsp. *canadensis,* 'Bob Gordon' elderberry rust infection study in 2016. W= 'Wyldewood' elderberry plants, B= 'Bob Gordon' elderberry plants, S= *Carex frankii (*Frank's sedge).

RESULTS

Potential infection periods of P. sambuci. From the time elderberry plants were uncovered to 19 May 2016, there were 30 potential infection periods with ≥ 3 h of continuous leaf wetness and mean maximum hourly temperatures ranging from 9 to 17˚ C (Table 1). Also, 269 mm of rainfall was recorded during 13 precipitation events. A few rust pustules were first observed on four of 72 'Bob Gordon' elderberry plants on 5 Apr. 2016. However, by 29 Apr. 2016, 59 of 72 plants were infected with rust, with a total of 304 pustules. Rust infection continued and 63 of 72 elderberry plants with 350 total pustules were recorded on 19 May 2016.

Table 1. Continuous elderberry leaf wetness periods with corresponding mean maximum hourly temperatures and precipitation recorded at the Horticulture and Agroforestry Research Center near New Franklin, MO during spring 2016.

Date[z]	Continuous leaf wetness (no. of hours)	Mean hourly temp. during wetness period (˚C)	Precipitation during leaf wetness period (mm)
3/12	3	12.5	3.1
3/12 to 3/13	18	13.2	5.6
3/13 to 3/14	13	10.9	0
3/14 to 3/15	13	11.4	0
3/31	7.5	13.1	5.1
4/6	3	13.3	0.5
4/10 to 4/11	13	11.9	17.5
4/16	5	10.8	0
4/16	10	10.8	0
4/17	11	12.3	0
4/18 to 4/19	16	16.2	58.7
4/19 to 4/20	11	15.0	35.1
4/20 to 4/21	13	9.7	0
4/22	9	8.9	0
4/23	11	11.4	0
4/25 to 4/26	10	16.2	0
4/26	3	15.4	0
4/27	3	15.8	1.9
4/27	4	12.4	0
4/29 to 4/30	15	14.2	34.5
5/1	8.5	12.0	0
5/6 to 5/7	9.5	12.2	0
5/7	3	17.8	0
5/8	7	14.3	0
5/8 to 5/9	16	17.1	0
5/9 to 5/10	11	16.0	6.9
5/10 to 5/11	14	16.1	1.0
5/12	5	14.4	0
5/15 to 5/16	10	11.2	17.3
5/16 to 5/17	22	10.4	81.8

[z]Rust pustules (≈ 5) were first observed on elderberry plants on 5 Apr. By 29 Apr., a total of 304 pustules were observed on 59 plants and 350 total pustules were observed on 19 May on 63 plants.

Rust infection of potted 'Bob Gordon' plants. During the growing season, 30 'Bob Gordon' elderberry plants were excluded from the study due to deer herbivory or cane dieback from an unknown disease. 'Bob Gordon' plants had relatively low levels of rust infection when evaluated on 29 Apr. 2016 (Table 2). However, elderberry plants spaced 10 cm from sedge had more infected leaves and leaflets, and a greater number of pustules per plant than those spaced at 20 cm and 30 cm. Also, the orientation of plants in each block (north, south, east, and west) did not affect the number of infected leaves or leaflets or pustules per plant (data not shown).

Table 2. Mean number of *P. sambuci* infected leaves, leaflets, and rust pustules on 'Bob Gordon' elderberry plants at three spacings from *Carex frankii* (Frank's sedge) plants on 29 Apr. 2016.[z]

Spacing from sedge (cm)	No. of infected leaves/plant	No. of infected leaflets/plant	No. of pustules/plant
10	3.3 a	5.8 a	7.6 a
20	1.7 b	2.5 b	3.5 b
30	2.8 ab	3.4 ab	3.9 b

[z]Means represent 24 replications of each spacing from sedge. Mean separation within columns by Fisher's protected LSD test ($P \leq 0.05$).

By 19 May 2016, the number of infected leaves, leaflets, and pustules per elderberry plant did not differ by plant spacing or orientation. However, when data for rust-infected plants were pooled, the number of pustules per plant ranged from one to 27 ($\overline{x} = 6$). Leaf number of rust-infected plants was also less than that of uninfected plants (Table 3).

The first dates of flowering were similar for all plants, occurring in the last week of June (Table 3). First and last harvest dates for rust-infected plants and uninfected plants were also similar. The mean number of cymes produced on rust-infected plants was three, averaging 761 berries, whereas the mean number of cymes for uninfected plants was two, averaging 930 berries. Mean berry weight from rust-infected plants was

11 mg less than that from uninfected plants (Table 3). On 19 Aug., leaf numbers were similar among infected and uninfected plants. However, shoot dry weight of rust-infected plants was greater than that of uninfected plants.

When berry puree characteristics were analyzed, there were no significant differences among treatments (Table 4). Numerically, soluble solid content was 0.6 ˚Brix lower and titratable acidity was 0.2 g/100 mL higher for rust-infected plants as compared to berry puree from uninfected plants (Table 4).

Table 3. Vegetative and fruiting characteristics of 'Bob Gordon' elderberry plants with or without *P. sambuci* rust infection.[z]

Treatment	Leaf no.[y]	First flowering date[x]	First harvest date[x]	Last harvest date[x]	Berry no.	Berry yield (g)	Mean berry wt. (mg)	Leaf no.[w]	Shoot dry wt.[v] (g)
Rust-infected	37 a	181 a	225 a	236 a	761 a	84 a	116 a	77 a	48.6 a
Uninfected	31 b	183 a	221 a	238 a	930 a	121 a	127 b	73 a	31.4 b

[z]Pooled data from rust-infected elderberry plants (n = 42) and non-infected plants (n = 12) were subjected to a t-test. Means followed by different letters are significantly different ($P \leq 0.05$). Mean separation within columns by Satterthwaite test when variances were unequal, or by a pooled test when variances were equal. Rust-infected plants averaged six pustules/plant.
[y]Number of leaves/plant on 19 May 2016.
[x]Values represent Julian days.
[w]Number of leaves/plant on 19 Aug. 2016.
[v]Shoots harvested 10 cm above media surface on 10 Oct. 2016.

