The Curious Poisoned Weed: Poison Ivy Ecology and Physiology

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ABSTRACT

Poison ivy (*Toxicodendron radicans* (L.) Kuntze) is a native perennial liana widely recognized for the production of urushiol, and the associated contact dermatitis it causes in humans. Poison ivy is predicted to become both more prevalent and more noxious in response to projected patterns of global change. Moreover, poison ivy is an important food source for avian species, and urushiol has numerous applications as a high-value engineering material. Thus, this curious weed has many avenues for future concern, and promise. Here, I address gaps in knowledge about poison ivy ecology and physiology so that we may better understand its weediness and utilize its benefits. I address three core areas: poison ivy establishment patterns; biotic interactions with multiple taxa; and the development of molecular tools for use in poison ivy. I found that the early life stage of seedling emergence is a critical linchpin in poison ivy establishment due largely to herbivore pressure from large grazers. I also describe the multifaceted relationship between poison ivy and avian frugivores that not only disperse the drupes of poison ivy but also aid in reduction of fungal endophytic phytopathogens. A survey of poison ivy urushiols yielded that while variation in urushiol congeners was high across individuals, relative congener levels were stable within individuals over a two month period. Lastly I demonstrate best practices for introducing and transiently expressing recombinant DNA in poison ivy as a step towards future reverse genetic procedures.

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GENERAL AUDIENCE ABSTRACT

Poison ivy is a native plant best known for its capacity to cause allergenic skin reactions in humans due to the chemical urushiol, which is found in all parts of the plant. While most people prefer to avoid this plant, poison ivy is an important food source for birds. In addition, urushiol has numerous applications as an engineered material. Despite these positive aspects, poison ivy is among those plants that are responding well to global change, such as increasing CO_2 levels and habitat fragmentation. Poison ivy has been shown to increase in size and produce more allergenic forms of urushiol under elevated CO_2 levels and there are concerns that poison ivy prefers the disturbed areas created by habitat fragmentation. These attributes suggest that poison ivy will become more prevalent and more noxious in the coming years. Thus, this curious weed has many avenues for both future concern and promise. To aid in our ability to manage poison ivy in the future, I used a combination of field, greenhouse, and laboratory studies to study the ecology of poison ivy. I investigated the early stages of the poison ivy life cycle, and the relationship between poison ivy and the animals that interact with it. I found that the earliest life stages of poison ivy are a critical linchpin for poison ivy survival due largely to large animals like deer eating the seedlings. I also describe the multifaceted relationship between poison ivy and birds, which not only disperse the seeds of poison ivy but also aid in reducing pathogens associated with the seeds. I surveyed the amounts and types of urushiols that poison ivy produces and found them to be highly variable from plant to plant, but relatively stable over time within a plant. Lastly, I demonstrate best practices for transient transgene

expression in poison ivy leaves as a step towards future genetic studies. These studies help expand our understanding of a problematic weed, and pave the way for future studies in weed ecology and in the utilization of urushiol in positive applications, showing that even poison ivy can be of benefit to the environment and humans.

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Chapter 1. Literature Review

The Anacardiaceae family is filled with members that cause allergenic contact dermatitis (Gladman 2006). The more common allergenic species of this family include *Toxicodendron radicans* (L.) Kuntze, eastern poison ivy, along with Rydberg poison ivy (*T. rydbergii* (Small ex Rydb.) Greene), poison oak (*T. diversilobum* (Torr. & A. Gray) Greene), poison sumac (*T. vernix* (L.) Kuntze), cashew (*Anacardium occidentale* L.), mango (*Magnifera indica* L.), and the Japanese lacquer tree (*T. vernicifluum* (Stokes) F. Barkley) (Kuntze 1891, Barkley 1937, Gillis 1971). Mango can cause contact dermatitis through the production of alkylresorcinols (Calvert et al. 1996, Oka et al. 2004). Cashew is capable of producing a similar reaction through the production of the related compound, anacardic acid (Rosen and Fordice 1994, Paramashivappa et al. 2001). The genus *Toxicodendron*, comprises plants that produce urushiol, which is responsible for millions of cases of allergenic dermatitis per year in the United States alone (Pariser et al. 2003).

Within *Toxicodendron*, five species are native to the United States: poison sumac (*T. vernix*), two species of poison oak (*T. diversilobum* and *T. toxicarium*), and two species of poison ivy (*T. rydbergii* and *T. radicans*). The poison ivy species are the most common, with *T. radicans* considered by some to be the most widespread member of the Anacardiacea family in the United States (Guin et al. 1981, Habeck 1988). *Toxicodendron radicans,* can be differentiated from *T. rydbergii* partially due to differences in range. While *T. radicans* is a liana found throughout the eastern US, *T. rydbergii* exists primarily north of the 44th parallel, though it can persist on mountain tops as far south as Mexico (Gillis 1971, Guin et al. 1981, Crosby 2004). *Toxicodendron radicans,* here within referred to simply as poison ivy except when comparing subspecies, encompasses nine subspecies; seven being native to North America, one to China, and one to Japan (Gillis 1971, Guin et al. 1981).

Poison ivy is distributed along most of the Eastern and Midwestern United States (Guin et al. 1981), and is the poison ivy most familiar to the public (Crosby 2004). Poison ivy has deciduous, pinnately trifoliate compound leaves (Figure 1.1). However, leaf shape can vary substantially both within and between subspecies. Leaves may be notched/lobed or have smooth margins, a trait that may vary on a single branch depending on leaf age. Leaves can have hairs/trichromes present ranging from pubescent in *T. radicans* ssp. *pubens*, to nearly glabrous such as in *T. radicans* ssp. *verrucosum* (Guin et al. 1981). Commonly the plant is found as a woody climbing vine, or liana, using aerial roots to climb into the canopy, although ground creeping and shrub-like growth habitats may occur, especially in cases where there is no structure for support (Gillis 1971).

Poison ivy is dioecious, with males and females that are often challenging to sex in the field. Both sexes produce a paniculate inflorescence that develops into 2-4mm diameter yellow, green, or white flowers (Gillis 1971, see Figure 1.2). Males tend to flower first, with females following approximately one week later. The flowering period lasts approximately three weeks (personal observations). The exact age at which plants will begin producing flowers is not well characterized. Once pollinated, each female flower will develop into a singular drupe.

The mature drupes of poison ivy comprise a brittle yellow exocarp surrounding a cream waxy mesocarp with black striations, speculated to be resin ducts/canals. The endocarp is tan and hard, allowing it to survive the digestion of avian species (Gillis 1971). The lipid content of the fruits is quite high at 47%, although the small size, 0.25-0.7cm diameter and a mean weight of 0.891g (Gillis 1971), translates into a low caloric yield (0.13KJ) on a per drupe basis (Stiles and White 1986). Poison ivy is reported to be bird dispersed, which could lead to both changes in seed viability and dispersal patterns. Yellow-rumped warblers and grey catbirds will preferentially feed

on poison ivy seeds, especially in the winter months (Suthers et al. 2000). In addition, there are over 50 observed avian associates of poison ivy that consume the fruits (Martin et al. 1951, Senchina 2008). Other known dispersal agents include squirrels. While typically the squirrels act as seed predators, cutting the endocarp and eating the embryo, any drupes collected by the squirrels only to be dropped on the way to their destination would be free to germinate (Penner et al. 1999). Rodents have also been noted to eat poison ivy seed, but likely function more exclusively as granivores due to their feeding habits (Martin et al. 1961).

The viability of seeds following avian digestion remains poorly characterized, and how that viability compares to seeds that do not pass through a bird—both of which are important for dispersal and establishment. The closest system that has been studied is the digestion of *Toxicodendron rydbergii* seeds by roughed grouse done by Penner et al (1999). This investigation found no significant difference in germination rate between drupes where mesocarp was mechanically removed and those digested by roughed grouse. Importantly, both samples were cold stratified by storing outside during the winter months of Manitoba, Canada (Penner et al. 1999). The necessity of cold stratification follows closely with early investigations of poison ivy where it was found that drupes needed some degree of cold treatment in order to break dormancy. In these germination tests, seeds collected from various populations were placed in a petri plate containing wet filter paper for six weeks at 1°C, followed by four weeks at room temperature (Gillis 1971). One additional study found success with a cold treatment for the purpose of poison ivy seed germination (Talley et al. 1996).

However, the necessity of a cold treatment has been brought into question by a recent study of poison ivy germination under laboratory conditions. In this study, investigations using poison ivy drupes collected from Virginia analyzed the efficacy of numerous combinations of seed

treatments to elicit germination. Cold treatment at 4°C was shown to be ineffective (<10%), whether seeds were stored for 2, 4, or 6 weeks prior to returning to room temperature. The germination rate of seeds simply treated by a water rinse with no subsequent cold treatment were not significantly different to any of the various durations of cold storage tested. Benhase and Jelesko (2013) were able to show that a combination of mechanical scarification, sulfuric acid scarification and bleach surface sterilization yielded the highest germination rates in the laboratory, up to 67% (Benhase and Jelesko 2013).

The drupes of poison ivy are suspected to have endophytic fungi that are closely associated with the mesocarp layer. Work done by Benhase and Jelesko showed that treatments of mechanical removal of the exocarp and part of the mesocarp layer, followed by sulfuric acid and a 50% bleach treatment only yielded a 25% drupe sterility rate, meaning some microbes may be present within the remaining drupe mesocarp tissues (Benhase and Jelesko 2013). Further, one fungal endophyte left after this bleach treatment will cause blighting on germinated seedlings if left unchecked. The source of this seedling blighting was determined to be a fungal phytopathogen of poison ivy seedlings *Colletotrichum fioriniae.* Koch's postulates were established for *C. fioriniae* isolated from the drupes of poison ivy causing wilt and blight symptoms in as little as three weeks post inoculation of axenic poison ivy seedlings (Kasson et al. 2014a). These results, coupled with lower fungal colony forming units isolated from seeds passing through the digestive tract of birds compared to their untreated counterparts (Jelesko Lab, unpublished data) suggests an additional benefit of avian frugivory of poison ivy past their apparent benefits to dispersal.

Poison ivy is palatable to a wide range of herbivores. Urushiol seemingly has no effect on North American mammal species. Goats, deer and cattle feed on poison ivy. In fact, goats can be used as a biocontrol for poison ivy, although not very selective or practical (Senchina 2008).

Known arthropod herbivores of poison ivy are abundant (Habeck 1988), and in fact some insects such as the poison-ivy sawfly (*Arge humeralis*) show characteristics that could make them candidates for poison ivy biocontrol (Regas-Williams and Habeck 1979). Although there has been some sporadic interest in using herbivores as biocontrol agents of poison ivy, there is no reported quantitative measure of the impact that herbivory has on poison ivy establishment.

While reproduction of poison ivy can occur vegetatively through rhizomatous growth, seeds are of course necessary for colonization of distant habitats. To this author's knowledge, no study exists where poison ivy seeds have been deliberately planted and habitat preferences investigated. As such, all evidence of poison ivy's proclivity to one habitat or another is the result of observations of resident populations. Poison ivy is capable of readily colonizing areas after fire disturbance, can persist in a wide range of soil types (Gillis 1971, Catling et al. 2002), and has a broad selection of suitable tree hosts on which it can climb (Talley et al. 1996). An observational study conducted by Gillis did not find higher incidence of poison ivy in forest edge habitats (Gillis 1971). However, other surveys contradict this (Fraver 1994, Londré and Schnitzer 2006), especially in instances when disturbance creates edge habitats (Mulligan and Junkins 1977, Buron et al. 1998, Allen et al. 2005). Ultimately, all evidence of poison ivy capacity to colonize specific habitats is purely *post facto* observational, though understanding habitat preferences would facilitate invasion risk assessment.

Poison ivy is among those vines responding positively to climate change. As part of the Duke Free-Air CO_2 Enrichment (FACE) experiment, resident lianas of poison ivy were subjected to elevated levels of atmospheric carbon dioxide. The average biomass of lianas in CO_2 treated plots (200µmol CO_2/mol over ambient levels) was 75% greater and water use efficiency 51% higher than those in control plots. Further, CO_2 enrichment affected urushiol metabolism. Plants

in CO_2 enriched plots saw a 153% increase in the more allergenic forms of urushiol compared to control plants (Mohan 2006, Ziska et al. 2007). Thus, as global change continues to augment both medical and ecological impacts of poison ivy, we need a better understanding of its ecology, particularly the potential for poison ivy to spread to new habitats.

Urushiol is a mixture of various alk(en)yl-catechol congeners, a class of alkylphenols (Symes and Dawson 1953). Urushiols can exist either as a 3-pentadecyl catechol or as a 3-heptadecyl catechol (Figure 1.3). The hydrocarbon tail can be unsaturated or consist of one to three double bonds (Markiewitz and Dawson 1965a). The relative abundance of urushiol congeners differs between the members of *Toxicodendron* genus. In poison ivy, the 3-pentadecyl mono, di and triolefins are most common, while in poison oak the 3-heptadecyl mono, di and triolefins are most common. The double bonds of the olefins are found in the same locations in both the 3-pentadecyl and 3-heptadecyl species at the 8, 11, 13 positions of the hydrocarbon tail (Sunthankar and Dawson 1954, Markiewitz and Dawson 1965a).

The Japanese lac tree (*Toxicodendron vernicifluum*), has a long history in the production of lacquerware products in Asian cultures (Lu et al. 2013). Lacquerware production is based upon the sap from the Japanese lac tree (the Japanese word for this sap is “urushi”) that is spread over wooden objects to form a high luster waterproof natural polymeric coating. The sap is largely comprised of urushiol and lesser amounts of polysaccharides and proteins. One of these proteins is the enzyme laccase that is responsible for oxidation-activated urushiol polymerization through semi- and ortho-quinone intermediates that undergo Michael addition reactions (Kumanotani 1978). The black staining caused by the oxidative polymerization of urushiol can also be used to color textiles (Senchina 2006).

Research into the lacquerware curing processes and the chemical structure of urushiol shows promise as an advanced bio-produced engineering material (Xia et al. 2009, Zheng et al. 2009, Zheng et al. 2014). The chemistry of the ortho-hydroxyls enables the chelation of various metals, and the production of urushiol-copper–co-polymers with anticorrosive properties (Xia et al. 2009). In other applications, urushiol was used to produce super-hydrophobic films fabricated through layer-by-layer assembly (Zheng et al. 2009). These inherent chemical properties hold enormous opportunities for a variety of high performance material science applications.

Of course, the most widely known consequence of human interaction with urushiol is allergenic delayed contact dermatitis resulting from contact with poison ivy/oak/sumac plant material (Howell 1944, Johnson et al. 1972, Epstein 1987, Kawai et al. 1991, Pariser et al. 2003). Interestingly, the allergenic dermatitis reaction is largely restricted to humans. Domesticated animals such as dogs and cats not only show no allergenicity, they may also serve as vectors spreading the oily urushiol to their owners (Crosby 2004). In a few studies, it has been shown that sensitization, and subsequent desensitization to urushiol is possible in guinea pigs, and to a lesser degree in mice and rats, although these results were only attainable after consistent and repeated high exposure (Watson et al. 1981, Dunn et al. 1982a, Murphy et al. 1983b, Watson 1986).

Despite this clinical information and largely anecdotal information on other animals, the ecological function of urushiol in natural habitats is largely unknown (Landsteiner and Jacobs 1936, Gillis 1971, Crosby 2004). Urushiol is found in all poison ivy tissues (Billets et al. 1976, Craig et al. 1978), suggesting it is constitutively produced. The allergenic response in humans at first suggests that urushiol is an anti-herbivory compound, though the lack of a comparable allergenic response in extant wild vertebrate animals questions that hypothesis (Senchina 2005,

2008). Similarly, we have commonly observed signs of apparent insect herbivory in wild poison ivy populations—also questioning the assertion that urushiol an effective antiherbivory defense.

Herbivory also results in tissue damage manifesting as wounds. Thus, another hypothesis is that urushiol is a critical component in a generalized wound response resulting from either herbivory or abiotic mechanical damage (broken limbs due to windstorms, or falling objects). Urushiol polymerizes into a water-proof polymer matrix in the presence of the enzyme laccase, thus sealing an open wound (Kumanotani 1978). Further, urushiol possesses anti-microbial properties (Suk et al. 2011). It is formally possible that urushiol could act as a sealant of wounded plant tissues effectively closing open plant tissues by providing an antibiotic-coating to exposed damaged tissues. Understanding the ecological role of urushiol remains an open area of research.

Dewick (1997) proposed that urushiol synthesis begins with a starter palmitoyl-CoA that is then extended with three malonyl-CoA units by a polyketide synthase (PKS) activity (Dewick 1997, Figure 1.4). Through subsequent keto-reduction, aldol condensation cyclization, dehydroxylation, and aromatization, these starters could yield anacardic acid, which Dewick presumed to be the immediate precursor to urushiol. In a similar fashion, an octadec(en)yl-CoA fatty acid starter would yield urushiols of C_{17} chain lengths. The extension of a fatty acid- CoA starter into a presumed tetraketide intermediate is proposed to be accomplishable by a PKS enzyme. There are three classes of PKS*'s*; Type I, II, and III. Plant type III PKS enzymes are responsible for the catalysis of many complex assemblies of secondary metabolites. In plants, the archetypal type III PKS enzyme functions as chalcone synthase (CHS) in the biosynthesis of flavonoids (Austin and Noel 2003, Abe et al. 2004a).