Table 4. Mean soluble solids, pH, and titratable acidity of berry puree from 'Bob Gordon' elderberry plants with or without *P. sambuci* rust infection.[z]

Treatment	Soluble solids (°Brix)	pH	Titratable acidity (g/100 mL as citric acid)
Rust-infected	10.6 a	5.0 a	0.68 a
Uninfected	11.2 a	5.1 a	0.48 a

[z]Pooled data from rust-infected elderberry plants (n = 42) and non-infected plants (n = 12) were subjected to a t-test. Means followed by similar letters are not significantly different ($P \leq 0.05$). Mean separation within columns by Satterthwaite test when variances were unequal, or by a pooled test when variances were equal. Rust-infected plants averaged six pustules/plant.

P. sambuci inoculation of potted 'Ozark' plants. During the growing season, 16 'Ozark' elderberry plants were excluded from the study due to deer herbivory and cane dieback from an unidentified disease. When elderberry plants were inoculated with rust using sections of rust-infected sedge tissue in April, there were no significant differences in infection severity on elderberry plants among inoculation treatments at any evaluation date. One to five pustules ($\overline{x} = 2.6$) per plant were observed on 19 May 2016.

Leaf numbers were similar among plants (only varying by one leaf) on 16 May 2016. When other vegetative and fruiting characteristics were analyzed, only the first flowering date was significant with rust-inoculated plants flowering nine days earlier than non-inoculated plants (Table 5). Plants with rust had a harvest period that was five days shorter than non-inoculated plants. Both rust-inoculated and non-inoculated plants had a mean cyme number of three per plant. Numerically, plants with rust produced more fruit and larger berries than those without rust. By 19 Aug. 2016, rust-inoculated plants generally had fewer leaves than non-inoculated plants but the shoot dry weights only varied by ≈ 2.5 g on 10 Oct. 2016. Berry puree characteristics were similar between the treatments, but soluble solids content of berry puree from rust-inoculated plants was generally lower (0.9 ˚Brix), than that from non-inoculated plants (Table 6).

Table 5. Vegetative and fruiting characteristics of 'Ozark' elderberry plants with or without *P. sambuci* rust inoculation.[z]

Treatment	Leaf no.[y]	First flowering date[x]	First harvest date[x]	Last harvest date[x]	Berry no.	Berry yield (g)	Mean berry wt. (mg)	Leaf no.[w]	Shoot dry wt. (g)[v]
Rust-inoculated	43 a	179 a	227 a	239 a	1129 a	109 a	165 a	61 a	36.3 a
Non-inoculated	44 a	188 b	235 a	252 a	621 a	70 a	117 a	80 a	38.8 a

[z]Pooled data from rust-infected elderberry plants (n = 12) and non-infected plants (n = 4) were subjected to a t-test. Means followed by different letters are significantly different ($P \leq 0.05$). Mean separation within columns by Satterthwaite test when variances were unequal, or by a pooled test when variances were equal. Rust-infected plants averaged 2.6 pustules/plant.
[y]Number of leaves/plant on 19 May 2016.
[x]Values represent Julian days.
[w]Number of leaves/plant on 19 Aug. 2016.
[v]Shoots harvested 10 cm above media surface on 10 Oct. 2016.

Table 6. Mean soluble solids, pH, and titratable acidity of berry puree from 'Ozark' elderberry plants with or without *P. sambuci* rust inoculation.[z]

Treatment	Soluble solids (°Brix)	pH	Titratable acidity (g/100 mL as citric acid)
Rust-inoculated	10.9 a	4.6 a	0.70 a
Non-inoculated	11.8 a	4.6 a	0.72 a

[z]Pooled data from rust-infected elderberry plants (n = 12) and non-infected plants (n = 4) were subjected to a t-test. Means followed by similar letters are not significantly different ($P \leq 0.05$). Mean separation within columns by Satterthwaite test when variances were unequal, or with a pooled test when variances were equal. Rust-infected plants averaged 2.6 pustules/plant.

Natural rust infection of 'Wyldewood' elderberry canes at a commercial elderberry planting. Ranges of pustule numbers per cane for low, medium, and high infection levels were 10 – 99, 100 – 199, and 200 – 300, respectively, on 31 May 2016. Although pustule number varied, significant differences in vegetative and fruiting were not found among the three levels of rust infection. When data were pooled from rust-infected canes, the mean number of pustules was 137 pustules on 31 May 2016. Rust pustules were also observed on flower pedicels before bloom through harvest on 'Wyldewood' elderberry canes in the field planting (Figs. 2 and 3). Leaf numbers per cane were similar for all plants on 31 May (Table 7). Rust-infected canes averaged 8 cymes/cane and non-infected canes averaged 9 cymes/cane. First and last fruit harvest dates for rust-infected canes were earlier than those for uninfected canes. Berry number and berry yield were reduced by rust infection; however, mean berry weights were similar among all canes. On 26 Aug. 2016, rust-infected plants had two fewer leaves and had ≈ 28 g less dry weight than uninfected plants, but results were not statistically significant.

When berry puree was evaluated, soluble solids and pH were similar among canes, but berry puree from rust-infected canes was 0.8 ˚Brix lower than that from uninfected plants. Titratable acidity of juice from rust-infected plants was lower than that of puree from uninfected plants.

Fig. 2. Rust pustules on ‘Wyldewood’ elderberry pedicels before bloom.

Fig. 3. Rust pustules on ‘Wyldewood’ elderberry pedicels during harvest.

Table 7. Vegetative and fruiting characteristics of 'Wyldewood' canes with and without *P. sambuci* rust infection.[z]

Treatment	Leaf no. [y]	First harvest date[x]	Last harvest date[x]	Berry no.	Berry yield (g)	Mean berry wt. (mg)	Leaf no.[w]	Shoot dry wt. (g)[v]
Rust-infected	56 a	217 a	225 a	1118 a	47 a	43.2 a	37 a	58.4 a
Uninfected	49 a	222 b	228 b	2105 b	88 b	40.8 a	39 a	86.3 a

[z]Pooled data from rust-infected elderberry canes (n = 33) and non-infected canes (n = 10) were subjected to a t-test. Means followed by different letters are significantly different ($P \leq 0.05$). Mean separation within columns by Satterthwaite test when variances were unequal, or with a pooled test when variances were equal. Rust-infected canes averaged 137 pustules/cane.

[y]Number of leaves/cane on 31 May 2016.

[x]Values represent Julian days.

[w]Number of leaves/canes on 26 Aug. 2016.

[v]Shoots harvested 10 cm above media surface on 13 Oct. 2016.

Table 8. Mean soluble solids, pH, and titratable acidity of berry puree from 'Wyldewood' elderberry canes with or without *P. sambuci* rust infection.[z]

Treatment	Soluble solids (°Brix)	pH	Titratable acidity (g/100 mL as citric acid)
Rust-infected	10.9 a	4.5 a	0.68 a
Uninfected	11.7 a	4.4 a	0.78 b

[z]Pooled data from rust-infected elderberry canes (n = 33) and non-infected canes (n = 10) were subjected to a t-test. Means followed by different letters are significantly different ($P \leq 0.05$). Mean separation within columns by Satterthwaite test when variances were unequal, and pooled test when variances were equal. Rust-infected canes averaged 137 pustules/cane.

DISCUSSION

Foliar symptoms of *Puccinia sambuci* rust (i.e., pustules) were first observed on 'Bob Gordon' elderberry plants as early as 5 April (Table 1). In a commercial planting, many rust pustules were observed later (29 May) on foliage and stems of 'Wyldewood' elderberry, with the initial infection occurring earlier. In some cases, stem distortion occurred when numerous rust pustules were present and may have limited cyme development. The number of pustules on leaflets and stems for each cane or plant were summed in these studies, but severity of stem distortion was not evaluated. Rust pustules were also observed on flower pedicels before bloom through harvest on 'Wyldewood' elderberry canes in the field planting (Figs. 2 and 3). Rust infection on pedicels has not been previously reported and likely contributes to loss of flowers and subsequent fruit loss.

Rust infection from *P. sambuci* occurs when germinated basidiospores from sedge plants penetrate susceptible elderberry tissue under favorable environmental conditions, including high humidity or adequate moisture on the plant tissue for a long enough time over a suitable range of temperatures. For the initial rust infection observed on 5 Apr.,

there were five potential infection periods (Table 1). Temperatures during these periods ranged from 10.9 to 13.2° C and leaf wetness durations ranged from 3 to 18 h. Of these potential infection periods, conditions occurring on 12 Mar. to 13 Mar. may have resulted in significant pustule development since 5.6 mm of precipitation occurred with 18 h of leaf wetness and mean hourly temperature was 13.2° C.

Number of plants infected and number of pustules (both pycnia and aecia) increased throughout April and May. From 6 Apr. to 27 Apr., there were 14 potential infection periods with five of them occurring during precipitation events. Conditions during 18 Apr. to 19 Apr. may have resulted in significant infection since 58.7 mm of precipitation occurred with 16 h of leaf wetness and mean hourly temperature was 16.2° C. From 29 Apr. to 19 May, 50 more pustules were recorded on elderberry plants when evaluated on 19 May, indicating that additional infection may have occurred at hourly temperatures as high as 17.8° C. Based on the results of the study, *P. sambuci* infection on elderberry plants can occur at temperatures between 9 and 18° C with adequate relative humidity and moisture. On other plants, such as asparagus, pycnia and aecia of *Puccinia asparagi* can develop on stems with at least 3 h of continuous wetting with temperatures ranging from 10 to 30° C (Beraha et al., 1960). Maximum infection intensity of *P. asparagi* aecia occurred when mean temperatures remained between 19 and 22° C for several days (Beraha et al., 1960). Both *P. sambuci* and *P. asparagi* occur during early spring, however the similarity between these fungal species is unknown and has not been investigated.

Few *P. sambuci* pustules were observed when ‘Ozark’ elderberry plants were inoculated and incubated at 17° C for 48 h. The reason for low pustule numbers may be

due to few teliospores on sedge leaves, poor teliospore and basidiospore germination, leaf age, or the presence of a thick cuticle on elderberry leaves at the time of infection. French and Lightfield (1990) reported teliospore germination of *P. punctiformis* (Strauss) on Canadian thistle (*Cirsium arvense* L.) was significantly affected by temperature with maximum germination at 16 to 20° C. Similarly, Morin et al. (1992) reported that teliospore germination and subsequent *P. xanthii* (Schwein) basidiospore production occurred at 20° C. After 3 to 6 h, *P. xanthii* basidiospores germinated at 16 to 28° C and produced thin germ tubes which directly penetrated through the cuticle and epidermis of cocklebur (*Xanthium occidentale* Bertol.) leaves (Morin et al., 1992). In the present study, incubation temperature of inoculated 'Ozark' elderberry plants was 17° C, which may be near the minimum temperature for teliospore and basidiospore germination. Melander and Craigie (1927) reported that thick epidermal cell walls of barberry (*Berberis vulgaris* L.) leaves reduced basidiospore penetration from *P. graminis* f. sp. *tritici* Pers. Thus, the mature middle leaflets inoculated on 'Ozark' elderberry plants may have had thicker epidermal cells that limited basidiospore germ tube penetration. Low humidity or lack of moisture was not likely a limiting factor to *P. sambuci* infection on 'Ozark' plants during incubation as Warmund (2015) achieved high pustule numbers (> 690 pustules/plant) using a similar procedure in which elderberry plants were misted with water before smearing infected sedge leaves on host leaflets and then incubating inoculated plants in bags at 18° C for 48 h. Thus, low spore numbers or thickness of the cuticle of mature 'Ozark' leaflets may have the limited basidiospore germ tube penetration into elderberry leaflets.

Elderberry plant proximity to the alternate host plants also influenced the incidence of rust infection, with those located closest to the sedge developing more pustules. These results suggest that rust infection can be reduced by elimination of the alternate host within or nearby elderberry plants. However, because *Carex* species have rhizomes, eradication by mechanical means is difficult (Hilty, 2016).