There are several lines of evidence that support Dewick's proposal that the first step in alkyl-phenol biosynthesis is comprised of a type III PKS. Indeed, in rice a type III PKS enzyme

called alkyresorcinol synthase, utilizes a fatty acid (FA)-CoA starter to both condense and then aromatize a tetraketide intermediate into alkyl-resorcyclic acid (Matsuzawa et al. 2010), which then undergoes an energetically favorable decarboxylation step to yield alkyl-resorcinol. Similar chemistries have been observed in stilbene and chalcone synthases (Schröder 1997, Austin and Noel 2003). The potential for type III PKS-like enzymes to utilize FA-CoA starter molecules and then undergo cyclization and aromatization was confirmed by feeding FA-CoA molecules together with malonyl-CoA to a recombinant CHS enzyme that resulted in both FA-tetraketides and alkyl-pyrones (Abe et al. 2004b). Along similar lines, two plant type III PKS-like enzymes utilize a FA-CoA starter molecule, but require an additional a polyketide cyclase (PKC) (Gagne et al. 2012) enzyme to complete the cyclization/aromatization step(s), resulting in alkyl-di-hydroxy-phenols (Taura et al. 2009, Taura et al. 2016). Thus, the formation of alkyl-phenols likely originate from FA-CoA starters that are elongated by type III PKS-like enzymes to form a FA-tetraketide intermediate that is subsequently cyclized and aromatized by either of two potential pathways (see Figure 1.4).

One possibility for cyclization of the FA-tetraketide is directly by the type III PKS to form alkylresorcyclic acid. However, this reaction would yield a product with meta-hydroxyl groups rather than ortho-hydroxyls. Removal of the 5-hydroxyl may be possible through an hydroxybenzoyl-CoA reductase (HBCR)-like enzyme found in certain Pseudomonas strains that reductively dehydroxylates a benzoate molecule. It is unknown whether this chemistry is possible in plants, as the reaction requires a high activation energy that is only met under anaerobic conditions and require conjugation with a Coenzyme A molecule to lower the activation energy for the reaction to occur (Glöckler et al. 1989, Brackmann and Fuchs 1993). If this reaction occurs in plants, then a thioesterase would be required to remove the CoA portion to yield anacardic acid.

As mentioned above, an alternative pathway for cyclization and aromatization of the FA-tetraketide intermediate is via the subsequent action of a polyketide cyclase (PKC) enzyme. Such is the case for *Cannabis sativa*, in which reactions catalyzed by a type III *PKS* and a polyketide cyclase (PKC) results in the formation olivatolic acid from a hexanol-CoA starter (Gagne et al. 2012). Similarly, in Rhododendron orsellinic acid is formed by the concerted action of the PKS orcinol synthase extending acetyl-CoA into a tetraketide which is cyclized and aromatized by a PKC enzyme to form orsellinic acid (Taura et al. 2016). If this is the case in poison ivy, then removal of the 5-hydroxyl group would occur by the presumed action of a polyketide reductase (PKR) to reduce the 3-keto group in the FA-tetraketide to a hydroxyl, prior to aromatization. Plant polyketide reductase (PKR) enzymes capable of reducing a polyketide to a hydroxyl group have been characterized in *Medicago sativa* and *Papaver somniferum* (Sallaud et al. 1995, Unterlinner et al. 1999).

Regardless of which pathway leads to cyclization and aromatization, both pathways predict the formation of anacardic acid as an intermediate in urushiol biosynthesis (Giessman 1967, Dewick 1997). The pathway from anacardic acid to urushiol remains enigmatic. The decarboxylation of anacardic acid to cardinol is energetically favorable but would require stereochemical specific hydroxylation at the 2-position of cardinol. Alternatively, it is also formally possible that anacardic acid could be concertedly decarboxylated and hydroxylated by a single enzyme analogous to the bacterial NahG-like hydroxylase (Zhang et al. 2013). However, NahG is a category of enzyme that is not known to be present in plants, although it is possible that an analogous enzyme activity arose independently by convergent evolutionary processes that would not show protein sequence similarity. Thus, our understanding of the biochemical steps

between anacardic acid and urushiol are the most poorly understood, largely because there is little precedent for these reactions in the literature.

References:

Abe, I., Y. Utsumi, S. Oguro, and H. Noguchi. 2004a. The first plant type III polyketide synthase that catalyzes formation of aromatic heptaketide. FEBS Lett **562**:171-176.

Abe, I., T. Watanabe, and H. Noguchi. 2004b. Enzymatic formation of long-chain polyketide pyrones by plant type III polyketide synthases. Phytochemistry **65**:2447-2453.

Agrawal, A. A. 2011. Current trends in the evolutionary ecology of plant defence. Functional Ecology **25**:420-432.

Allen, B. P., R. R. Sharitz, and P. C. Goebel. 2005. Twelve years post-hurricane liana dynamics in an old-growth southeastern floodplain forest. Forest Ecology and Management **218**:259-269.

Andrieu, A., J. C. Breitler, C. Sire, D. Meynard, P. Gantet, and E. Guiderdoni. 2012. An in planta, Agrobacterium-mediated transient gene expression method for inducing gene silencing in rice (Oryza sativa L.) leaves. Rice.

Angelo, A., and R. L. Ory. 1983. Lipid degradation during seed deterioration. Phytopathology **73**.

Arcos-Ortega, G. F., R. A. Chan-Kuuk, W. A. Gonzalez-Kantun, R. Souza-Perera, Y. E. Nakazawa-Ueji, E. Aviles-Berzunza, G. Godoy-Hernandez, M. A. Lawton, and J. J. Z. Aguilar. 2010. Agrobacterium tumefaciens-transient genetic transformation of Habanero pepper (Capsicum chinense Jacq.) leaf explants. Electronic Journal of Biotechnology.

Austin, M. B., and J. P. Noel. 2003. The chalcone synthase superfamily of type III polyketide synthases. Natural Product Reports **20**:79-110.

Ausubel, F. A., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 2006. Current Protocols in Molecular Biology. John Wiley & Sons, New York.

Baer, H., and R. T. Bowser. 1963. Antibody production and development of contact skin sensitivity in guinea pigs of various ages. Science **140**:1211-1212.

Baer, H., M. Hooton, H. Fales, A. Wu, and F. Schaub. 1980. Catecholic and other constituents of the leaves of Toxicodendron radicans and variation of urushiol concentrations within one plant. Phytochemistry **19**:799-802.

Bakshi, S., A. Sadhukhan, S. Mishra, and L. Sahoo. 2011. Improved Agrobacterium-mediated transformation of cowpea via sonication and vacuum infiltration. Plant Cell Reports **30**:2281-2292.

Barkley, F. A. 1937. A monographic study of Rhus and its immediate allies in North and Central America, including the West Indies. Annuls of the Missouri Botanical Garden **37**:265-498.

Bengtsson, M., A.-C. Bäckman, I. Liblikas, M. I. Ramirez, A.-K. Borg-Karlson, L. Ansebo, P. Anderson, J. Löfqvist, and P. Witzgall. 2001. Plant Odor Analysis of Apple: Antennal Response of Codling Moth Females to Apple Volatiles during Phenological Development. Journal of Agricultural and Food Chemistry **49**:3736-3741.

Benhase, E. B., and J. G. Jelesko. 2013. Germinating and culturing axenic poison ivy seedlings. HortScience **48**:1525-1529.

Billets, S., J. C. Craig, M. D. Corbett, and J. F. Vickery. 1976. Component analysis of the urushiol content of poison ivy and poison oak. Phytochemistry **15**:533-535.

Boman, J. S., and B. B. Casper. 1995. Differential postdispersal seed predation in disturbed and intact temperate forest. American Midland Naturalist:107-116.

Bowser, R. T., and H. Baer. 1963. Contact Sensitivity and Immunologic Unresponsiveness in Adult Guinea Pigs to a Component of Poison Ivy Extract, 3-N-Pentadecylcatechol. J Immunol **91**:791-794.

Bowser, R. T., R. L. Kirschstein, and H. Baer. 1964. Contact sensitivity in Rhesus monkeys to poison ivy extracts and fluorodinitrobenzene. Pro Soc Exp Biol Med **117**:763-766.

Brackmann, R., and G. Fuchs. 1993. Enzymes of anaerobic metabolism of phenolic compounds. 4-Hydroxybenzoyl-CoA reductase (dehydroxylating) from a denitrifying Pseudomonas species. European Journal of Biochemistry **213**:563-571.

Buron, J., D. Lavigne, K. Grote, R. Takis, and O. Sholes. 1998. Association of Vines and Trees in Second-Growth Forest. Northeastern Naturalist **5**:359-362.

Calvert, M. L., I. Robertson, and H. Samaratunga. 1996. Mango dermatitis: allergic contact dermatitis to *Mangifera indica*. Australas J Dermatol **37**:59-60.

Carey, M. P., B. L. Sanderson, K. A. Barnas, and J. D. Olden. 2012. Native invaders – challenges for science, management, policy, and society. Frontiers in Ecology and the Environment **10**:373-381.

Catling, P. M., A. Sinclair, and D. Cuddy. 2002. Plant community composition and relationships of disturbed and undisturbed alvar woodland. Canadian Field-Naturalist **116**:571-579.

Condon, M. A. S., T. W.; Strain, B. R. 1992. Allocation Patterns in Two Tropical Vines in Response to Increased Atmospheric CO_2. Functional Ecology **6**.

Corbett, M. D., and S. Billets. 1975. Characterization of poison oak urushiol. Journal of pharmaceutical sciences **64**:1715-1718.

Cornille, A., J. G Underhill, A. Cruaud, M. Hossaert-McKey, S. D Johnson, K. Tolley, F. Kjellberg, S. Noort, and M. Proffit. 2011. Floral volatiles, pollinator sharing and diversification in the fig-wasp mutualism: Insights from Ficus natalensis, and its two wasp pollinators (South Africa).

Craig, J. C., C. W. Waller, S. Billets, and M. A. Elsohly. 1978. New GLC analysis of urushiol congeners in different plant parts of poison ivy, Toxicodendron radicans. Journal of pharmaceutical sciences **67**:483-485.

Crosby, D. G. 2004. The poisoned weed: plants toxic to skin. Oxford University Press.

Curtis, M. D., and U. Grossniklaus. 2003. A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol **133**:462-469.

Daniell, H., J. Vivekananda, B. L. Nielsen, G. N. Ye, K. K. Tewari, and J. C. Sanford. 1990. Transient foreign gene expression in chloroplasts of cultured tobacco cells after biolistic delivery of chloroplast vectors. Proc Natl Acad Sci U S A **87**:88-92.

Dewick, P. M. 1997. Medicinal natural products: a biosynthetic approach. John Wiley & Sons, Chichester, UK.

Drummond, M. H., M. P. Gordon, E. W. Nester, and M. D. Chilton. 1977. Foreign DNA of bacterial plasmid origin is transcribed in crown gall tumours [*Agrobacterium tumefaciens*]. Nature **269**:535-536.

Dunn, I. S., D. J. Liberato, R. G. Dennick, N. Castagnoli, and V. S. Byers. 1982a. A murine model system for contact sensitization to poison oak or ivy urushiol components. Cellular Immunology **68**:377-388.

Dunn, I. S., D. J. Liberato, R. G. Dennick, N. Castagnoli, and V. S. Byers. 1982b. A murine model system for contact sensitization to poison oak or ivy urushiol components. Cellular Immunology **68**:377-388.

ElSohly, M. A., P. D. Adawadkar, C.-Y. Ma, and C. E. Turner. 1982. Separation and characterization of poison ivy and poison oak urushiol components. Journal of Natural Products **45**:532-538.

Epstein, W. L. 1987. Plant-induced dermatitis. Annals of Emergency Medicine **16**:950-955.

Epstein, W. L. 1994. Occupational poison ivy and oak dermatitis. Dermatologic Clinics **3**:511-516.

Fraver, S. 1994. Vegetation Responses along Edge-to-Interior Gradients in the Mixed Hardwood Forests of the Roanoke River Basin, North Carolina. Conservation Biology **8**:822-832.

Gagne, S. J., J. M. Stout, E. Liu, Z. Boubakir, S. M. Clark, and J. E. Page. 2012. Identification of olivetolic acid cyclase from *Cannabis sativa* reveals a unique catalytic route to plant polyketides. PNAS **109**:12811-12816.

Gartner, B. L., C. Wasser, E. Rodriguez, and W. L. Epstein. 1993. Seasonal variation of urushiol content in poison oak leaves. Dermatitis **4**:33-36.

Gayer, K. D., and J. W. Burnett. 1988. Toxicodendron dermatitis. Cutis **42**:99-100.

Giessman, T. A. 1967. The biosynthesis of phenolic plant products. Page 1209 *in* P. Bernfeld, editor. The biogenesis of natural compounds. Pergamon Press Ltd.

Gillis, W. T. 1971. The systematics and ecology of poison-ivy and the poison-oaks (Toxicodendron, Anacardiaceae). Rhodora **73**:72-159.

Gladman, A. C. 2006. Toxicodendron dermatitis: poison ivy, oak, and sumac. Wilderness & environmental medicine **17**:120-128.

Glöckler, R., A. Tschech, and G. Fuchs. 1989. Reductive dehydroxylation of 4-hydroxybenzoyl-CoA to benzoyl-CoA in a denitrifying, phenol-degrading Pseudomonas species. FEBS Lett **251**:237-240.

Gross, M., H. Baer, and H. M. Fales. 1975. Urushiols of poisonous Anacardiaceae. Phytochemistry **14**:2263-2266.

Guin, J. D., W. T. Gillis, and J. H. Beaman. 1981. Recognizing the toxicodendrons (poison ivy, poison oak, and poison sumac). Journal of the American Academy of Dermatology **4**:99-114.

Habeck, D. H. 1988. Insects associated with poison ivy and their potential as biological control agents. Pages 6-11 *in* Proceedings of the VII international symposium on biological control of weeds.

Hammerschmidt, R. 1999. Phytoalexins: What have we learned after 60 years? Annual Review of Phytopathology **37**:285-306.

Hill, G. A., V. Mattacotti, and W. D. Graha. 1934a. The toxic principle of the poison ivy. Journal of the American Chemical Society **56**:2736-2738.

Hill, G. A., V. Mattacotti, and W. Graham. 1934b. The toxic principle of the poison ivy. Journal of the American Chemical Society **56**:2736-2738.

Howell, J. B. 1944. Poison ivy smoke. Archives of Dermatology **50**:306-307.

Huang, M., A. M. Sanchez-Moreiras, C. Abel, R. Sohrabi, S. Lee, J. Gershenzon, and D. Tholl. 2012. The major volatile organic compound emitted from Arabidopsis thaliana flowers, the sesquiterpene (E)-β-caryophyllene, is a defense against a bacterial pathogen. New Phytologist **193**:997-1008.

Jelesko, J. G., K. Carter, W. Thompson, K. Kinoshita, and W. Gruissem. 2004. Meiotic recombination between paralogous *RBCSB* genes on sister chromatids of *Arabidopsis thaliana*. Genetics **166**:947-957.

Jelesko, J. G., R. Harper, M. Furuya, and W. Gruissem. 1999. Rare germinal unequal crossing-over leading to recombinant gene formation and gene duplication in Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA. **96**:10302-10307.

Johnson, R. A., H. Baer, C. H. Kirkpatrick, C. R. Dawson, and R. G. Khurana. 1972. Comparison of the contact allergenicity of the four pentadecylcatechols derived from poison ivy urushiol in human subjects. Journal of Allergy and Clinical Immunology **49**:27-35.

Kapila, J., R. DeRycke, M. VanMontagu, and G. Angenon. 1997. An Agrobacterium-mediated transient gene expression system for intact leaves. Plant Science **122**:101-108.

Kasson, M., J. Pollok, E. Benhase, and J. Jelesko. 2014a. First report of seedling blight of eastern poison ivy (*Toxicodendron radicans*) by *Colletotrichum fioriniae* in Virginia. Plant disease **98**:995-995.

Kasson, M. T., J. R. Pollok, E. B. Benhase, and J. G. Jelesko. 2014b. First report of seedling blight of Eastern poison ivy (*Toxicodendron radicans*) by *Colletotrichum fioriniae* in Virginia. Plant disease **98**:995-996.

Kawai, K., M. Nakagawa, F. M. Liew, and H. Yasuno. 1991. Hyposensitization to urushiol among Japanese lacquer craftsmen: results of patch tests on students learning the art of lacquerware. Contact Dermatitis **25**:290-295.

Kessler, A. 2015. The information landscape of plant constitutive and induced secondary metabolite production. Current Opinion in Insect Science **8**:47-53.

Kim, S. S., E. Grienenberger, B. Lallemand, C. C. Colpitts, S. Y. Kim, A. Souza Cde, P. Geoffroy, D. Heintz, D. Krahn, M. Kaiser, E. Kombrink, T. Heitz, D. Y. Suh, M. Legrand, and C. J. Douglas. 2010. LAP6/POLYKETIDE SYNTHASE A and LAP5/POLYKETIDE SYNTHASE B encode hydroxyalkyl alpha-pyrone synthases required for pollen development and sporopollenin biosynthesis in *Arabidopsis thaliana*. Plant Cell **22**:4045-4066.