Results from my studies indicate that the effect of *P. sambuci* on vegetative growth of elderberry plants varies with pustule numbers. At low levels of infection (≤ 6 pustules/plant), leaf number on 'Bob Gordon' and 'Ozark' was not affected by *P. sambuci* (Tables 3 and 5). However, 'Bob Gordon' elderberry plants had more leaves early in the growing season and may have been more vigorous plants than the controls at the time of infection, resulting in infected plants with higher shoot weight on 10 Oct. (Table 3). Alternatively, a mean infection of six pustules per plant may not have been great enough to reduce shoot dry weight (Table 3). When leaf numbers of inoculated and non-inoculated 'Ozark' plants were nearly the same early in the growing season (19 May), rust-infected plants had 19 fewer leaves on 19 Aug. and 2 g less shoot dry weight on 10 Oct. (Table 5).

When the number of leaves per cane were evaluated on 'Wyldewood' plants, no statistical differences were detected among rust-infected ($\bar{x} = 137$ pustules/cane) and uninfected canes on 31 May or 26 Aug.). Rust-infected canes of 'Wyldewood' elderberry plants had seven more leaves than controls when evaluated on 31 May, but rust-infected plants had two less leaves than the control on 26 Aug. Thus, rust-infected canes lost nearly twice as many leaves (19) compared with uninfected canes (10) by the end of the growing season, indicating that rust enhanced premature defoliation on plants

(Table 7). Rust-infected canes also had ≈ 28 g less shoot dry weight than controls on 13 Oct. (Table 7). Because shoot dry weights were limited to tissue removal at 10 cm above the potting medium surface and plants varied in the number of stems, statistical differences among rust-infected and uninfected plants may not have been detected. In an earlier study, Warmund (2015) found that plant dry weight of 'Bob Gordon' elderberry was reduced when pustule numbers averaged 690 pustules/plant.

The fruiting response of elderberry plants also varied by the level of rust infection. When pustule numbers were low (≤ 2.6 pustules/plant), berry numbers and yield were not significantly different among rust-infected and control plants (Table 5). 'Ozark' plants used in this experiment were young (less than two-years-old) and canes were pruned in spring 2016, which may explain the variation in harvested berries. For 'Bob Gordon' plants averaging six pustules/plant, there was a 31% reduction in berry yield. 'Wyldewood' canes averaging 137 pustules/cane had 47% fewer berries and total fruit weight at harvest (Table 7). Using a typical plant spacing at 1.2 x 3.1 m (2,688 plants/ha), berry yields reported herein, and a selling price of $4.40/kg fruit (M. Warmund, personal communication), the estimated loss of income due to rust infection is $440/ha for 'Bob Gordon' and $2,295/ha for 'Wyldewood' (assuming 5 canes/plant with similar berry weight).

Soluble solid contents of berry puree from elderberry plants were not statistically different in any of the 2016 experiments. However, soluble solid contents of puree from rust-infected plants was generally 0.6 to 0.9° Brix lower than that of puree from control plants (Tables 4, 6, and 8). Due to the naturally low soluble solids content of elderberry fruit, sweeteners are added during processing (Warmund et al., 2016). Thus, reduced

soluble solids in fruit harvested from rust-infected plants would require additional sweetening, therefore increasing the cost of production of processed elderberry products. Titratable acidity was not statistically different between rust-infected and control 'Bob Gordon' and 'Ozark' plants. However, titratable acidity of berry puree from rust-infected 'Wyldewood' canes was 0.11 g/mL greater than that of puree from uninfected control canes, indicating that puree quality was adversely affected at a high level of rust infection (Table 8).

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CHAPTER 3: ELDERBERRY LEAF SPOT STUDY

INTRODUCTION

In Missouri, leaf spot lesions on elderberry are generally observed in the summer when temperatures are warm and relative humidity is high. Several leaf spot diseases have been reported for elderberry and their causal agents including, *Ascochyta wisconsina* Davis, *Phaeoramularia catenospora* Atk., *Cercospora depazeoides* Desm., *Cerosporella prolificans* Ellis & Holw., *Phyllosticta sambuci* Desm., *Mycosphaerella* sp., *Ramularia sambucina* Sacc., and *Septoria sambucina* Peck. (Charlebois et al., 2010). However, since elderberry is a relatively new specialty crop, few of these organisms have been identified in Missouri.

Colletotrichum is an important genus of plant pathogenic fungi that parasitize sagricultural crops worldwide such as, cereals and grasses, cucurbits, eggplant, tomato, strawberry, sugarcane, mango, avocado, olive, coffee, etc. (Agrios, 2005). Based upon economic importance, *Colletotrichum* species are considered the eighth most injurious plant pathogenic fungi in the world (Cannon et al., 2012). Anthracnose, a common disease associated with *Colletotrichum*, appears as blackened sunken necrotic lesions caused by black acervuli on stems, leaves, and fruit (Freeman et al., 1998). *Colletotrichum* symptoms including leaf spots have been reported on hosts, such as American sweetgum (Garibaldi et al., 2016), walnut (Zhu et al., 2015), orange (Cheng et al., 2014), and apple (Crusius et al., 2002).

Infection and disease development of *Colletotrichum* is promoted by warm, wet, and humid conditions (Coates et al., 2014). *Colletotrichum* species produce asexual conidia which are disseminated by splash from frequent precipitation, irrigation, or heavy

dews, especially when plants are overcrowded (Than et al., 2008). *Colletotrichum* can also be disseminated by the introduction of infected transplants (Hyde et al., 2009; Cannon et al., 2012). The duration of surface leaf wetness has the greatest influence on conidial germination, infection, and growth of *Colletotrichum* within the host (Than et al., 2008). Conidia germinate on susceptible host tissue and form an infection structure called an appressoria which mechanically penetrates host plant epidermal walls and releases compounds, such as glycerol and melanin that aide in increasing turgor pressure for penetration (Agrios 2005; Kubu et al., 2000). Initially, *Colletotrichum* infection begins a biotrophic nutrition strategy, however, a delayed necrotrophic nutrition strategy follows resulting in death of plant cells and pathogenic lesions (O'Connell et al., 2000; Perfect et al., 1999). The production of host-induced virulence effectors promotes the growth of the fungus within plant tissues (Cannon et al., 2012).

Symptoms appear several days to weeks after infection as *Colletotrichum* species have a latent period which is temperature dependent with higher temperatures resulting in shorter latent periods (King et al., 1997). Yakoby et al. (2000) reported that *C. gloeosporioides* Penz. secreted ammonia in host tissue, which resulted in a pH increase in the infected area and enabled production of pectate lyase, a cell wall-degrading enzyme which promotes fungal pathogenicity. *C. acutatum* J.H. Simmonds in apple and *C. coccodes* Wallr. in tomato also produced ammonia and increased pH in the infected tissue, resulting in maceration of plant cell walls and enhanced virulence (Prusky et al., 2001). Acervuli develop on lesions on infected plant tissues and produce additional conidia, causing secondary infection of additional plants throughout the growing season (Coates et al., 2014).

Colletotrichum species survive and overwinter by producing micro-sclerotia in and on dead host tissues of crop residues and seeds (Than et al., 2008). Some *Colletotrichum* species produce a *Glomerella* telemorph (i.e., sexual stage) which most often develops on dead host tissues (Cannon et al., 2012). The disease cycle of *Colletotrichum* will repeat under favorable environmental conditions during the following growing season with the production of conidia or ascospores if a *Glomerella* telemorph is present.

The systematics of *Colletotrichum* species are considered unsatisfactory due to discrepancies in morphological distinctions among species, as well as ambiguity of species lifestyles, and incorrectly-named sequences in GenBank (Hyde et al., 2009). Symptoms from *Colletotrichum* species observed on plant material and in culture vary widely in morphological features and vary with environmental conditions, making morphology based identification especially difficult (Cannon et al., 2012).

C. acutatum has been reported on elderberry (*Sambucus nigra*) fruit, but no other plant organs in Switzerland (Michel et al., 2013). In 2015, *Colletotrichum* was isolated from necrotic lesions on elderberry leaflets from 'Bob Gordon' elderberry in Missouri (J.D. Mihail, unpublished data). Therefore, the objectives of this study were to document *Colletotrichum* species infecting American elderberry in Missouri and determine which species are associated with elderberry leaf spot.

MATERIALS AND METHODS

Fungal isolations from leaf spots on elderberry plants. Fungi were isolated from symptomatic leaf spots occurring on two-year-old 'Bob Gordon' elderberry plants

growing in containers (3.74 L) in a nursery area at the Horticulture and Agroforestry Research Center (HARC) on 7 June 2015. Leaf spots were angular, purple-colored lesions with necrotic centers. Each lesion was cut from a leaflet (≈ 5 mm x 5 mm), containing both the diseased tissue and a surrounding margin of green tissue. Infected tissues were then surface sterilized in 70% ethanol for 30 to 45 s and rinsed twice for 30 s in sterile deionized water. Lesions were blotted dry with a sterile paper towel, cut in half, and both pieces of tissue were placed in 60-mm-diameter petri dishes (Fisher Scientific, Hanover Park, IL) with half-strength potato dextrose agar (PDA). The medium was prepared by dissolving 19.5 g potato dextrose agar (Remel, Thermo Fisher Scientific, Lenexa , KS) in 1 L deionized water supplemented with 0.5 g streptomycin sulfate (Sigma Chemical Co, St. Louis, MO) to exclude bacteria. Plates were then incubated for 2 to 3 d in an inverted position at ≈ 23° C under fluorescent lighting (75 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with a 10 h photoperiod until hyphal growth was ≈ 3 mm from the diseased tissue. Hyphae were then transferred from the cultures and plated in Van Tieghem cells (Tuite, 1969) in 60-mm-diameter petri dishes containing water agar streptomycin (WAS) media. WAS media was prepared by dissolving 20 g technical agar (Difco™, Becton, Dickinson and Company, Sparks MD), in 1 L deionized water, with 0.5 g streptomycin sulfate. After hyphae had grown 3 mm from the Van Tieghem cell, they were transferred and plated in 95-mm-diameter petri dishes containing half-strength PDA as previously described.

Fungal isolates were maintained in pure culture on half-strength PDA at ≈ 23° C under fluorescent lighting (75 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with a 10 h photoperiod. On 1 Feb. 2016, isolates were sent to the University of Missouri Plant Diagnostic Clinic for DNA

sequencing. Actively growing mycelium were cultured on full-strength PDA (39 g potato dextrose agar and 1 L deionized water) plates covered with cellophane and, mycelia were scraped from the cellophane were then frozen at -80° C. Genomic DNA was extracted using the Easy-DNA kit (Invitrogen Corp., Carlsbad, CA) from the frozen mycelia. The internal transcribed spacer (ITS) region was amplified with PCR using primer pairs ITS5 (5' -GAAGTAAAAGTCGTAACAAGG -3') (Ristanio et al., 1998) and ITS4 (5' -TCCTCCGCTTATTGATATGC -3') (Gardes and Burns, 1993). Each PCR consisted of 10 µL of MyTaq red reaction buffer (Bioline Inc., Taunton, MA), 200 nM of each primer, 0.25 µL of *Taq* polymerase (Bioline Inc.), and 50 ng of genomic DNA (Cottrill et al., 2016). Thermal cycling conditions involved initial denaturation at 95° C for 10 min followed by 40 cycles of 95° C for 30 s, 50° C for 30 s, and 72° C for 1 min, and a final extension step at 72° C for 10 min. Purification of amplified isolated DNA was performed using the ExoSAP-IT kit (Thermo Fisher Scientific, Lenexa, KS). Purified PCR products were Sanger-sequenced by the University of Missouri DNA sequencing facility. Base calls were checked manually and reads assembled in SeqMan Pro (DNASTAR Inc., Madison, WI). The assembled contig was subjected to BLAST analysis against the National Center for Biotechnology Information nucleotide database (Sharma et al., 2014) and isolates were putatively identified as *Colletotrichum.* *Colletotritchum* cultures were sent by overnight mail to the USDA ARS Systematic Mycology & Microbiology Lab in Beltsville, MD on 9 Feb. 2016 for DNA analysis by amplifying ITS regions to determine the species. Putative results indicated that the culture was *Colletotrichum salicis.*

On 20 Nov. 2015, elderberry plants were covered with a polyethylene foam blanket and plastic sheeting for winter protection. Plants were uncovered on 11 Mar. 2016. Dormant oil (Damoil®; Drexel Chemical Company, Memphis, TN) was applied to plants to control overwintering eriophyid mites (*Phyllocoptes wisconsinensis* Kiefer) at 7.5 mL a.i.·L^{-1} on 14 Mar. 2016. Elderberry plants were fertilized with 50 g 15N-9P-12K controlled-release fertilizer (Osmocote®; Scotts Company, Marysville, OH) on 21 Mar. 2016 and pruned to six nodes on 28 Mar. 2016. From 22 Mar to 25 Jul. 2016, spirodiclofen (Envidor® 2 SC; Bayer, Research Triangle Park, NC) was applied at 240 g/L a.i. at 10 to 14 d intervals for mite control.