Kim, S. Y., C. C. Colpitts, G. Wiedemann, C. Jepson, M. Rahimi, J. R. Rothwell, A. D. McInnes, M. Hasebe, R. Reski, B. T. Sterenberg, and D. Y. Suh. 2013. Physcomitrella PpORS, basal to plant type III polyketide synthases in phylogenetic trees, is a very long chain 2'-oxoalkylresorcinol synthase. Journal of Biological Chemistry **288**:2767-2777.

King, J. L., J. J. Finer, and L. K. McHale. 2015. Development and optimization of agroinfiltration for soybean. Plant Cell Reports **34**:133-140.

Klein, T. M., M. Fromm, A. Weissinger, D. Tomes, S. Schaaf, M. Sletten, and J. C. Sanford. 1988. Transfer of foreign genes into intact maize cells with high-velocity microprojectiles. Proc Natl Acad Sci U S A **85**:4305-4309.

Klein, T. M., E. D. Wolf, R. Wu, and J. C. Sanford. 1987. High-Velocity Microprojectiles for Delivering Nucleic-Acids into Living Cells. Nature **327**:70-73.

Kumanotani, J. 1978. Laccase-catalyzed polymerization of urushiol in precisely confined Japanese lacquer system. Die Makromolekulare Chemie **179**:47-61.

Kuntze. 1891. Revisio Generum Plantarum, Liepzig.

Kurtz, A. P., and C. R. Dawson. 1971. Synthesis of compounds structurally related to poison ivy urushiol. 4. 3-(1-Alkyl)alkylcatechols of varying side-chain shape and flexibility. J Med Chem **14**:733-737.

Landsteiner, K., and J. Jacobs. 1936. Studies on the sensitization of animals with simple chemical compounds. II. The Journal of Experimental Medicine **64**:625.

Laurance, W. F., A. S. Andrade, A. Magrach, J. L. C. Camargo, J. J. Valsko, M. Campbell, P. M. Fearnside, W. Edwards, T. E. Lovejoy, and S. G. Laurance. 2014. Long-term changes in liana abundance and forest dynamics in undisturbed Amazonian forests. Ecology **95**:1604-1611.

Londré, R. A., and S. A. Schnitzer. 2006. The distribution of lianas and their change in abundance in temperate forests over the past 45 years. Ecology **87**:2973-2978.

Lu, R., T. Yoshida, and T. Miyakoshi. 2013. Oriental Lacquer: A Natural Polymer. Polymer Reviews **53**:153-191.

Ma, C.-Y., M. A. Elsohly, and J. K. Baker. 1980. High-performance liquid chromatographic separation of urushiol congeners in poison ivy and poison oak. Journal of Chromatography A **200**:163-169.

Majima, R. 1922. Uber den hauptbestandteil des Japan-lacs, VIII. Mitteilung: stellung der doppelbindungen in der seitenkette des urushiols und beweisfuhrung, dab das urushiol eine mischung ist. Berichte der Deutschen Chemischen Gesellschaft **55(b)**:172-191.

Majima, R., and S. Cho. 1907. Ueber einen hauptbestandteil des japanischen lackes. Berichte der Deutschen Chemischen Gesellschaft **40**:632.

Malhi, Y. 2012. The productivity, metabolism and carbon cycle of tropical forest vegetation. Journal of Ecology **100**:65-75.

Mankin, S. L., G. C. Allen, and W. F. Thompson. 1997. Introduction of a plant intron into the luciferase gene of Photinus pyralis. Plant Molecular Biology Reporter **15**:186-196.

Markiewitz, K. H., and C. R. Dawson. 1965a. On the isolation of the allegenically active components of the toxic principle of poison ivy. Journal of Organic Chemistry **30**:1610-1613.

Markiewitz, K. H., and C. R. Dawson. 1965b. On the Isolation of the Allegenically Active Components of the Toxic Principle of Poison Ivy. The Journal of Organic Chemistry **30**:1610-1613.

Martin, A. C., H. S. Zim, and A. L. Nelson. 1951. American wildlife & plants. Dover Publications, Inc., New York, New York, USA.

Martin, A. C., H. S. Zim, and A. L. Nelson. 1961. American wildlife & plants: a guide to wildlife food habits: the use of trees, shrubs, weeds, and herbs by birds and mammals of the United States. Courier Corporation.

Matsuzawa, M., Y. Katsuyama, N. Funa, and S. Horinouchi. 2010. Alkylresorcylic acid synthesis by type III polyketide synthases from rice *Oryza sativa*. Phytochemistry **71**:1059-1067.

Matthew J. Kauffman, and John L. Maron. 2006. Consumers Limit the Abundance and Dynamics of a Perennial Shrub with a Seed Bank. The American Naturalist **168**:454-470.

McKown, A. D., J. Klápště, R. D. Guy, R. Y. Soolanayakanahally, J. La Mantia, I. Porth, O. Skyba, F. Unda, C. J. Douglas, Y. A. El-Kassaby, R. C. Hamelin, S. D. Mansfield, and Q. C. B. Cronk. 2017. Sexual homomorphism in dioecious trees: extensive tests fail to detect sexual dimorphism in Populus. Scientific Reports **7**:1831.

Mitich, L. W. 2000. Kudzu [Pueraria lobata (Willd.) Ohwi]. Weed Technology **14**:231-235.

Mo, R. L., Y. M. Huang, S. C. Yang, Q. L. Zhang, and Z. R. Luo. 2015. Development of Agrobacterium-mediated transient transformation in persimmon (Diospyros kaki Thunb.). Scientia Horticulturae **192**:29-37.

Mohan, J. E., L. H. Ziska, W. H. Schlesinger, R. B. Thomas, R. C. Sicher, K. George, and J. S. Clark. 2006. Biomass and toxicity responses of poison ivy (*Toxicodendron radicans*) to elevated atmospheric CO2. Proc Natl Acad Sci U S A **103**:9086-9089.

Mohan, J. E. Z., L. H.; Schlesinger, W. H. ; Thomas R. B.; Sicher, R. C.; George, K.; and Clark, J. S. 2006. Biomass and toxicity responses of poison ivy(Toxicodendron radicans) to elevated atmospheric CO2. PNAS **103**.

Mulligan, G. A., and B. E. Junkins. 1977. THE BIOLOGY OF CANADIAN WEEDS: 23. Rhus radicans L. Canadian Journal of Plant Science **57**:515-523.

Murcia, C. 1995. Edge effects in fragmented forests: implications for conservation. Trends in Ecology & Evolution **10**:58-62.

Murphy, J. C., E. S. Watson, and E. C. Harland. 1983a. Toxicological evaluation of poison oak urushiol and its esterified derivative. Toxicology **26**:135-142.

Murphy, J. C., E. S. Watson, and E. C. Harland. 1983b. Toxicology evaluation of poison oak urushiol and its esterified derivative. Toxicology **26**:135-142.

Neilson, E. H., J. Q. D. Goodger, I. E. Woodrow, and B. L. Møller. 2013. Plant chemical defense: at what cost? Trends in plant science **18**:250-258.

Oka, K., F. Saito, T. Yasuhara, and A. Sugimoto. 2004. A study of cross-reactions between mango contact allergens and urushiol. Contact Dermatitis **51**:292-296.

Paramashivappa, R., P. P. Kumar, P. J. Vithayathil, and A. S. Rao. 2001. Novel Method for Isolation of Major Phenolic Constituents from Cashew (*Anacardium occidentale* L.) Nut Shell Liquid. Journal of Agricultural and Food Chemistry **49**:2548-2551.

Pariser, D. M., R. I. Ceilley, A. M. Lefkovits, B. E. Katz, and A. S. Paller. 2003. Poison ivy, oak and sumac. Dermatology Insights **4**:26-28.

Penner, R., G. E. E. Moodie, and R. J. Staniforth. 1999. The dispersal of fruits and seeds of Poison-ivy, *Toxicodendron radicans*, by Ruffed Grouse, *Bonasa umbellus*, and squirrels, *Tamiasciurus hudsonicus* and *Sciurus carolinensis*. Canadian Field-Naturalist **113**:616-620.

Popay, I., and R. Field. 1996. Grazing animals as weed control agents. Weed Technology **10**:217-231.

Putz, F. E. 1995. Vines in treetops: consequences of mechanical dependence. Forest canopies.

Rackemann, F. M., and F. A. Simon. 1934. The sensitization of guinea pigs to poison ivy. Science **79**:399.

Regas-Williams, K. A., and D. H. Habeck. 1979. Life history of a poison-ivy sawfly Arge humeralis (Beauvois)(Hymenoptera: Argidae). Florida Entomologist:356-363.

Rosen, T., and D. B. Fordice. 1994. Cashew nut dermatitis. South Medical Journal **87**:543-546.

Sallaud, C., J. El-Turk, C. Breda, D. Buffard, I. de Kozak, R. Esnault, and A. Kondorosi. 1995. Differential expression of cDNA coding for chalcone reductase, a key enzyme of the 5-deoxyflavonoid pathway, under various stress conditions in *Medicago sativa*. Plant Science **109**:179-190.

Sanford, J. C., F. D. Smith, and J. A. Russell. 1993. Optimizing the biolistic process for different biological applications. Methods Enzymol **217**:483-509.

Savatin, D. V., G. Gramegna, V. Modesti, and F. Cervone. 2014. Wounding in the plant tissue: the defense of a dangerous passage. Frontiers in Plant Science **5**:470.

Schafer, M., and P. M. Kotanen. 2003. The influence of soil moisture on losses of buried seeds to fungi. Acta Oecologica **24**:255-263.

Schnitzer, S. A., and F. Bongers. 2011. Increasing liana abundance and biomass in tropical forests: emerging patterns and putative mechanisms. Ecology Letters **14**:397-406.

Schnitzer, S. A., and W. P. Carson. 2010. Lianas suppress tree regeneration and diversity in treefall gaps. Ecology Letters **13**:849-857.

Schröder, J. 1997. A family of plant-specific polyketide synthases: facts and predictions. Trends in plant science **2**:373-378.

Senchina, D. S. 2005. Beetle Interactions with Poison Ivy and Poison Oak (Toxicodendron P. Mill. sect. Toxicodendron, Anacardiaceae). The Coleopterists Bulletin **59**:328-334.

Senchina, D. S. 2006. Ethnobotany of poison ivy, poison oak, and relatives (Toxicodendron spp., Anacardiaceae) in America: Veracity of historical accounts. Rhodora **108**:203-227.

Senchina, D. S. 2008. Fungal and animal associates of Toxicodendron spp.(Anacardiaceae) in North America. Perspectives in Plant Ecology, Evolution and Systematics **10**:197-216.

Senchina, D. S., and K. S. Summerville. 2018. Great diversity of insect floral associates may partially explain ecological success of poison ivy (Toxicodendron radicans subsp. negundo [Greene] Gillis, Anacardiaceae). The Great Lakes Entomologist **40**:3.

Shurtleff, W., and A. Aoyagi. 1977. The Book ofKudzu. Brookline, MA: Autumn Press.

Simberloff, D., L. Souza, M. A. NUnez, M. N. Barrios-Garcia, and W. Bunn. 2012. The natives are restless, but not often and mostly when disturbed. Ecology **93**:598-607.

Stiles, E. W., and D. W. White. 1986. Seed deposition patterns: influence of season, nutrients, and vegetation structure. Pages 45-54 Frugivores and seed dispersal. Springer.

Suk, K. T., S. K. Baik, H. S. Kim, S. M. Park, K. J. Paeng, Y. Uh, I. H. Jang, M. Y. Cho, E. H. Choi, M. J. Kim, and Y. L. Ham. 2011. Antibacterial Effects of the Urushiol Component in the Sap of the Lacquer Tree (*Rhus verniciflua* Stokes) on *Helicobacter pylori*. Helicobacter **16**:434-443.

Sunthankar, S. V., and C. R. Dawson. 1954. The structural identification of the olefinic components of Japanese Lac urushiol. Journal of the American Chemical Society **76**:5070-5074.

Suthers, H. B., J. M. Bickal, and P. G. Rodewald. 2000. Use of successional habitat and fruit resources by songbirds during autumn migration in central New Jersey. The Wilson Bulletin **112**:249-260.

Symes, W. F., and C. R. Dawson. 1953. Separation and structural determination of the olefinic components of poison ivy urushiol, cardanol and cardol. Nature **171**:841-842.

Symes, W. F., and C. R. Dawson. 1954. Poison ivy "urushiol". Journal of the American Chemical Society **76**:2959-2963.

Talley, S. M., R. O. Lawton, and W. N. Setzer. 1996. Host preferences of *Rhus radicans* (Anacardiaceae) in a southern deciduous hardwood forest. Ecology **77**:1271-1276.

Taura, F., M. Iijima, E. Yamanaka, H. Takahashi, H. Kenmoku, H. Saeki, S. Morimoto, Y. Asakawa, F. Kurosaki, and H. Morita. 2016. A Novel Class of Plant Type III Polyketide Synthase Involved in Orsellinic Acid Biosynthesis from Rhododendron dauricum. Frontiers in Plant Science.

Taura, F., S. Tanaka, C. Taguchi, T. Fukamizu, H. Tanaka, Y. Shoyama, and S. Morimoto. 2009. Characterization of olivetol synthase, a polyketide synthase putatively involved in cannabinoid biosynthetic pathway. FEBS Lett **583**:2061-2066.

Theoharides, K. A., and J. S. Dukes. 2007. Plant invasion across space and time: factors affecting nonindigenous species success during four stages of invasion. New Phytologist **176**:256-273.

Unterlinner, B., R. Lenz, and T. M. Kutchan. 1999. Molecular cloning and functional expression of codeinone reductase: the penultimate enzyme in morphine biosynthesis in the opium poppy *Papaver somniferum*. Plant Journal **18**:465-475.

USDA. 2017. The PLANTS Database [Online]. Greensboro, NC 27401-4901. Available: http://plants.usda.gov/core/profile?symbol=TORA2 [Accessed 5/21/2019].

van der Heijden, G. M. F. P., J. S.; Schnitzer, S. A. 2015. Lianas reduce carbon accumulation and storage in tropical forests. PNAS **112**.

van der Heijden, G. M. F. S., S. A;. Powers, J. S.; Phillips, O. L. . 2013. Liana Impacts on Carbon Cycling, Storage and Sequestration in Tropical Forests. Biotropica **45**.

Vander Wall, S. B., K. M. Kuhn, and M. J. Beck. 2005. SEED REMOVAL, SEED PREDATION, AND SECONDARY DISPERSAL. Ecology **86**:801-806.

VanderGheynst, J. S., H. Y. Guo, and C. W. Simmons. 2008. Response surface studies that elucidate the role of infiltration conditions on Agrobacterium tumefaciens-mediated transient transgene expression in harvested switchgrass (Panicum virgatum). Biomass & Bioenergy **32**:372-379.

Voinnet, O., S. Rivas, P. Mestre, and D. Baulcombe. 2003. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J **33**:949-956.

Wang, Y. C., T. M. Klein, M. Fromm, J. Cao, J. C. Sanford, and R. Wu. 1988. Transient expression of foreign genes in rice, wheat and soybean cells following particle bombardment. Plant Mol Biol **11**:433-439.

Watson, E. S. 1986. Toxicodendron hyposensitization programs. Clin Dermatol **4**:160-170.

Watson, E. S., J. C. Murphy, and M. A. ElSohly. 1983. Immunologic studies of poisonous Anacardiaceae: oral desensitization to poison ivy and oak urushiols in guinea pigs. J Invest Dermatol **80**:149-155.

Watson, E. S., J. C. Murphy, P. W. Wirth, M. A. Eisohly, and P. Skierkowski. 1981. Immunological studies of poisonous Anacardiaceae: production of tolerance in guinea pigs using 3-n-pentadecylcatechol-"modified" autologous blood cells. J Pharm Sci **70**:785-789.

Weaver, M. A., R. E. Hoagland, and C. D. Boyette. 2015. Kudzu response to foliar applied herbicides. American Journal of Plant Sciences **6**:856.

Weisberg, A. J., G. Kim, J. H. Westwood, and J. G. Jelesko. 2017. Sequencing and De Novo Assembly of the *Toxicodendron radicans* (Poison Ivy) Transcriptome. Genes (Basel) **8**.

Xia, J. R., Y. L. Xu, B. H. Hu, and J. H. Lin. 2009. A rapid approach to urushiol-copper(I) coordination polymer under UV irradiation. Progress in Organic Coatings **65**:510-513.

Zhang, K., R. Halitschke, C. Yin, C. J. Liu, and S. S. Gan. 2013. Salicylic acid 3-hydroxylase regulates Arabidopsis leaf longevity by mediating salicylic acid catabolism. Proc Natl Acad Sci U S A **110**:14807-14812.

Zheng, X. L., J. B. Weng, Q. M. Huang, B. H. Hu, T. Qiao, and P. Deng. 2009. Fabrication of a stable poly(vinylpyrrolidone)/poly(urushiol) multilayer ultrathin film through layer-by-layer assembly and photo-induced polymerization. Colloids and Surfaces a-Physicochemical and Engineering Aspects **337**:15-20.

Zheng, X. L., J. B. Weng, S. Y. Li, H. Q. Liu, B. H. Hu, Y. H. Li, X. F. Meng, and H. R. Ruan. 2014. Anticorrosive ultrathin film derived from bio-based urushiol-Ti by layer-by-layer self-assembly. Chemical Engineering Journal **245**:265-275.

Ziska, L. H., R. C. Sicher, K. George, and J. E. Mohan. 2007. Rising atmospheric carbon dioxide and potential impacts on the growth and toxicity of poison ivy (*Toxicodendron radicans*). Weed Science **55**:288-292.