Inoculation of 'Bob Gordon' elderberry plants with Colletotrichum. Forty-eight *C. salicis* sub-cultures were prepared on 16 May 2016 using Czapek-Dox V8 agar (CDV8). CDV8 media was prepared by dissolving 45.4 g Czapek agar (Difco™, Becton, Dickinson and Company, Sparks, MD) and 10 g technical agar with 200 mL clarified V8 (Campbell Soup Company, Camden, NJ) and 800 mL deionized water. Petri dishes were then inverted and placed at ≈ 23° C under fluorescent lighting (75 µmol·m^{-2}· s^{-1}) with a 10 h photoperiod.

Three concentrations of *C. salicis* inoculum were prepared from the plates. Conidia were retrieved by flooding each plate with 15 mL of sterile deionized water for 10 min, gently agitating the culture with a sterile bent glass rod, and then pipetting the suspended spore solution into a sterile beaker. Concentrations were determined and adjusted using a hemacytometer (Fisher Scientific, Hanover Park, IL). Low, medium, and high conidial concentrations were 1.67 x 10^3, 5.0 x 10^4, and 5.0 x 10^5 conidia·mL^{-1}, respectively.

Immediately before plant inoculation, ≈ 22 µL surfactant (Tween 20; Croda Inc., New Castle, DL) was added to each conidial solution and an aerosol pressurized sprayer (Sigma-Aldrich, St. Louis, MO) was used to apply 30 mL of inoculum onto each elderberry plant on 9 Jun. 2016. Eight plants were inoculated at each concentration of conidia and eight plants were used as non-infected controls. Immediately after inoculation, *C. salicis* treated and non-treated elderberry plants were placed on an inverted 8.5 L polyethylene container and sealed in 208 L polyethylene bags containing 10 L of water to maintain a humid environment for conidial germination and infection. Bagged plants were incubated at 26° C for 36 h. After incubation, plants were arranged in a randomized complete block design in the nursery area at HARC. Sixteen 'Ozark' elderberry plants were interspersed among the 'Bob Gordon' plants for pollination. Overhead irrigation was used to irrigate throughout the growing season.

Pathogenicity and re-isolation. Two leaflets exhibiting leaf spot symptoms were sampled from each of the inoculated plants and photographed on 28 Jun. 2016. Fungal isolations were performed as previously described to achieve pure cultures. On 25 July 2016, the number of symptomatic leaf spots per plant were recorded for all elderberry plants. Two leaflets per plant were collected and two lesions from each leaflet were used for the re-isolation process as previously described. All the re-isolated cultures were kept in the dark at ≈ 23° C and monitored for conidial production. Cultures plated on half-strength PDA from the 25 Jul. 2016 isolation exhibiting *Colletotritchum* conidia were sent by overnight mail to the USDA ARS Systematic Mycology & Microbiology Lab in Beltsville, MD on 29 Aug. 2016 for DNA analysis by amplifying ITS regions to determine the genus and species.

RESULTS

Leaf spot identification. One inoculated and two control plants were lost during this study due to an irrigation system malfunction. Nineteen days after inoculation of *C. salicis* isolates, leaf spot symptoms were observed on nine of the inoculated plants. Symptoms were angular, purple-colored leaf spot lesions with necrotic centers (Fig. 4). However, inoculation treatments did not result in varying numbers of symptomatic leaf spots. After re-isolation of fungi from elderberry leaflets, four of nine cultures produced conidia consistent with *Colletotrichum* as described by Weir et al. (2012). All three inoculation treatments produced *Colletotrichum* conidia in culture after 20 d of growing on half-strength PDA. Colony color was light grey with light brown concentric bands and slight shades of salmon and pink towards the center. Conidia were one-celled, hyaline, and cylindrical with round ends when examined with a compound microscope at 30x magnification (Fig. 5). Sclerotia and melanized hyphae were also present on the cultures. Other fungal organisms were isolated from lesions on other infected elderberry plants, but they appeared morphologically dissimilar from the putative *Colletotrichum* cultures.

On 25 Jul. 2016, 22 of 23 inoculated plants had leaf spot lesions. Five of six control plants also had leaf spot lesions, indicating natural infections occurred. Only one culture from a control plant produced *Colletotrichum*-like colonies. Fungi from the high inoculation treatment produced conidia during the second re-isolation.

The USDA ARS Systematic Mycology & Microbiology Lab putatively identified fungi from the inoculated and non-inoculated plants as *Colletotrichum kahawae* subsp.

ciggaro (Fig. 6), and *Colletotrichum aenigma* (Fig. 7), respectively, based on ITS sequencing.

Fig. 4. *Colletotrichum* lesions on infected elderberry leaflet.