Zotz, G., N. Cueni, and C. Körner. 2006. In situ growth stimulation of a temperate zone liana (Hedera helix) in elevated CO2. Functional Ecology **20**:763-769.

Figures:

Figure 1.1: Poison ivy seedling, 3 months after germination.

Figure 1.2: Male flowers. Picture taken in the summer of 2016 at the Virginia Tech Golf Course.

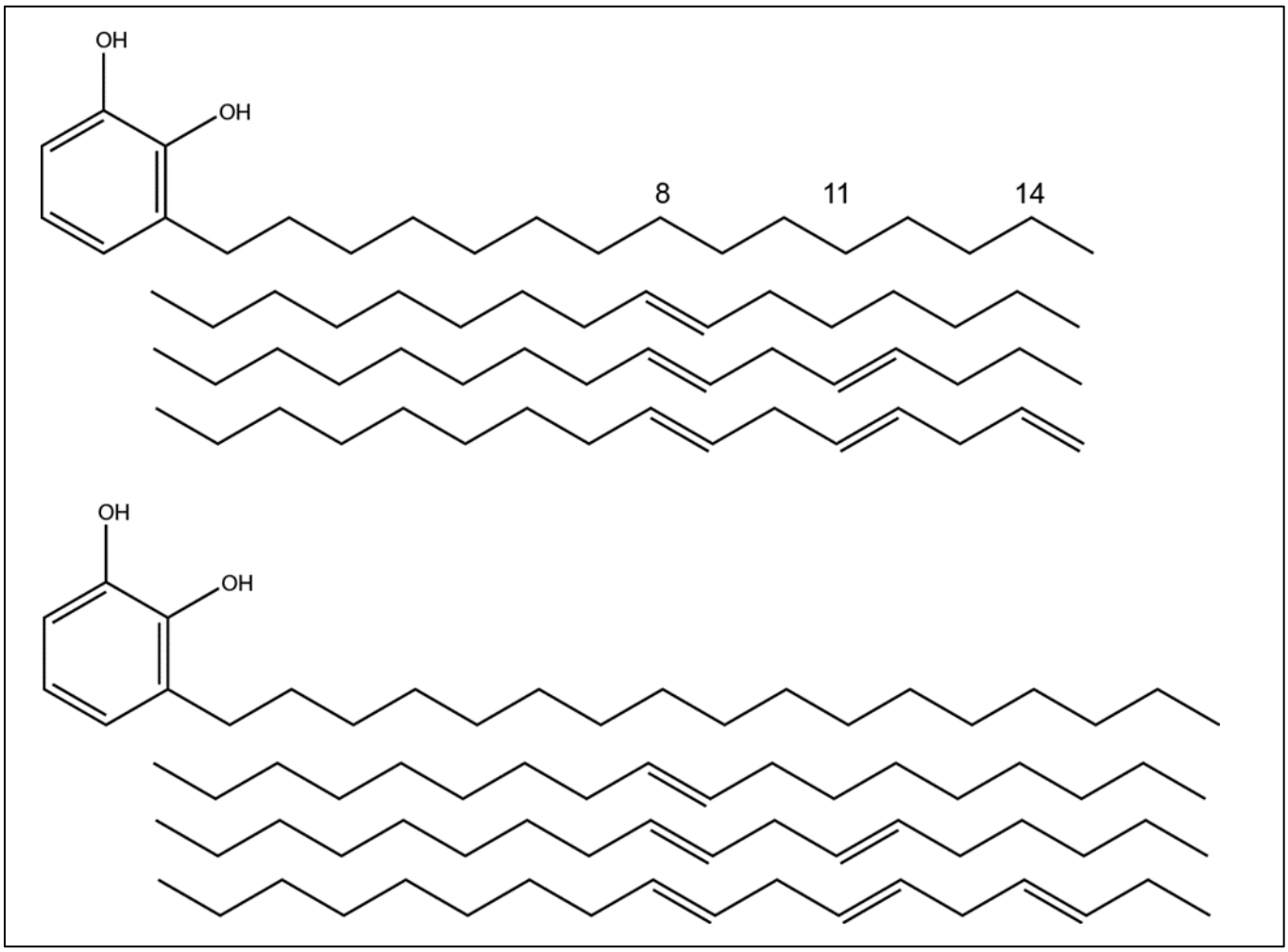

Figure 1.3: **Urushiol congeners found in poison ivy.** Double bonds may either be cis or trans, resulting in slight retention time variation in GC-MS analysis.

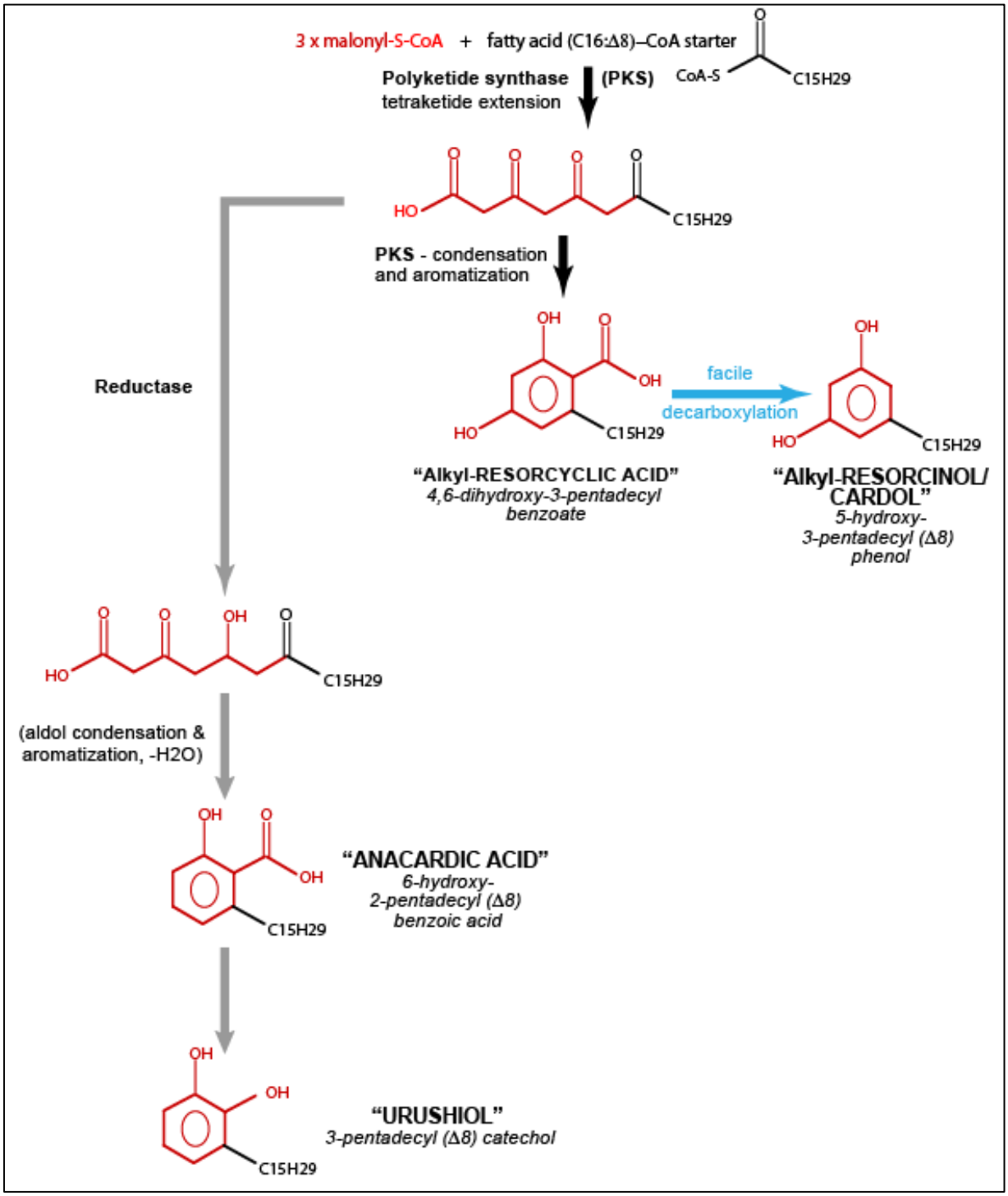

Figure 1.4: **Putative urushiol biosynthetic pathways proposed by Dewick (1997).**

Chapter 2. Investigating the benefits of avian digestion for poison ivy drupes.

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Abstract:

Poison ivy (*Toxicodendron radicans* (L.) Kuntz) is a native weed known for the contact dermatitis symptoms its product urushiol can cause in humans. However, poison ivy drupes also represent an important winter food source for many birds that inhabit or migrate through its range. While it is clear that poison ivy drupes are bird-dispersed, other potential benefits of avian-digestion of the drupes such as priming for germination or phytosanitation are not known, which we explore here. We demonstrated that avian digestion results in reduced fungal colony forming units, thus likely reducing fungal pathogen pressure from the phytopathogen *Colletotrichum fioriniae*, which is known to cause seedling blight in poison ivy. Likely, this is the result of removal of the mesocarp layer of the drupes during avian digestion. The effects of avian digestion on poison ivy drupe germination remains unclear as digestion by some birds, such as grey cat birds and cardinals, were associated with higher germination rates than untreated drupes, but still unable to surpass more than 10% germination under laboratory and greenhouse conditions. Overall, avian frugivory appears to play an additional, although probably small, role in poison ivy establishment beyond drupe dispersal.

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Introduction:

Poison ivy (*Toxicodendron radicans* (L.) Kuntze) is a native weed best known for the allergenic contact dermatitis experienced by those unfortunate enough to be exposed to the urushiol present in this plant species. Urushiol is the generic term for a collection of alk(en)yl-catechol congeners that can have either a C15 or C17 alk(en)yl chain (Majima and Cho 1907, Majima 1922, Hill et al. 1934b, Symes and Dawson 1953, Markiewitz and Dawson 1965b). While urushiol is an effective deterrent for humans, there is a dearth of reports of native wildlife or domesticated animals showing any urushiol-induced/associated allergenic contact dermatitis symptoms. By way of example, goats are effective at eating undesired poison ivy but do not show any aversion or adverse effects of eating poison ivy (Popay and Field 1996). Dogs and cats can brush against poison ivy transferring urushiol to their fur without evidence of allergenic contact dermatitis, but then transfer the urushiol from their fur to their human companions where it results in allergenic dermatitis (Epstein 1994, Crosby 2004). Whether herbivores of poison ivy are somehow able to inactivate the allergen or they are otherwise insensitive to its allergenic effects remains very poorly understood.

Attempts to develop laboratory animal models for human urushiol allergenic dermatitis focused mostly on Guinea pigs and to a lesser degree mice (Rackemann 1934, Dunn, 1982). Guinea pigs display urushiol-specific sensitization resulting in substantial allergenic dermatitis symptoms (Rackemann and Simon 1934). However, unlike humans that show long term immunological memory to urushiol exposure, Guinea pig immunological sensitivity to urushiol greatly diminishes beginning at six months of age (Baer and Bowser 1963, Bowser and Baer 1963). Mice can be sensitized to urushiol, but the allergenic dermatitis symptoms are dramatically less than those observed in either humans or Guinea pigs (Dunn et al. 1982b).

Interestingly, Rhesus macaques are about 1,000-fold less sensitive to urushiol-induced contact dermatitis compared to humans (Bowser et al. 1964).

However, the applicability of these laboratory animal studies to native fauna in natural settings is unclear, and it appears that many native species are able to interact with the plant with no ill effects. Upwards of 50 avian associates of poison ivy have been observed interacting with the plant for either food or nesting material (Martin et al. 1951, Senchina 2008). Goats, deer, squirrels, insects of various orders, and dogs among many other animals show no ill effects to direct exposure to urushiol/poison ivy (Popay and Field 1996, Penner et al. 1999, Senchina 2005). The varied and numerous classes of animals observed interacting, either through herbivory or simply physical contact, suggests that urushiol is not a potent or broadly effective defensive compound against native herbivores.

The drupes of poison ivy may be an important food source for some bird species (Suthers 2000, Stiles 1986). Poison ivy drupes are bird dispersed, and the drupes can survive the digestion by birds (Gillis 1971). The drupes are composed of three layers; a brittle yellow exocarp, waxy mesocarp with black striations and a dense hard endocarp that protects the embryo within. Due to the mesocarp, the lipid content of the fruits is quite high at 47% (Gillis 1971) and the caloric yield has been estimated to be 0.13KJ (Stiles and White 1986). This is somewhat offset by their small size with an average diameter of 0.25-0.7cm and a mean weight of 0.891g (Gillis 1971). Yellow-rumped warblers and grey catbirds will preferentially feed on poison ivy seeds especially in the winter months (Suthers et al. 2000). Squirrels may also disperse drupes but typically act as seed predators with some accidental dropping of the fruits (Penner et al. 1999).

While these observations suggest birds as a primary dispersal vector, only a single study, done on the closely related species, Rydberg's poison ivy (*T. rydbergii*), specifically focused on

the effect of avian digestion on the viability of drupes post-dispersal, in which roughed grouse were the primary avian frugivores. No difference in germination rates resulted between drupes where mesocarp was removed and those digested by roughed grouse (Penner et al. 1999). This may suggest that avian frugivory serves solely as a dispersal mechanism and not as a mechanism to break the dormancy of the drupes of *T. rydbergii*. Past this example, the viability of poison ivy drupes following avian digestion remains largely unknown, and how that viability compares to drupes that do not pass through a bird—both of which are important for poison ivy dispersal and establishment.

Importantly, the study by Penner involved the cold stratification of *T. rydbergii* drupes by storing them outside during the entire winter months of Manitoba, Canada (Penner et al. 1999). The necessity of cold stratification was demonstrated in another study done on *T. radicans* (Gillis 1971). Despite this, the most recent inquiry into poison ivy germination found that cold treatment at 4°C was ineffective (<10% germination) whether drupes were stored for two, four, or six weeks prior to germination (Benhase and Jelesko 2013). Benhase and Jelesko (2013) found that a combination of mechanical and sulfuric acid scarification yielded the highest laboratory germination rate of nearly 45%. Importantly, this study also noted that physical removal of the mesocarp prior to sulfuric acid treatment led to greater dissolution of the outer layers of the endocarp. They proposed that acid-induced pitting of the brachysclereid and osteosclereid cell layers of the endocarp stimulated embryo imbibition, breaking the physical dormancy of the drupes and allowing them to germinate (Benhase and Jelesko 2013). Together, these studies suggest that poison ivy drupes may require a combination of stratification and dissolution of the mesocarp with subsequent pitting of the endocarp to promote germination. The latter two processes suggest that passage of poison ivy drupes through the avian digestion tract may provide

manifold benefits towards promoting poison ivy seedling germination. However, this hypothesis was previously untested.

One last possible benefit poison ivy drupes may receive through avian digestion is the reduction or change of the drupe microbiome. An additional component of the germination protocol developed by Benhase and Jelesko included a 50% bleach treatment. This rather harsh sterilization procedure was only able to yield a 25% drupe sterility rate. The authors postulated that some microbes may be present within drupe tissues as endophytes (Benhase and Jelesko 2013). The drupes that were not effectively sterilized were contaminated with a common fungal species. This fungal species, *Colletotrichum fioriniae,* was later isolated from the drupes of poison ivy and found to be the source of wilt and blight symptoms in the germinating poison ivy seedlings (Kasson et al. 2014). Therefore, avian digestion may reduce fungal pathogen pressure by removal of fungal infected mesocarp tissue similar to the in vitro acid scarification used by Benhase and Jelesko to stimulate in vitro seedling germination.

This study seeks to test several hypotheses arising from the proposed mutualism between poison ivy and its avian dispersal agents. The present study serves to evaluate three key areas relating only to the plant member of the proposed mutualism: 1) how does the removal of the exocarp and mesocarp layers affect the numbers of culturable fungi and bacteria associated with the drupes? 2) to what degree does avian digestion remove the exocarp and mesocarp layers of the drupes? 3) does avian digestion prime the drupes for germination under lab conditions and in soil?

Methods:

Extraction of culturable microbes from drupes:

Drupes were placed into sterilized 1.2mL library tubes along with three 3mm glass beads and 0.5mL of sterile DI water. These tubes were agitated using a Mini-Beadbeater-96 (Biospec Products Inc., Bartlesville, OK) for 2 min. The liquid was allowed to settle for 5 min so that removed mesocarp and exocarp tissue would settle out from the supernatant. The liquid portion was transferred to a sterile 1.5mL centrifuge tube from which eight ten-fold serial dilutions were preformed. Using either LBA media for assessing bacteria or PDA media (Kasson et al. 2014) for fungi, 100µL of each dilution was plated. The drupe was removed from the library tube and placed on 0.5x MS media and incubated in the dark at room temperature to assess seedling germination.

Exocarp removal:

To assess the effect of the presence or removal of the exocarp effect on total colony forming units (CFU), the previously described culturable microbe extraction method was used. Drupes were assessed visually for the presence of the exocarp. Drupes used were collected from the Virginia Tech Golf Course (37.227841, -80.432291) in 2014 and another collection from 2015, with 25 drupes per treatment. Originally, bacteria CFU counts were conducted 2 days post inoculation (dpi), however in some cases, bacteria were growing quite slowly, resulting in no visible colonies on the plate. Thus bacterial and fungal CFU's were counted 4dpi after incubating at room temperature, however this change was made after some bacterial plates were past 4dpi. Data from these drupes were therefore excluded from analysis (n=20, approximately 5 per treatment). Observations of fungal colony morphology were assessed visually, however we did not characterize beyond the presence or absence of *Colletotrichum sps*. Fungal and bacterial CFU data was natural log transformed and fit using a two-way ANOVA with exocarp presence

and collection year as predictors in JMP 13 (SAS Institute Inc., Cary NC). Germination, defined as the emergence of the radicle from the endocarp, was assessed for each drupe.

Digestion by captive house finches:

Poison ivy drupes were offered to captive house finches (*Haemorhous mexicanus*) by placing the drupes within feeding dishes available to 11 birds. Drupes were offered once per day for a period of 120 min. The drupes that passed through the birds were collected from paper placed beneath each cage after each feeding session. These drupes were scored visually using a dissecting scope for the remaining mesocarp cover and placed into three bins of 0-25% remaining, 25-50%, and 50-100% remaining. Drupe culturable fungi load and germination was assessed for 45 digested drupes as previously described. These were compared to 25 drupes that did not pass through birds. CFU data was natural log transformed and fit against the predictor of bird digestion using a one way ANOVA. Alternatively, remaining mesocarp cover was fit as the predictor. Undigested drupes were assigned to another mesocarp cover class in addition to the three previously described. Analysis was performed in JMP 13 (SAS Institute Inc., Cary NC).

Feeding of drupes to wild birds:

Wooden perches were constructed at the Glade Road Research Facility in Blacksburg, VA. Perches were approximately 1.8m tall with a 0.6m cross member at the top to which intact whole poison ivy panicles were secured to bent wires to approximate a branch. Two perches were constructed 17m apart to encourage birds to fly from one perch to the other, and thus increasing their overall time spent on the perches. To collect digested drupes, an elevated net was placed inside an 86cm x 50cm plastic tub installed below each perch. Poison ivy panicles were collected from the Virginia Tech Golf Course (37.227841, -80.432291) in 2015. Capture efficiency, defined as the percentage of drupes that were retrieved, and remaining drupes left on

attached panicles were tracked. Video data from camcorders (Sony, model DCR-SR47) used during daylight hours (approximately from 9AM to 6PM) served to identify which bird species were feeding on the drupes. Video data was collected at least twice per month. Drupes collected from the perches were dried in open petri plates and stored at room temperature.

Germination of drupes post-avian digestion:

Drupes were either left untreated, acid scarified (Benhase and Jelesko 2013), or randomly chosen from the pool of wild avian digested drupes prior to plating on 0.5x MS media petri plates. As analysis of the culturable fungal loads of these drupes was not preformed, there was no need to subject these drupes to bead beating. Avian digested drupes were collected from the feeding perches available to wild birds. One modification was made to the Benhase and Jelesko protocol. Instead of water washes following treatment with bleach, the drupes were removed from the bleach and immediately plated. This resulted in a much higher sterility rate, typically >95%. Twenty-five seeds were plated on a numbered grid to allow for identification of individual seeds. There were eight replications per treatment (N=600). Once plated, each drupe was scored in terms of the mesocarp cover present by using a dissecting microscope (Figure 2.1) according to the following scale: 1) No mesocarp cover present; 2) >5% coverage; 3) 5-25% coverage; 4) 25-50% coverage; 5) 50-75% coverage; 6) 75-100% coverage. This scale was generated using the reference seeds shown Figure 2.1, and was used only to assess mesocarp cover visible on one side of the drupe. Plated drupes were stored in the dark at room temperature. After four days of incubation drupes were scored for the presence or absence of fungal growth. Germination was assessed daily from July 2nd to August 2nd of 2016. Germination data was modeled using a logistic regression containing treatment as the predictor. For the acid-scarified drupes only, germination was fitted against mesocarp cover using a logistic regression, as germination rate

was too low to assess this for the other treatments. Statistical analyses were performed using JMP 13 Pro (SAS Institute Inc., Cary NC).

Effects of soil microbial community on germination:

Soil from Kentland Research Farm (37°11'49.6"N 80°34'54.9"W) was collected and dried. Once dried, soil was sieved in 0.625cm hardware cloth to remove rocks and larger organic material before sifting in 2mm sieve. Half of this soil was sterilized by autoclaving for 60 minutes on two consecutive days. Additionally, sand was autoclaved in the same fashion. Soil and sand were mixed in a 1:1 ratio before filling $5cm^3$ pots. Fifty untreated, acid-scarified or avian digested drupes, collected from the feeding perches, were planted and assigned to trays in a complete random design. Drupes were planted on August 15^{th}, 2016 in a greenhouse. Pots were watered three times a day for 10 min with a 16/8 light hour day and a mean ambient temperature of 21°C. Seedling emergence was tracked on a weekly basis. Weeds were hand-removed, and trays were shifted on the growth tables when emergence data was collected. Emergence data was modeled using a logistic regression containing seed treatment and soil sterilization as fixed effects as well as the interaction between the two main effects. The interaction term was not significant (P-value >0.05) and was removed from the final model. All analyses were performed using JMP 13 Pro (SAS Institute Inc., Cary NC).

Results:

Exocarp removal reduced total culturable microbe load:

Removal of the exocarp layer reduced bacterial and fungal CFUs by approximately 1.8 and 2.7-fold respectively (Table 2.1, Figure 2.2). Fungal counts were generally much lower than bacterial counts but were much closer in the collection of drupes from 2015 compared to 2014. Drupes from the 2014 collection had bacterial counts on average 32-fold higher than the 2015

collection. Fungal CFUs were diametric to this with an approximately 19-fold reduction in 2014 to 2015. This may suggest that there is some inhibitory effect between the two classes of microbes. Colony morphology resembling *Colletotrichum fioriniae* (Kasson et. al., 2014) was observed on all fungal growth plates. None of the drupes in this study germinated in the germination assays. This might have been a consequence of the bead beating procedure used to extract the microbes from the drupes. The bead beating of drupes was a rather violent procedure, so it is possible that the embryos of these drupes may have been inadvertently rendered inviable, resulting in the observed lack of germination.

Avian drupe digestion by captive house finches reduced both mesocarp cover and fungal CFU:

Digestion by birds reduced fungal CFUs by an average of 164-fold (Table 2.2, Figure 2.3) compared to the untreated drupes. Fungal colony morphology resembling *Colletotrichum fioriniae* was observed as the dominant colony morphology from inoculation derived from both avian digested drupes and untreated controls. When evaluated by remaining mesocarp cover over the endocarp rather than simply avian digestion, reduction in remaining mesocarp translated to lower fungal counts (Table 2.2, Figure 2.3). Drupes that passed through the house finches generally resembled those that are mechanically and chemically scarified according the Benhase and Jelesko protocol with some key distinctions. Firstly, while treatment by either method will ubiquitously result in the removal of the readily friable exocarp layer, the artificial acid scarification appeared to be more effective at removing the mesocarp layer. The exact degree to which avian digestion resulted in removal of the mesocarp was evaluated in more detail with the drupes that passed through wild birds. Drupes treated with concentrated sulfuric acid incur pitting of the endocarp brachysclereid and osteosclereid endocarp layers which allows water to

reach the embryo thus triggering germination (Benhase and Jelesko 2013). Such brachysclereid and osteosclereid pitting was not readily observed on the avian digested drupes.

Observations of wild birds feeding on poison ivy drupes:

Drupes were removed quickly from the panicles (upwards of 250 drupes per day) by birds during the summer months, but the drupe removal rate decreased significantly by November and remained low throughout winter (Table 2.3). Grey catbirds (*Dumetella carolinensis*) were the most frequent visitors (64% of all avian visitors), followed by northern cardinals (*Cardinalis cardinalis,* 29%) and rarely by other species in the winter months (Table 2.3). Drupe capture efficiency remained high through November (average of 18.5%), but dramatically reduced along with bird visits from November to February (Table 2.3). Drupes were still removed during the late winter months but it was unclear what animal species were responsible for drupe removal. Additionally, in the winter months cracked endocarps lacking embryos were found frequently and made up the majority of the recovered drupes during this period. Generally, time spent feeding at the perches lasted less than one minute though this time increased in instances where more than one individual was visiting the perch. Grey catbirds almost exclusively swallowed drupes quickly after removal from the panicle, and fed on multiple drupes, and spent an average of 45s at the perch per visit. Cardinals were slower eaters, taking an average of 67s and typically only fed on one drupe per visit.

Germination of drupes post wild bird digestion:

Almost 10% (18 drupes of 200) of the drupes germinated after digestion by wild birds visiting the feeders. In contrast, 70% of acid-scarified drupes (n=200) germinated, while only one untreated drupe germinated (n=200) (Table 2.4). Fungal contamination was nearly

ubiquitous in both untreated and wild avian-digested drupes (~99%), while only a single drupe was contaminated after acid scarification with bleach treatment. Remaining mesocarp cover was correlated with drupe treatment ($R^2 = 0.45$, P-value = <0.0001). Untreated drupes retained their full mesocarp layer however no drupes with exocarp intact were used. Acid-scarified drupes and avian digested treatments were both successful in removing some, but generally not all of the mesocarp layer. However, avian digestion was less efficient with ~20% of drupes retaining an intact mesocarp layer (Figure 2.4). Acid-scarified drupes with less intact mesocarp layers were more likely to germinate (Table 2.4).

Effects of soil microbial community germination:

Emergence rate across all treatments was low and did not vary with soil sterilization (Table 2.5). Acid-scarified drupes preformed best with 27 of 100 drupes producing germinated seedlings, followed by avian-digested drupes with 9 seedlings. No untreated drupes produced germinated seedlings. No evidence of poison ivy seedling blight caused by *C. fioriniae* was observed.

Discussion:

Despite the interest in poison ivy and it's avian associates (see Senchina, 2008), the only prior study to look at the effect of avian digestion on a poison ivy species was conducted on the closely related Rydberg's poison ivy, *Toxicodendron rydbergii* (Penner et al. 1999). The germination results from our *T. radicans* drupes that were eaten by visiting wild birds were more or less in line with the germination rates that Penner (1999) observed with *T. rydbergii* drupes that passed through ruffed grouse. Penner (1999) observed a mean germination rate of 15% (to our 10%) though drupes were treated differently after collection from the bird feces. In the present study, drupes were collected, dried, and stored at room temperature until germination

assays were conducted. In the Penner study, drupes were stored outside during the winter months, thus undergoing cold stratification. Whether this had any effect on germination rate is questionable (see Benhase and Jelesko, 2013).

Penner's control drupes preformed quite differently to ours. Across all of our assays, only a single untreated drupe out of 325 germinated compared to the ~14% success rate observed by Penner. This led to the conclusion by Penner that digestion by rough grouse was not successful in priming poison ivy drupes for germination. Our results may appear to be suggesting the same conclusion, but both studies are limited in that they are not exact representations of what may be occurring in nature. Results from another study conducted by Dickinson et al. (see Chapter 3) demonstrated that untreated poison ivy drupes were capable of producing seedlings at a 25% success rate when planted in a Virginia forest in May. In that same study, acid-scarified drupes were also planted but produced seedling on average two weeks earlier. We suspect this was due to a combination of removal of the mesocarp and pitting of the endocarp speeding up the germination of the acid-scarified drupes. Presumably, the mesocarp and endocarp layers of poison ivy drupes are likely degraded in the soil, which avian digestion may contribute towards by removing some during digestion as we have demonstrated here with both wild birds and house finches. Drupes become available to the birds in the fall. Thus if avian-digestion immediately primes the drupes for germination, the newly germinated seedlings would likely die due to frost and freezing over the winter. Possibly there are other germination cues outside of physical dormancy such as temperature that prevent this from occurring that have not yet been elucidated for poison ivy drupes.

In contrast to the drupes passed through wild birds, germination attempts on drupes that passed through house finches were completely unsuccessful. This could be due to a variety of

reasons. Primarily, drupes that passed through house finches received a secondary treatment of bead beating to extract the culturable microbes from the mesocarp. This process may have killed the embryo in the drupes, but we did not evaluate this possibility. Secondly, drupes that passed through the wild birds were exposed to the elements, including some rain. Further, we are not sure if wild house finches normally feed on poison ivy (see Senchina 2008 for a review of known avian associated of poison ivy), thus captive house finches may not have been the best candidate for studies on avian frugivore-induced poison ivy germination. In contrast, grey catbirds are known to feed on poison ivy drupes (Martin et al. 1961). To our knowledge, northern cardinals have not previously been observed feeding on poison ivy. They were absent from our observation in the winter months, so it is unclear if poison ivy represents a normal cardinal food source, or whether they were simply opportunistic in feeding on a novel food source during the summer. Lastly, while our observations show that only cardinals and grey catbirds fed on poison ivy during the periods where we collected the avian-digested drupes for germination assays, we do not know which drupes passed through which bird species. Further investigations interested in the interaction between specific frugivores and poison ivy would necessitate more direct feeding approaches such as capturing the birds.

Our approach to feeding wild birds had some inherent limitations. Not all bird species will feed on perches. To mitigate this, we attached intact panicles to bent wires to approximate a branch. This would still not be appropriate for ground feeders that may be primarily feeding on drupes that dropped from the panicles or on creeping-habit poison ivy plants. Observations of birds feeding on poison ivy are already numerous, though scattered in the literature (see Senchina 2008 for a review). The results of our observations of feeding primarily serve to identify which birds fed on the poison ivy drupes we collected for our germination assays.

However, we did notice some interesting patterns over the 9-month period feeding was occurring. Feeding was very high in the summer months when poison ivy drupes would not normally be available. The exact cause of this is not clear, though two possibilities seem likely. One possibility may have been the novelty of that type of food source during summer, especially to grey catbirds which will preferentially eat poison ivy drupes during winter (Suthers et al. 2000). Further, feeding during the winter months may have slowed simply because there were other sources of poison ivy drupes available from the poison ivy lianas common in our study area.

We demonstrated that avian digestion can approximate the levels of mesocarp removal achieved by acid-scarification and that removal of this layer is associated with lower numbers of fungi. While the pathogen *C. fioriniae* was not fully removed from the drupes, the 164-fold reduction in overall fungal CFU's suggests a substantial reduction in overall culturable fungal loads, including fungal pathogen pressure. If the exocarp is not already removed prior to avian digestion, it would certainly not be present post digestion. Removal of this layer was also associated with reduced total fungal CFU's. We did not observe any seedling blight in either sterilized on non-sterilized soil in any of our drupe treatments. This evaluation was limited by assessing seedling emergence rather than germination directly. An approach that would allow for scoring germination, in as close to natural soil conditions with control over the microbiome may be logistically impossible. Our two-pronged approach with the control of laboratory conditions followed by our greenhouse approach resulted in very similar germination/emergence rates for drupes digested by wild birds.

This series of experiments demonstrates the multifaceted benefits poison ivy receives due to digestion of its drupes by avian frugivores. In addition to their role as dispersers (Martin et al.

1961, Penner et al. 1999, Suthers et al. 2000), avian frugivores reduce the total culturable fungi present within the mesocarp and exocarp of the fruits. This is accomplished by the removal of the exocarp and mesocarp layers. Whether or not avian digestion primes poison ivy drupes for germination remains an open question. Poison ivy drupes that passed through wild birds (mostly catbirds and cardinals) did show an increase in poison ivy seedling germination over a longer incubation time of 31 days, albeit to a much lesser degree that acid scarification methods, suggesting that avian digestion alone incompletely breaks the physical dormancy of the drupes. In exchange, birds receive food high in lipid content and no apparent negative effects from urushiol (Gillis 1971, Stiles and White 1986). Thus, while poison ivy weed management may be of interest to reduce human allergenic dermatitis, poison ivy remains an important native food source for many animals.

References:

Baer, H., and R. T. Bowser. 1963. Antibody production and development of contact skin sensitivity in guinea pigs of various ages. Science **140**:1211-1212.

Benhase, E. B., and J. G. Jelesko. 2013. Germinating and culturing axenic poison ivy seedlings. HortScience **48**:1525-1529.

Bowser, R. T., and H. Baer. 1963. Contact Sensitivity and Immunologic Unresponsiveness in Adult Guinea Pigs to a Component of Poison Ivy Extract, 3-N-Pentadecylcatechol. Journal of Immunology **91**:791-794.

Bowser, R. T., R. L. Kirschstein, and H. Baer. 1964. Contact sensitivity in Rhesus monkeys to poison ivy extracts and fluorodinitrobenzene. Pro Soc Exp Biol Med **117**:763-766.

Dunn, I. S., D. J. Liberato, R. G. Dennick, N. Castagnoli, and V. S. Byers. 1982. A murine model system for contact sensitization to poison oak or ivy urushiol components. Cellular Immunology **68**:377-388.

Gillis, W. T. 1971. The systematics and ecology of poison-ivy and the poison-oaks (Toxicodendron, Anacardiaceae). Rhodora **73**:72-159.

Hill, G. A., V. Mattacotti, and W. Graham. 1934. The toxic principle of the poison ivy. Journal of the American Chemical Society **56**:2736-2738.

Kasson, M., J. Pollok, E. Benhase, and J. Jelesko. 2014. First report of seedling blight of eastern poison ivy (*Toxicodendron radicans*) by *Colletotrichum fioriniae* in Virginia. Plant disease **98**:995-995.

Majima, R. 1922. Uber den hauptbestandteil des Japan-lacs, VIII. Mitteilung: stellung der doppelbindungen in der seitenkette des urushiols und beweisfuhrung, dab das urushiol eine mischung ist. Berichte der Deutschen Chemischen Gesellschaft **55(b)**:172-191.