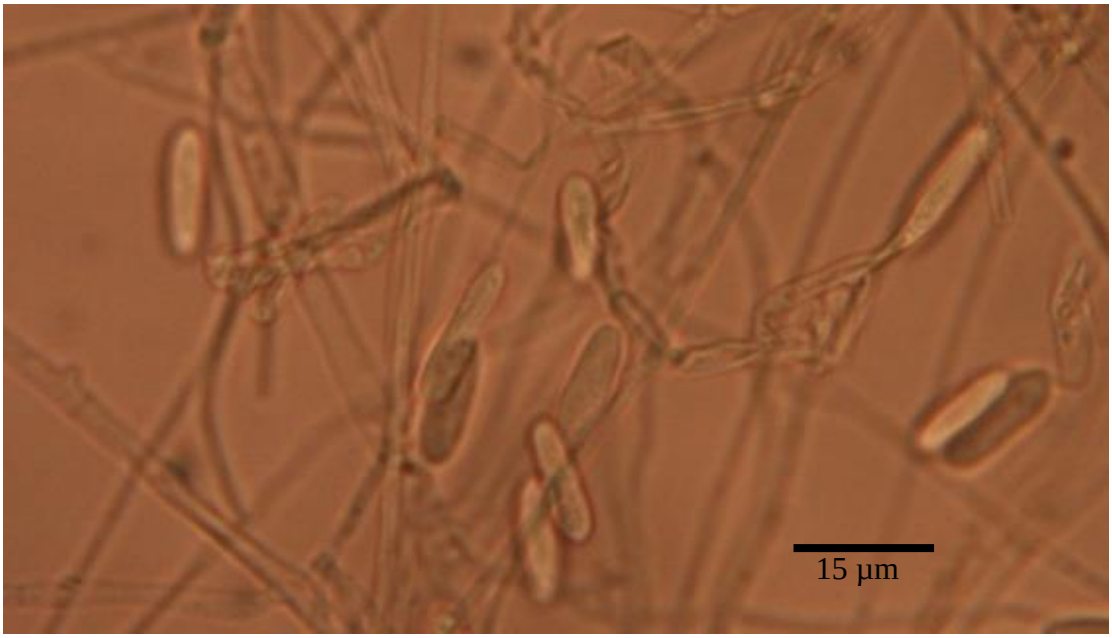

Fig. 5. *Colletotrichum* conidia spores as seen under the compound microscope.

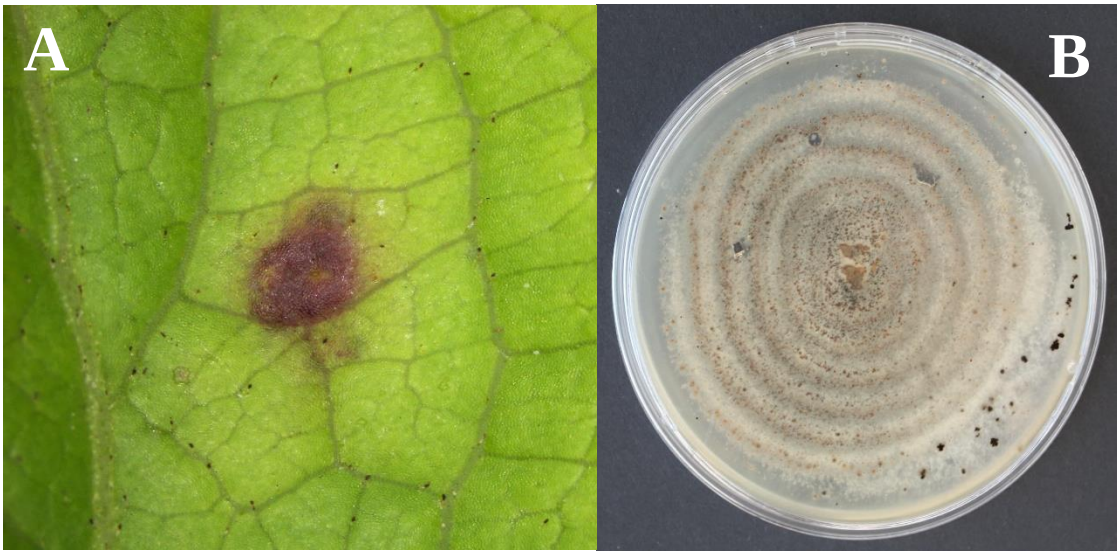

Fig.6. (A) Elderberry foliar symptoms of *Colletotrichum kahawae* subsp. *ciggaro* and (B) its colony morphology on half strength potato dextrose agar.

Fig. 7. (A) Elderberry foliar symptoms of *Colletotrichum aenigma* and (B) its colony morphology on half strength potato dextrose agar.

DISCUSSION

On 25 Jul. 2016, 22 of 23 inoculated plants had leaf spot lesions. Due to the close proximity of infected plants and control plants in the nursery area, four out of five non-inoculated control plants developed leaf spot symptoms during the growing season.

Three putative fungal pathogens were tentativley identified from leaf spots occurring on elderberry during the 2015 and 2016 growing season including, *Colletotrichum salicis, C. kahawae* subsp. *ciggaro,* and *C. aenigma.* Although 'Bob Gordon' elderberry plants were inoculated with *C. salicis,* other *Colletotrichum* species were re-isolated from leaf spot lesions on infected plants, indicating background infection occurred. Also, the use of daily overhead irrigation likely enhanced disease infection from fungi other than *C. salicis. Colletotrichum* conidia are most often dispersed from acervuli on lesions during warm, wet conditions by rain splashing. Thus, the use of overhead irrigation for elderberry plants likely caused initial and secondary infections to occur on inoculated and non-inoculated plants.

Interestingly, the two *Colletotrichum* species isolated from leaf spots in 2015 and 2016 vary phylogenetically. *C. salicis* belongs to the acutatum clade and both *C. kahawae* subsp. *ciggaro* and *C. aenigma* belong to the gloeosporioide clade (Cannon et al., 2012; Weir et al., 2012). All three species of *Colletotrichum* produced visually-similar lesions on elderberry leaflet tissue, appear similar in colony morphology, and produce visually-similar conidia, making it difficult to distinguish the species based upon symptom and isolate morphology. Molecular techniques have been adopted to characterize and identify taxa within *Colletotrichum;* however, accurate identification of *Colletotrichum* species has its limitations due to ambiguity of species lifestyles and incorrectly-named sequences in GenBank (Hyde et al., 2009). Therefore, although species of *Colletotrichum* were sequenced using ITS, it is difficult to classify these species with 100% certainty due to taxonomic and phylogenetic confusion within the genus.