Majima, R., and S. Cho. 1907. Ueber einen hauptbestandteil des japanischen lackes. Berichte der Deutschen Chemischen Gesellschaft **40**:632.

Markiewitz, K. H., and C. R. Dawson. 1965. On the Isolation of the Allegenically Active Components of the Toxic Principle of Poison Ivy. The Journal of Organic Chemistry **30**:1610-1613.

Martin, A. C., H. S. Zim, and A. L. Nelson. 1951. American wildlife & plants. Dover Publications, Inc., New York, New York, USA.

Martin, A. C., H. S. Zim, and A. L. Nelson. 1961. American wildlife & plants: a guide to wildlife food habits: the use of trees, shrubs, weeds, and herbs by birds and mammals of the United States. Courier Corporation.

Penner, R., G. E. E. Moodie, and R. J. Staniforth. 1999. The dispersal of fruits and seeds of Poison-ivy, *Toxicodendron radicans*, by Ruffed Grouse, *Bonasa umbellus*, and squirrels, *Tamiasciurus hudsonicus* and *Sciurus carolinensis*. Canadian Field-Naturalist **113**:616-620.

Popay, I., and R. Field. 1996. Grazing animals as weed control agents. Weed Technology **10**:217-231.

Rackemann, F. M., and F. A. Simon. 1934. The sensitization of guinea pigs to poison ivy. Science **79**:399.

Senchina, D. S. 2005. Beetle Interactions with Poison Ivy and Poison Oak (Toxicodendron P. Mill. sect. Toxicodendron, Anacardiaceae). The Coleopterists Bulletin **59**:328-334.

Senchina, D. S. 2008. Fungal and animal associates of Toxicodendron spp.(Anacardiaceae) in North America. Perspectives in Plant Ecology, Evolution and Systematics **10**:197-216.

Stiles, E. W., and D. W. White. 1986. Seed deposition patterns: influence of season, nutrients, and vegetation structure. Pages 45-54 Frugivores and seed dispersal. Springer.

Suthers, H. B., J. M. Bickal, and P. G. Rodewald. 2000. Use of successional habitat and fruit resources by songbirds during autumn migration in central New Jersey. The Wilson Bulletin **112**:249-260.

Symes, W. F., and C. R. Dawson. 1953. Separation and structural determination of the olefinic components of poison ivy urushiol, cardanol and cardol. Nature **171**:841-842.

Talley, S. M., R. O. Lawton, and W. N. Setzer. 1996. Host preferences of *Rhus radicans* (Anacardiaceae) in a southern deciduous hardwood forest. Ecology **77**:1271-1276.

Tables:

Table 2.1: Summary statistics for endocarp removal and drupe collection year on bacterial and fungal CFU counts.

	Log Bacterial CFUs (Exocarp)			Log Fungal CFUs (Exocarp)		
	SS	*F*	*P*	*SS*	*F*	*P*
Exocarp Removal	10.23	7.4	< **0.001**	41.23	37.01	< **0.001**
Collection Year	276.94	200.23	< **0.001**	150.14	134.78	< **0.001**

Table 2.2: Summary statistics for mesocarp removal and avian digestion on fungal CFU counts.

	Log Fungal CFUs (Avian Digestion)		
	SS	*F*	*P*
Mesocarp Cover	631.89	161.81	< **0.001**
Digestion	577.18	271.13	< **0.001**

Table 2.3: Summary of wild bird feeding patterns on poison ivyfrom May 2016- Feburaury 2017.

Date Range	Drupes				Birds				Recordings
	Set Out	Recovered	Untouched	Capture Efficeincy (%)	Catbird	Cardinal	Other	Average Feeding Time (s)	
5/11-5/24	904	107	17	12.06	48	23	0	55.69	7
5/25-6/29	738	161	0	21.82	1	0	0	13.00	1
7/18-8/4	213	66	28	35.68	1	1	1	56.33	2
8/11-8/26	230	47	0	20.43	0	0	0	NA	2
8/26-11/16	250	43	0	17.20	0	0	2	101.50	1
11/16-12/12	240	11	0	4.58	1	0	0	53.00	2
1/17-2/9	450	9	0	2.00	2	0	2	80.75	3

Table 2.4: Summary statistics for drupe treatment on germination of drupes. Additionally, summary statistics for mesocarp cover on germination of acid-scarified drupes only. Excluded terms denoted by (-).

	Germination (All Drupes)		Germination (Acid-Scarified)	
	X^2	*P*	X^2	*P*
Drupe Treatment	304.690	< **0.001**	-	-
Mesocarp Cover	-	-	15.93	**0.007**

Table 2.5: Summary statistics for drupe treatment and soil sterilization on germination of drupes.

	Germination	
	X^2	*P*
Soil Sterilization	0.110	**0.742**
Drupe Treatment	41.980	< **0.001**

Figures:

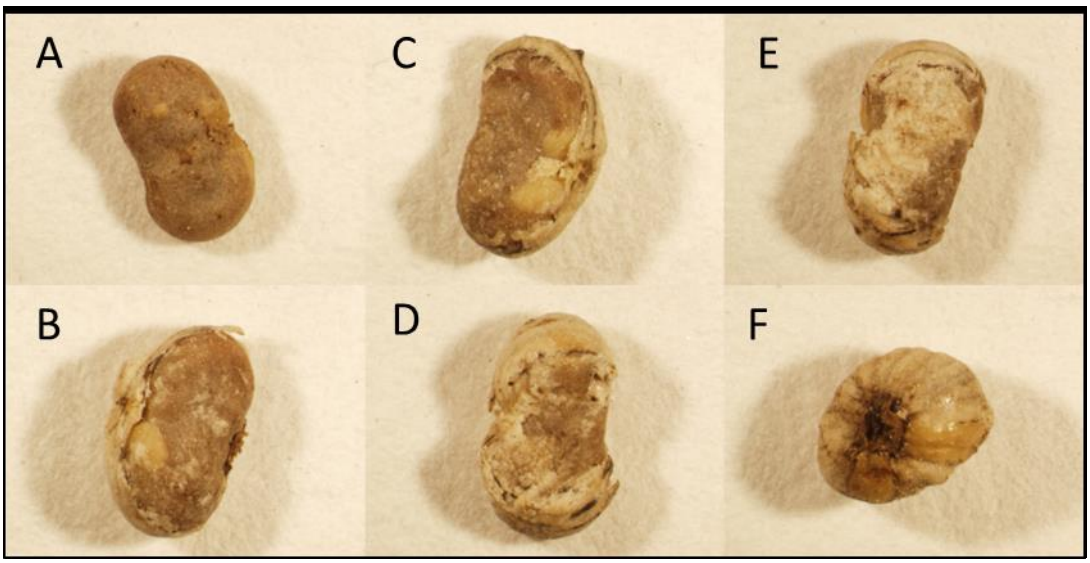

Figure 2.1: Poison ivy drupes post-avian digestion. The digestion removes variable amounts of mesocarp. These seeds were used as a reference for developing a scale for determining mesocarp cover left after digestion. **A)** No mesocarp cover present **B)** >5% coverage **C)** 5-25% coverage **D)** 25-50% coverage **E)** 50-75% coverage **F)** 75-100% coverage.

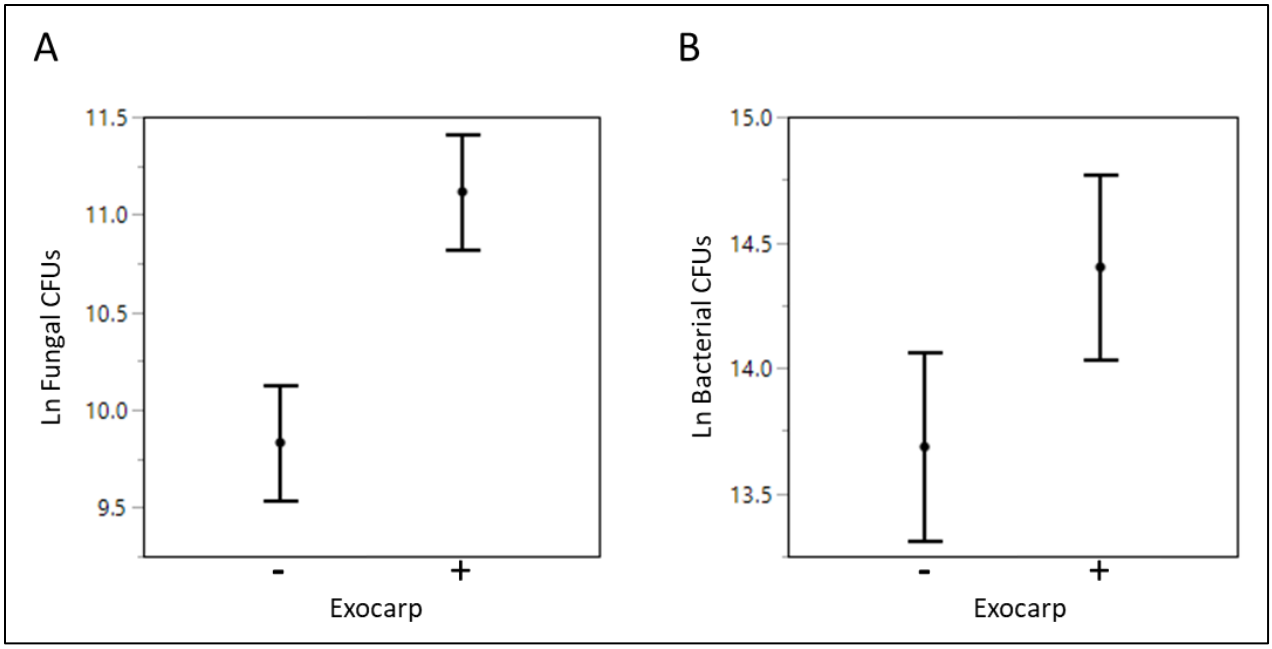

Figure 2.2: Removal of the exocarp drastically reduced the amount of bacterial and fungal CFUs. A) Natural log (Ln) fungal LS mean CFUs cultured from drupes with (+) and without (-) exocarp from both collection years with 95% CI. **B)** Ln bacterial LS mean CFUs cultured from drupes with and without exocarp from both collection years with 95% CI.

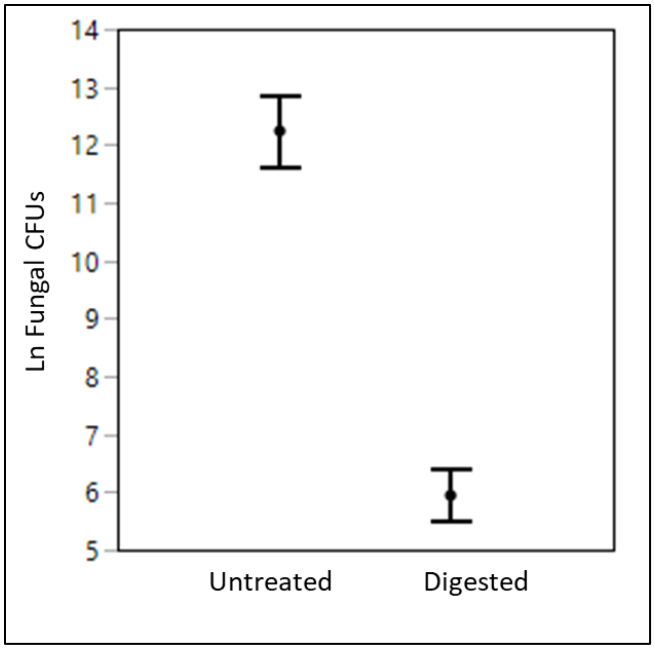

Figure 2.3: Natural log (Ln) fungal LS mean CFUs cultured from drupes either digested by house finches or untreated from both collection years with 95% CI.

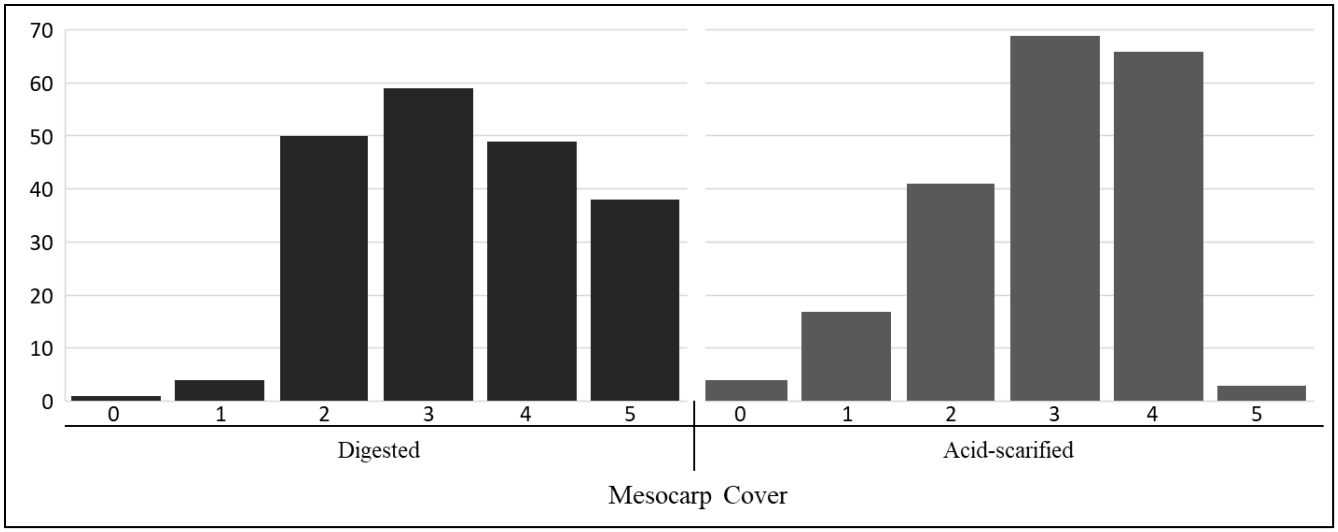

Figure 2.4: Histograms of mesocarp cover classes for avian-digested and acid-scarified poison ivy drupes. Avian-digestion was generally less effective than acid-scarification at removing the mesocarp layer.

Chapter 3. Habitat predictions and establishment limitations of a problematic native liana.

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Abstract:

Poison ivy *(Toxicodendron radicans* (L.) Kuntze) is a North American perennial liana best known for its capacity to cause contact allergenic dermatitis in humans due to the natural product urushiol. Like many lianas, poison ivy seems to respond favorably to several patterns of global change including increased atmospheric CO_2 and habitat disturbance. Further, while a native weed, poison ivy displays some characteristics of an invasive species. The present study aimed to facilitate a better understanding of poison ivy's invasible habitat, population demography, and biotic interactions. Through the lens of invasion stages, we evaluated the colonization and establishment patterns of poison ivy in forest interior and edge habitats. Contrary to previous evaluations on poison ivy germination, we found that untreated drupes established at higher rates than those that underwent simulated avian digestion. While poison ivy initially colonized forest interiors at a higher rate than edge habitats, these seedlings were less likely to survive. This was attributed to higher herbivory pressure; however once established poison ivy was tolerant of most herbivory, except that of large grazers such as deer. Thus, the early life stage of seedling emergence is a critical linchpin in poison ivy establishment. Reduced herbivory pressure of emergent liana seedlings may represent a potentially overlooked mechanism of the rapid increased abundance of lianas in recent decades.

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Introduction:

Lianas, or woody vines, play an important role in forests as competitors of their tree hosts (Schnitzer and Bongers 2011, van der Heijden 2015). As structural parasites, lianas are able to allocate more resources towards the production of leaves, leading to a myriad of advantages, including increased competition for light and structural damage to their host's branches (Putz 1995). Those lianas that can also creep along the ground with offshoots or stolons from a main vine can compete for physical space by smothering slower growing tree seedlings (Schnitzer and Carson 2010). These advantages are further facilitated by increased atmospheric CO_2 levels, as lianas accumulate relatively more biomass than other woody plants (Condon 1992, Mohan 2006, Zotz et al. 2006). Additionally, increased forest fragmentation and management practices are increasing disturbance and forest edges, both of which benefit liana recruitment and establishment (Laurance et al. 2014).

At an ecosystem level, lianas can have dramatic impacts. The invasive kudzu (*Pueraria lobata*) is capable of producing vast dense mats covering many hectares comprising tens of thousands of individual kudzu plants (Mitich 2000). Uncontrolled for a century, a single hectare of kudzu is predicted to spread to 5,250 hectares in the habitat of the Deep South of the United States (Shurtleff and Aoyagi 1977). Thousands of trees are killed annually due to its spread and in Mississippi alone, kudzu accounts for an estimated 54 million dollars per year in timber losses (Weaver et al. 2015). In tropical forests, native lianas are associated with reductions in net forest primary-production (Malhi 2012, van der Heijden 2013). A recent study found that due to increased tree mortality attributed to lianas, tropical forests may be capturing up to 76% less carbon per year than possible (van der Heijden 2015).

Increasingly, we are seeing evidence that some native lianas are responding favorably to human-mediated environmental change. These so-called native invaders exhibit similar ecological and economic impacts as well as the spread and abundance associated with non-native invaders (Carey et al. 2012, Simberloff et al. 2012). For example, the term native invader has been used to describe *Vitis* spp. in the US due to their ability to alter community structure and fire regimes in temperate forests. However, we currently lack basic ecological and population demographic information on most lianas, in particular for temperate lianas. This information will become increasingly important to understand shifting species geographic distributions and how recipient plant communities respond to global change.

Toxicodendron radicans (L) Kuntze (poison ivy) is a perennial liana native to the Eastern US that is best known for its capacity to cause contact dermatitis in humans due to the chemical urushiol found throughout the plant (Epstein 1987, 1994). Evident of poison ivy's emerging role as a weedy species, it is a regulated noxious weed in Minnesota, where it must be managed on any lands open to the public for business or commerce (USDA 2017). Poison ivy is also a listed noxious weed list in Ontario, Quebec, and Manitoba (OMAFRA 1999). Following introduction to Bermuda, the University of Florida and the Commonwealth Institute of Biological Control of Bermuda briefly pursued a biocontrol program in the 1970-1980s (Regas-Williams and Habeck 1979, Habeck 1988).

Like other lianas, poison ivy has been shown to have enhanced performance under elevated CO_2 —though poison ivy stands out for multiple reasons. First, after five years at elevated CO_2 levels, poison ivy accumulated biomass 5-fold more quickly than other woody plants (Mohan 2006). Further, under these conditions poison ivy produced more allergenic forms of urushiol, suggesting that the clinical severity of the dermatological rashes will likely worsen with climate

change (Mohan 2006, Ziska 2007). Additionally, poison ivy appears to favor anthropogenically disturbed and edge habitats (Allen et al. 2005, Londré and Schnitzer 2006), which are also likely to continue to expand with urbanization and habitat fragmentation (Murcia 1995). Poison ivy may also be particularly adept at capitalizing on instances where tree canopy coverage is compromised. Allen et al. (2005) found evidence that poison ivy grew more rapidly on trees with severe limb damage, likely a result of decreased shading. Thus, as global change continues to augment both medical and ecological impacts of poison ivy, a better understanding of its invasible habitat, population demography, and biotic interactions are needed.

Here we aim to facilitate more accurate predictions of the population dynamics and potential expansion, and limitations, of poison ivy to new habitats. Specifically, we will address four key questions: 1) do drupes that pass through a simulated animal gut establish at higher rates than untreated drupes; 2) do seedling establishment rates vary between forest edge and interior habitats?; 3) do specific habitat characteristics predict establishment rates?; and 4) what impacts do seed predation and herbivory have on poison ivy establishment? This work aims to not only provide key insights into the life history of a problematic and expanding native invader, but also to serve as a foundation to understanding the establishment of lianas. Further, we also aim to demonstrate the utility of applying the fundamentals of invasion biology to evaluating native invaders.

Methods:

Establishment of poison ivy in forest interior and edge habitats

Plant material: Drupes for this experiment were collected from the Virginia Tech Golf Course (37.227841, -80.432291) in fall 2015, and were either left untreated ("Untreated") or mechanically and chemically scarified ("Treated") (Benhase and Jelesko 2013). One modification was made to

the Benhase protocol: rather than washing the residual bleach from the drupes with water, the drupes were instead immediately plated onto 0.5x MS media plates, ensuring higher sterility. The "treated" drupes were included to simulate a drupe that was bird dispersed, having the exocarp and mesocarp partially removed (Penner et al. 1999, Benhase and Jelesko 2013). Thus, we were testing whether recruitment varied among drupes that were gravity ("untreated") or bird ("treated") dispersed.

Planting: In addition to testing for drupe conditions effects as outlined above, we were also interested in testing whether poison ivy emergence and establishment varied among edge and interior forest habitats. This study was conducted at the Kentland Research Farm (37°11'49.6"N 80°34'54.9"W) where five replicate sites were chosen that had both an interior and edge habitat. Within each habitat, we used a stratified random design of 10 $1m^2$ plots, with five receiving untreated and five receiving treated drupes. In each plot, 25 drupes were gently depressed into the soil in a 5x5 grid spaced 20cm apart, with a 20cm long bamboo skewer marking the placement of each drupe. In total, 2500 drupes (5 sites x 2 habitats x 10 plots x 25 drupes) were planted on May 18-20^{th}, 2016.

Data Collection: Seedling emergence and survival was tracked weekly from May-August and then biweekly from September-October, 2016. In following years (2017-2018), data collection began once plants broke dormancy (~April) and concluded in October, or until leaves senesced. In July-August of each year, percent light transmittance using an AccuPAR model LP-80 ceptometer, soil moisture using a Dynamax TH300 probe, and percent bare ground was collected per plot. Soil samples were collected on August 26^{th} 2016 for each habitat and analyzed by the Virginia Tech Soil Testing Lab. At the end of the third growing season (2018), we recorded plant height and leaf

number, and cut all aboveground biomass at the soil surface, dried, and weighed for final biomass of all remaining plants.

Analysis: Emergence data from 2016 was modeled using a logistic regression with habitat, treatment, site, and the interaction between habitat and treatment as fixed effects. Additionally, to evaluate the effect of plot-level abiotic factors, emergence data was converted to a percentage at the plot scale, followed by a reverse stepwise linear regression model selection. The initial full model included the following fixed effects: treatment, percent bare ground, percent soil moisture, soil pH, and their full factorial interactions, and site. Percent emergence was arcsine square root transformed to meet model assumptions. Percent bare ground and percent light transmittance were correlated (correlation 0.698, P-value = <0.0001), so only the metric of bare ground was included. High order non-significant effects were removed step-wise until no non-significant interactions remained. Survival data was modeled using a logistic regression containing habitat, treatment, and site as fixed effects as well as the interaction between habitat and treatment. Few remaining plants at the end of the study (n=18) necessitated that Firth bias-adjusted estimates be used. All analyses were performed using JMP 13 Pro (SAS Institute Inc., Cary NC).

Herbivore Exclusion

Field Sites: This study was designed to isolate the effects of various herbivores on poison ivy seedlings. The herbivore exclusion plots were constructed at three of the sites described above, at least 25m from the interior forest establishment plots. Each site contained three replicate blocks in which transplanted poison ivy seedlings were protected from herbivores of various sizes. Each block consisted of four 1.5m^2 plots, within which poison ivy seedling were transplanted into the central 1m^2. The plots were randomly assigned to one of four treatments: 1) no exclusion, or all herbivores allowed; 2) exclusion of large vertebrates; 3) exclusion of large and small vertebrates;

and 4) exclusion of all vertebrate herbivores and insects. Treatment 1 was outside all fencing thus available to all herbivores. Surrounding treatments 2-4 was 2.13m tall deer fencing with a 1.27cm mesh size, in order to exclude deer and other large mammals. For treatment 2 we cut 25.4cm holes at ground level, allowing for smaller animals such as rabbits and rodents to enter, while keeping large animals out. For treatments 3-4, a secondary fence of hardware cloth with a 0.635cm mesh size was buried 30cm, to exclude all vertebrates, but allow insects (Matthew J. Kauffman and John L. Maron 2006). Lastly, treatment 4 was treated monthly with the insecticide Sevin, mixed 120mL per liter of water (TechPac LLC, Atlanta, Georgia) to prevent insect herbivory.

Planting: Drupes from the same collection as above were propagated in growth chambers as previously described (Benhase and Jelesko 2013). After approximately seven weeks, plants were transferred to a greenhouse for one week to reduce transplant shock, and then planted in the field July 10th, 2017, with five seedlings transplanted per plot. Transplants were organized with one at each of the four corners with the fifth in the center. Transplants that died within five days of planting were replaced.

Data Collection: Prior to planting, we recorded height and leaf number of each poison ivy seedling to account for size asymmetries at planting. Survival was recorded weekly for each individual, and any noticeable leaf damage recorded. Damage was initially classified as caused by either disease or herbivores, and then the extent of that damage was recorded either as a percentage of a single compound leaf, or in the case of full removal, a count of missing leaves. This was conducted on the same schedule as the establishment study and culminated in October 2018. At the conclusion of the experiment, 65 weeks after transplanting, height and leaf number were recorded before aboveground plant material was collected, dried, and weighed for biomass. Game cameras were utilized to identify any key herbivores of poison ivy.

Analysis: Survival was modeled using a logistic regression with initial height as a covariate, exclusion treatment, and block nested within site as main effects. To evaluate the effect of herbivory exclusion on plant fitness, final plant height and natural log transformed aboveground biomass were modeled using least squares regression with main effects of exclusion treatment and block nested within site. To account for differences in plant size at planting, initial height was included as a covariate. In both models, a student's T-test was applied to evaluate differences in means between the levels of herbivore exclusion. Some plants experienced disease, possibly as a result of herbivory. As our primary concern for the preceding models was to test whether the exclusion treatment affected plant fitness, plants experiencing disease were included. However, disease incidence was still evaluated using a logistic regression with firth adjusted maximum likelihood. Exclusion treatment and block nested within site were included as main effects with initial height included as a covariate.

The incidence of herbivory occurring in 2018 was fit using a logistic regression to test whether plants that had experienced herbivory in 2017 were more or less likely to experience herbivory in the following year, which was included as a binomial predictor. Further, exclusion treatment, initial height, and block nested within site were included as fixed effects. This model did not include plants that suffered from disease as our focus was strictly to evaluate whether herbivory occurred. All analyses were performed using JMP 13 Pro (SAS Institute Inc., Cary NC).

Additionally, herbivory extent in 2018 was classified into seven levels according to the following: 0) no herbivory, 1) very low, classified as 1-25% of a leaf removed; 2) low, as 25-50% of a leaf removed; 3) moderate, as 50-99% of a leaf removed, 4) high, as 1 full leaf removed, 5) very high, as 2 or more full leaves removed, or 6) death of the plant. Herbivory extent was treated as an ordinal response and fitted using a cumulative link model using a logit link, with exclusion

treatment and block nested within site as fixed effects. This model did not include plants that suffered from disease. This analysis was conducted in R (3.4.3: R Core Team, 2017) using the *ordinal* package.

Post-dispersal drupe predation

This study was designed to identify the fate of poison ivy drupes to predation in order to better evaluate the emergence rates of the establishment study. On August 7th 2018, drupes from the same collection above were placed in 9.5cm^2 plates constructed of 0.635cm mesh hardware cloth and lined with shade cloth. Plates buried in the ground up to the lip of the plates, with 25 drupes per plate. One plate of drupes were placed either within a fencing of 0.625cm mesh hardware cloth, to exclude small and large vertebrates, or in open plots. This was repeated in three blocks at three of the sites from the above study (3 sites x 3 blocks x 2 treatments 18 dishes). After one week, plates were collected and remaining drupes counted.

Analysis: A generalized linear model with a Poisson distribution and log link function with site and exclusion and their interaction as fixed effects was used to analyze the numerical count data of missing drupes using JMP 13 Pro (SAS Institute Inc., Cary NC).

Results:

Establishment of poison ivy in forest interior and edge habitats

Seedling emergence from treated drupes began in early to mid-June, with an average of 26 days after planting. Untreated drupes typically did not emerge until the beginning of July, with an average of 41 days after planting. Despite the later start, untreated drupes resulted in twice as many seedlings as treated drupes (Figure 3.1, Table 3.1). Nearly twice as many poison ivy seedlings emerged in the forest interior habitat than the edge habitat (Figure 3.1). Additionally, more plants

emerged in plots with more bare ground and acidic soils (Table 3.1). Seedling emergence past the first year was exceedingly rare (n=11, 1.8%).

While 608 seedlings resulted from 2500 drupes (24%), only 3% (18 total plants) survived until the end of the study (29 months). Most seedlings died within two months after emergence, with 53 plants (8.8 %) remaining at the beginning of the first winter with another 17 plants not surviving the first winter. In most cases, plants that died in the first year did not show evidence of abiotic stress such as wilting or nutrient deficiency. Rather, seedlings, particularly at the cotyledon stage, were simply missing entirely, likely a result of herbivory, as was apparent on some seedlings that did survive. Plants in edge habitats were much more likely to survive in each year of the study (Figure 3.1). This resulted in more surviving plants in the edge habitat despite the 2:1 advantage interior plants had in initial emergence. The chances of survival did improve in the second and third years, with a 64% survival rate in the second year and a 49% survival rate in the third. Remaining plants were 12.5cm (±3.6) tall, had 2.6 (±1.9) leaves, and 0.325g (±0.17) aboveground biomass, however there was not enough data to evaluate differences between drupe treatments or habitat.

Seedling herbivore exclusion

Seedling survival across all treatments was high (82.5%), and did not vary across treatments (Table 3.3). Final biomass varied among exclusion treatments (P-value = 0.055), with plants open to all herbivores accumulating an average of 32% less biomass than herbivore excluded plants. Plants in the open plots were also shorter by 2.6cm (27%, Figure 3.2, Table 3.4). The incidence of disease did not vary by exclusion treatment with the largest difference between treatments being six diseased plants in the full exclusion treatment versus nine plants in both the no exclusion and small vertebrate exclusion treatments. The incidence of herbivory was also even

across the exclusion treatments (Table 3.3, min. 15 events, max 19 events). After accounting for the influence of disease, twice as many plants died from herbivory in the open plots (n=8) compared to the plants in the full exclusion treatment (n=4). Further, the full exclusion treatment had nearly double the amount of plants with no herbivory compared to those in the open plots (Figure 3.3, Table 3.5.), though overall exclusion treatment was not a strong predictor for herbivory extent (Table 3.6).

Post-dispersal drupe predation

Generally, few drupes were removed regardless of treatment. In one instance, 19 of 25 drupes were removed, and occurred to an unprotected seed holder thus may have been a rare instance of seed predation. After removal of this outlier, there were no differences in drupe predation among treatments (Table 3.2). After excluding this outlier, the next highest amount was the removal of 7 drupes, with an average of 3.2 (±2.3).

Discussion:

Invasion is classically considered to occur in four stages: transportation, colonization, establishment, and spread (Theoharides and Dukes 2007). Analogues of each of these stages can be attributed to native invaders. Colonization, defined here as the initiation of new plants from propagules, and establishment, defined here as the long-term survival of new plants, are directly applicable to native species. Unlike introduced non-native plants which are typically transported across large distances, native plants are already present and have experienced the local conditions for many generations. Thus for native species, transport and spread are inherently coupled and occur over shorter distances than those associated with non-native invasive species. Native species may either expand their range into novel landscapes, or become locally abundant, as a result of disturbances or other anthropogenic changes (Simberloff et al. 2012). However, we lack

information on the conditions likely to facilitate spread and establishment of most native species in response to these changes.

Dispersion, or transport/spread, of poison ivy propagules can occur either clonally over short distances or through avian endozoochory of the drupes (Suthers et al. 2000, Senchina 2008). Recent work suggests mechanical and chemical drupe scarification leads to higher germination rates (Benhase and Jelesko 2013), though drupe viability following bird digestion has not been examined thoroughly to date. However, our study demonstrates that neither (approximated) avian endozoochory, nor cold stratification suggested by earlier reports, are prerequisites for germination and subsequent seedling emergence (Gillis 1971). We found that untreated (raw) drupes produced seedlings at higher rates than those that were mechanically and chemically scarified, which contradicts previous laboratory studies that found enhanced germination of "treated" versus "raw" drupes (Benhase and Jelesko 2013). In a laboratory study, Benhase and Jelesko (2013) observed that drupe treatment with sulfuric acid lead to pitting of the endocarp, which they suspected allowed the embryo to imbibe water and subsequently germinate. This pitting may have led to germination under less favorable conditions in our field experiment, leading to failed seedling emergence. Alternatively, the treated drupes may have been compromised due to decreased defenses in the soil (Angelo and Ory 1983, Schafer and Kotanen 2003). However, we also found a higher seedling emergence rate in more acidic soils, possibly due to more complete or faster degradation of the mesocarp and endocarp. Further, we found essentially no long-term dormancy in poison ivy drupes as demonstrated by the relative lack of emergence of new seedlings in the study's second and third years. Our work provides only initial information on poison ivy drupe-soil dynamics, but at a minimum suggests that bird dispersal is not required for successful recruitment.

Once drupes have been dispersed, successful establishment is contingent on both abiotic and biotic interactions (Theoharides and Dukes 2007). Aside from microbial degradation, seed predation is the most common biotic filter limiting germination (Boman and Casper 1995). For example, Penner et al. (1999) noted that squirrels act as seed predators of *T. rydbergii*, crushing the drupes in their teeth and extracting the embryos, rendering them non-viable. Other rodents may be serving similar roles (Martin et al. 1961). Our evaluation of post-dispersal seed predation suggests squirrels, and other small rodents, that could climb over our exclusion fences, or birds are predating poison ivy drupes. However, we observed low seed predation (~12%), suggesting that under our conditions poison ivy drupes experienced little biotic resistance following dispersal. Further, secondary dispersal may result from attempted seed predation as observed previously with squirrels and *T. rydbergii* (Penner et al. 1999), and well documented in other systems (Vander Wall et al. 2005), offsetting the perceived loss in propagule pressure.

We expected to observe some seedling blight, especially in our untreated drupes due to the fungal pathogen *Colletotrichum fioriniae*. This pathogen has been isolated from the mesocarp of poison ivy drupes and readily causes blight and wilt symptoms on poison ivy seedlings in the laboratory (Benhase and Jelesko 2013, Kasson et al. 2014). No plants in our study demonstrated such symptoms. There are a few possibilities that may explain this. Firstly, our study design necessitated that we could only evaluate seedling emergence and not germination. It is possible that some drupes did germinate, but subsequently died from infection. Another possibility is that in soil the extant microbial community sufficiently lowers the pathogen pressure from *C. fioriniae* by posing competition and restriction of its growth.