C. salicis was first isolated from angular, purple-colored lesions with necrotic centers on elderberry leaflets sampled on 7 June 2015 from two-year-old 'Bob Gordon' elderberry plants. *C. salicis* has not previously been reported on elderberry. Known hosts of *C. salicis* include Asian pear (*Pyrus pyrifolia* Burman*)*, apple (*Malus domestica* Borkh.), and strawberry (*Fragaria x ananassa* Dutch.) in New Zealand, and tomato (*Solanum lycopersicum* L.) in Germany (Damm et al., 2012). Symptoms of this species have been described as fruit rot, anthracnose, and petiole lesions (Damm et al., 2012). Sexual morphs of this species have been identified as *Glomerella salicis* (Damm et al., 2012; Jayawardena et al., 2016).

In September 2013, *C. acutatum* was identified as the causal agent of anthracnose on black elderberry (*Sambucus nigra* L.) fruit in Switzerland (Michel et al., 2013). Isolates of *C. acutatum* from black elderberry grown on potato dextrose agar resulted in the growth of white to grey mycelium with salmon-colored spore masses, similar to the culture morphology of *C. salicis, C. kahawae* subsp. *ciggaro* and *C. aenigma* observed on half strength potato dextrose agar during the 2016 growing season. Small lesions were observed on individual berries grown at the commercial planting in New Bloomfield, Mo in 2016, however attempts at isolating the causal agent were inconclusive.

C. acutatum is one of the most severe diseases on strawberry (*Fragaria* x *ananassa*), resulting in major crop losses (Freeman and Nizani 1997; Leandro et al., 2001). Lesions on petioles and leaves are observed with *C. acutatum* infection of strawberry, as well as flower blight and fruit rot. Most often, *C. acutatum* is introduced to strawberry fields by infected plant material and can survive as a symptomless

endophyte in living strawberry tissue (Cannon et al., 2012; Hyde et al., 2009). It is unknown if *Colletotrichum* is endophytic in elderberry.

While this is the first report of *C. kahawae* subsp. *ciggaro* on elderberry, coffee (*Coffea Arabica* L.) is a commercially-important host for this fungus and is the causal agent of coffee berry disease, which is a major limiting factor of coffee production. The fungus infects green coffee berries, causing anthracnose-like symptoms and premature fruit drop (Pires et al., 2015). Yield losses up to 70 to 80% can result from infection if no control measures are used (Pires et al., 2015). Other known hosts of *C. kahawae* subsp. *ciggaro* include blueberry (*Vaccinium spp.*) and avocado (*Persea spp.*) (Weir et al., 2012). Sexual morphs have been identified as *Glomerella cingulate* var. *migrans* and *Glomerella rufomaculans* var. *vaccinia* (Weir et al., 2012).

Colletotrichum aenigma also has not been reported on elderberry. Known hosts of *C. aenigma* include Asian pear (*Pyrus pyrifolia)* in Japan, avocado (*Persea americana* Miller) in Israel (Weir et al., 2012), and dragon fruit (*Hylocereus undatus* Haw.) in Thailand (Meetum et al., 2015). Symptoms of infection by this species have been described as necrotic lesions on mature stems and fruits (Meetum et al., 2015). Weir et al. (2012), reported *C. aenigma* may have more white aerial mycelium in culture compared to other species, however this was not the case for colonies isolated in 2016. Although elderberry plants were inoculated with *C. salicis* and Koch's postulates were not fulfilled, premature defoliation on elderberry plants was observed in the 2016 study (presumably caused by *Colletotrichum* species). To prevent leaf spot diseases of elderberry, drip irrigation could be used to limit leaf wetness periods conducive to *Colletotrichum* infection.

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CHAPTER 4: SUMMARY AND CONCLUSIONS

Four studies were conducted to evaluate the effect of elderberry diseases in Missouri caused by fungal pathogens. In the first study, infection periods of elderberry rust from *Puccinia sambuci* occurred in early April at 9 to 18° C, ≥ 3 h leaf wetness, and ≥ 85% relative humidity. The greatest number of rust pustules/plant occurred when sedge was in close proximity to elderberry plants, suggesting that eradication of sedge within the plant rows would decrease pustule frequency and reduce the severity of infection.

Elderberry plant response to *P. sambuci* varied by rust-infection level. When young, potted elderberry plants averaged 3 to 6 rust pustules/plant, the number of leaves increased during the growing season and shoot dry weight was not adversely affected by rust. However, field-grown elderberry plants heavily infected with rust (137 pustules/cane) lost nearly twice as many leaves as controls during the growing season, indicating rust-induced defoliation. Shoot dry weight of these heavily infected canes was also 32% less than that of controls.

Fruiting characteristics of elderberry plants also varied, depending on the number of rust pustules. First and last harvest dates were advanced by the high level of rust infection on 'Wyldewood' elderberry canes, but not by low pustules numbers (< 6 pustules/plant) on 'Bob Gordon' or 'Ozark' plants. Similarly, berry yields were not significantly different at low infection levels, even though rust-infected 'Bob Gordon' plants had a 31% reduction in yield with an estimated $440/ha loss of income. Heavily-infected 'Wyldewood' canes had a significant loss in berry yield (47%) and potential

income ($2,295/ha), assuming a conservative estimate of five canes/plant and a selling price of $4.40/kg fruit.

Puccinia rust did not significantly alter soluble solids, pH, or titratable acidity of berry puree. However, in all experiments, soluble solids of berry puree from rust infected plants were 0.6 to 0.9 °Brix lower than that of puree from infected plants. Soluble solid content of elderberry fruit is already generally low, therefore processors of elderberry products would incur additional costs for sweeteners as a result of rust infected plants. Thus, elderberry rust affects economic returns of processors as well as growers.

In the final study, the causal organism for leaf spot disease was investigated. *Colletotrichum salicis* was isolated from necrotic lesions on elderberry leaflets in 2015. In 2016, elderberry plants were inoculated with *C. salicis* with leaf spots observed 19 days later. From diseased leaf spot lesions, fungal pathogens were re-isolated from inoculated plants as well as control plants. Two other *Colletotrichum* species *C. kahawae* subsp. *ciggaro* and *C. aenigma,* were recovered. Therefore, *Colltotrichum* species in background inocula naturally occur in Missouri. All three species appear similar in terms of symptoms, isolates, and spore morphology, making it difficult to determine the casual agent based upon morphology.