Once propagules are dispersed to a new location, fruits have persisted following predation, and the conditions for germination have been met, colonization is the next stage in the invasion

process. Poison ivy is cable of readily colonizing areas after fire disturbance and can persist in a wide range of soil types (Gillis 1971), and has broad host tree suitability (Talley et al. 1996). An observational study conducted by Gillis did not find higher incidence of poison ivy in forest edge habitats (Gillis 1971). However, other surveys contradict this (Fraver 1994, Londré and Schnitzer 2006), especially in instances when disturbance creates edge habitats (Mulligan and Junkins 1977, Buron et al. 1998, Allen et al. 2005). Ultimately, all evidence of larger abundances of poison ivy in edge habitats has been purely observational, thus our work represents the first manipulative empirical investigation.

We found that poison ivy colonization was highest in habitats where resident competition was lowest, using percent bare ground as a proxy. In our study system, this coincided with the forest interior habitats. However, while emergence rate was higher in the forest interior, survival was highest in edge habitats where plant cover was higher. The low survival in the more open interior habitats did not appear to be due to apparent disease or abiotic stress; rather the low survival was most likely due to herbivory. Thus, while barren ground with less competition was beneficial for emergence, being hidden within other plants may have been beneficial for long-term survival, eluding herbivores. While our study did not explicitly evaluate disturbance, edge habitats often occur as the result of disturbance such as canopy gaps following tree death. This pattern is observed in many native invasive plants, which typically only become invasive after disturbances such as changes in fire regimes (Simberloff et al. 2012). Poison ivy has demonstrated higher abundances following both canopy collapse (Allen et al. 2005), and post-fire recolonization (Gillis 1971).

Surprisingly few plants survived until the end of the study. Of the 2500 drupes planted and subsequent 608 seedlings that were found, only 18 survived. However, this could be offset in

several ways. Firstly, poison ivy is capable of reproducing clonally through either rhizomes or stolons. The capacity for poison ivy to reproduce in this manner has not been evaluated, though our personal observations of the sheer number of plantlets found under or near an older liana suggests this could be a major reproductive strategy. An older poison ivy liana is also capable of producing many drupes over its lifespan (Gillis 1971). Thus, a second method to overcome the observed low establishment rate is through the production of many drupes. In other words, like many plants, poison ivy hedges against seedling mortality through other life history strategies.

The establishment phase of invasion is primarily limited by biotic interactions (Theoharides and Dukes 2007). Herbivory of poison ivy can be significant, especially in winter months when other food sources are scarce (Gillis 1971, Penner et al. 1999, Senchina 2008). Urushiol has no apparent effect on non-human mammalian species; with goats, deer, and cattle observed feeding on poison ivy (Senchina 2008). Several arthropods are known to feed on the leaves of poison ivy (Habeck 1988), and in fact some insects such as the poison-ivy sawfly (*Arge humeralis*) show specificity that could make them candidates for biocontrol (Regas-Williams and Habeck 1979). This level of vertebrate and insect herbivory suggests that urushiol is not involved in herbivory defense, at least not as a general defense. Work done to establish an animal model for the observed dermatitis in humans has largely been unsuccessful with severity of reactions dramatically less than those in humans (Bowser et al. 1964, Dunn et al. 1982a), or no long term sensitivity (Baer and Bowser 1963). Thus, the applicability of these animal models to natural settings is lacking and there remains numerous cases of native fauna interacting with poison ivy without demonstrable urushiol sensitivity.

Despite observational reports of various herbivores of poison ivy, there exists no empirical evidence of the identity and impact of herbivores on poison ivy establishment (Senchina 2008). In

contrast to our establishment study, survival in our herbivore exclusion study was quite high at ~80%, even in the unprotected plots. This, compounded with higher survival rates in the later years of the establishment study, suggests that seedlings are most susceptible to herbivory in the first few months following germination. Poison ivy is woody, and our plants that experienced herbivory were usually able to recover and survive. The transplants used in the herbivore exclusion study were approximately two months post germination but because they were grown under ideal laboratory and greenhouse conditions, perhaps they were able to lignify more quickly. For these same reasons, it is unlikely that chemical defenses were higher in our transplants, due to being grown axenically for the first 5 weeks.

While most plants survived, plants open to all herbivores accumulated less biomass than plants with even minimal protection. Our observations recorded some large herbivores in our study area such as bears, turkeys, and raccoons, though these animals showed no interest in the poison ivy seedlings. However, deer were also quite numerous and we suspect them to be the primary herbivores responsible for the herbivory of the unprotected plants. Deer would also be capable of completely removing seedlings as was observed in the establishment study, though we did not confirm this. We suspect that insect herbivores of poison ivy must have been prevalent due to the abundance of herbivory events that resulted in very little removal of leaf material. Insect herbivores of poison ivy are numerous and span many families (Senchina 2005, 2008). This may explain why our insecticide treatment did not fully control insect herbivory.

Our results suggest that the early life stage of seedling emergence and establishment are critical linchpins in poison ivy population demography, and once established appear to be tolerant of most vertebrate herbivory. With many lianas able to outgrow other woody plants under elevated CO_2 levels (Mohan 2006), we might expect an increase in their abundance as more individuals are

able to escape herbivore pressure by climbing into the canopy. As demonstrated by lianas in tropical forests, these plant species are quite capable of competing with their tree hosts, resulting in tree deaths (van der Heijden 2013). Native lianas like poison ivy represent a relatively under-studied threat to forest and human health. We have demonstrated how poison ivy, and likely other lianas, are able to colonize disturbed habitats while also being hindered by large mammals such as deer at early life stages. Liana abundance has dramatically increased in recent decades, presumably through habitat fragmentation, land use changes, and elevated atmospheric CO_2 (Schnitzer and Bongers 2011). Reduced herbivory pressure may also be contributing towards their rapid expansion, representing a potentially overlooked mechanism that warrants further evaluation in other problematic lianas.

References:

Allen, B. P., Sharitz, R. R. & Goebel, P. C. 2005. Twelve years post-hurricane liana dynamics in an old-growth southeastern floodplain forest. *Forest Ecology and Management,* 218**,** 259-269.

Baer, H. & Bowser, R. T. 1963. Antibody production and development of contact skin sensitivity in guinea pigs of various ages. *Science,* 140**,** 1211-1212.

Benhase, E. B. & Jelesko, J. G. 2013. Germinating and culturing axenic poison ivy seedlings. *HortScience,* 48**,** 1525-1529.

Bowser, R. T., Kirschstein, R. L. & Baer, H. 1964. Contact sensitivity in Rhesus monkeys to poison ivy extracts and fluorodinitrobenzene. *Pro Soc Exp Biol Med,* 117**,** 763-766.

Buron, J., Lavigne, D., Grote, K., Takis, R. & Sholes, O. 1998. Association of Vines and Trees in Second-Growth Forest. *Northeastern Naturalist,* 5**,** 359-362.

Carey, M. P., Sanderson, B. L., Barnas, K. A. & Olden, J. D. 2012. Native invaders – challenges for science, management, policy, and society. *Frontiers in Ecology and the Environment,* 10**,** 373-381.

Condon, M. A. S., T. W.; Strain, B. R. 1992. Allocation Patterns in Two Tropical Vines in Response to Increased Atmospheric CO_2. *Functional Ecology,* 6.

Dunn, I. S., Liberato, D. J., Dennick, R. G., Castagnoli, N. & Byers, V. S. 1982. A murine model system for contact sensitization to poison oak or ivy urushiol components. *Cellular Immunology,* 68**,** 377-388.

Epstein, W. L. 1987. Plant-induced dermatitis. *Annals of Emergency Medicine,* 16**,** 950-955.

Epstein, W. L. 1994. Occupational poison ivy and oak dermatitis. *Dermatologic Clinics,* 3**,** 511-516.

Fraver, S. 1994. Vegetation Responses along Edge-to-Interior Gradients in the Mixed Hardwood Forests of the Roanoke River Basin, North Carolina. *Conservation Biology,* 8**,** 822-832.

Gillis, W. T. 1971. The systematics and ecology of poison-ivy and the poison-oaks (Toxicodendron, Anacardiaceae). *Rhodora,* 73**,** 72-159.

Habeck, D. H. Insects associated with poison ivy and their potential as biological control agents. Proceedings of the VII international symposium on biological control of weeds, 1988. 6-11.

Kasson, M., Pollok, J., Benhase, E. & Jelesko, J. 2014. First report of seedling blight of eastern poison ivy (*Toxicodendron radicans*) by *Colletotrichum fioriniae* in Virginia. *Plant disease,* 98**,** 995-995.

Laurance, W. F., Andrade, A. S., Magrach, A., Camargo, J. L. C., Valsko, J. J., Campbell, M., Fearnside, P. M., Edwards, W., Lovejoy, T. E. & Laurance, S. G. 2014. Long-term changes in liana abundance and forest dynamics in undisturbed Amazonian forests. *Ecology,* 95**,** 1604-1611.

Londré, R. A. & Schnitzer, S. A. 2006. The distribution of lianas and their change in abundance in temperate forests over the past 45 years. *Ecology,* 87**,** 2973-2978.

Malhi, Y. 2012. The productivity, metabolism and carbon cycle of tropical forest vegetation. *Journal of Ecology,* 100**,** 65-75.

Maron, J. L. & Crone, E. 2006. Herbivory: effects on plant abundance, distribution and population growth. *Proceedings of the Royal Society B: Biological Sciences,* 273**,** 2575-2584.

Maron, J. L. & Vila, M. 2001. When do herbivores affect plant invasion? Evidence for the natural enemies and biotic resistance hypotheses. *Oikos,* 95**,** 361-373.

Martin, A. C., Zim, H. S. & Nelson, A. L. 1961. *American wildlife & plants: a guide to wildlife food habits: the use of trees, shrubs, weeds, and herbs by birds and mammals of the United States,* Courier Corporation.

Matthew J. Kauffman & John L. Maron 2006. Consumers Limit the Abundance and Dynamics of a Perennial Shrub with a Seed Bank. *The American Naturalist,* 168**,** 454-470.

Mitich, L. W. 2000. Kudzu [Pueraria lobata (Willd.) Ohwi]. *Weed Technology,* 14**,** 231-235.

Mohan, J. E. Z., L. H.; Schlesinger, W. H. ; Thomas R. B.; Sicher, R. C.; George, K.; and Clark, J. S. 2006. Biomass and toxicity responses of poison ivy(Toxicodendron radicans) to elevated atmospheric CO2. *PNAS,* 103.

Mulligan, G. A. & Junkins, B. E. 1977. THE BIOLOGY OF CANADIAN WEEDS: 23. Rhus radicans L. *Canadian Journal of Plant Science,* 57**,** 515-523.

OMAFRA. 1999. Poison Ivy FactSheet [Online]. Available: http://www.omafra.gov.on.ca/english/crops/facts/99-015.htm [Accessed 4/23/2019]

Penner, R., Moodie, G. E. E. & Staniforth, R. J. 1999. The dispersal of fruits and seeds of Poison-ivy, *Toxicodendron radicans*, by Ruffed Grouse, *Bonasa umbellus*, and squirrels, *Tamiasciurus hudsonicus* and *Sciurus carolinensis*. *Canadian Field-Naturalist,* 113**,** 616-620.

Putz, F. E. 1995. Vines in treetops: consequences of mechanical dependence. *Forest canopies.*

Regas-Williams, K. A. & Habeck, D. H. 1979. Life history of a poison-ivy sawfly Arge humeralis (Beauvois)(Hymenoptera: Argidae). *Florida Entomologist***,** 356-363.

Schnitzer, S. A. & Bongers, F. 2011. Increasing liana abundance and biomass in tropical forests: emerging patterns and putative mechanisms. *Ecology Letters,* 14**,** 397-406.

Schnitzer, S. A. & Carson, W. P. 2010. Lianas suppress tree regeneration and diversity in treefall gaps. *Ecology Letters,* 13**,** 849-857.

Senchina, D. S. 2008. Fungal and animal associates of Toxicodendron spp.(Anacardiaceae) in North America. *Perspectives in Plant Ecology, Evolution and Systematics,* 10**,** 197-216.

Shurtleff, W. & Aoyagi, A. 1977. The Book ofKudzu. Brookline, MA: Autumn Press.

Simberloff, D., Souza, L., NUnez, M. A., Barrios-Garcia, M. N. & Bunn, W. 2012. The natives are restless, but not often and mostly when disturbed. *Ecology,* 93**,** 598-607.

Suthers, H. B., Bickal, J. M. & Rodewald, P. G. 2000. Use of successional habitat and fruit resources by songbirds during autumn migration in central New Jersey. *The Wilson Bulletin,* 112**,** 249-260.

Talley, S. M., Lawton, R. O. & Setzer, W. N. 1996. Host preferences of *Rhus radicans* (Anacardiaceae) in a southern deciduous hardwood forest. *Ecology,* 77**,** 1271-1276.

Theoharides, K. A. & Dukes, J. S. 2007. Plant invasion across space and time: factors affecting nonindigenous species success during four stages of invasion. *New Phytologist,* 176**,** 256-273.

USDA. 2017. *The PLANTS Database* [Online]. Greensboro, NC 27401-4901. Available: http://plants.usda.gov/core/profile?symbol=TORA2 [Accessed 5/21/2019].

van der Heijden, G. M. F. P., J. S.; Schnitzer, S. A. 2015. Lianas reduce carbon accumulation and storage in tropical forests. *PNAS,* 112.

van der Heijden, G. M. F. S., S. A;. Powers, J. S.; Phillips, O. L. 2013. Liana Impacts on Carbon Cycling, Storage and Sequestration in Tropical Forests. *Biotropica,* 45.

Weaver, M. A., Hoagland, R. E. & Boyette, C. D. 2015. Kudzu response to foliar applied herbicides. *American Journal of Plant Sciences,* 6**,** 856.

Ziska, L. H. S., R. C.; George, K.; and Mohan, J. E. 2007. Rising Atmospheric Carbon Dioxide and Potential Impacts on the Growth and Toxicity of Poison Ivy (Toxicodendron radicans). *Weed Science,* 55.

Zotz, G., Cueni, N. & Körner, C. 2006. In situ growth stimulation of a temperate zone liana (Hedera helix) in elevated CO2. *Functional Ecology,* 20**,** 763-769.

Tables:

Table 3.1: Summary statistics for each treatment on seedling emergence in the first year and survival over the course of the study. Additionally, summary statistics for site characteristics on percent seedling emergence per plot in the first year. F-statistics are shown for general linear models and X^2-statistic for logistic regressions. Excluded terms denoted by (-).

	Emergence		Survival		Percent Emergence		
	X^2	*P*	X^2	*P*	*SS*	*F*	*P*
Habitat	105.3	< **0.001**	11.5	< **0.001**	-	-	-
Treatment	90.4	< **0.001**	3.2	0.076	1.03	31.24	< **0.001**
Site	60.2	< **0.001**	17	**0.002**	0.53	4.05	**0.005**
Habitat x Treatment	2.5	0.116	0.6	0.446	-	-	-
% Bare Ground	-	-	-	-	0.4	12.04	< **0.001**
% Soil Moisture	-	-	-	-	0.06	1.71	0.194
Soil pH	-	-	-	-	0.23	7.02	**0.01**

Table 3.2: Summary statistics for site and protection treatment on the count of missing drupes from petri dishes.

	Missing Drupes	
	X^2	*P*
Protection	0.106	0.745
Site	0.179	0.914

Table 3.3: Summary statistics for each treatment on the incidence of herbivory and disease, and survival over the course of the study. Excluded terms denoted by (-).

	Incidence of Herbivory		Incidence of Disease		Survival	
	X^2	*P*	X^2	*P*	X^2	*P*
Treatment	1.13	0.770	0.71	0.871	3.59	0.309
Block[Site]	6.83	0.337	14.49	**0.025**	8.55	0.200
Site	27.26	< **0.001**	5.29	0.071	5.84	**0.054**
Initial Height	9.80	0.002	0.57	0.450	0.54	0.461
Herbivory in 2017	0.14	0.706	-	-	-	-

Table 3.4: Summary statistics for exclusion treatment on log plant above ground biomass and plant height.

	Log Biomass			Height		
	SS	*F*	*P*	*SS*	*F*	*P*
Treatment	4.41	2.6	**0.055**	213.7	4.59	**0.004**
Site	1.9	1.68	0.191	154.53	4.97	**0.008**
Initial Height	9.09	16.04	< **0.001**	702.76	45.21	< **0.001**
Block[Site]	3.75	1.1	0.363	141.81	1.52	0.176

Table 3.5: Contrasts showing effects of exclusion treatments on herbivory extent. A positive contrast indicates that plants in this treatment experienced more severe herbivory than those in the full exclusion treatment.

	Herbivory Extent		
	B± *se*	*z*	*P*
No Exclusion	1.019 ± 0.442	2.31	**0.021**
Large Vertebrate	0.562 ± 0.437	1.29	0.198
Small Vertebrate	0.350 ± 0.440	0.8	0.426

Table 3.6: Summary statistics for site, block nested within site and protection treatment on the extent of herbivory damage experienced by plants.

	Herbivory Extent	
	X^2	*P*
Treatment	5.367	0.147
Block[Site]	4.650	0.199
Site	0.687	0.709

Figures:

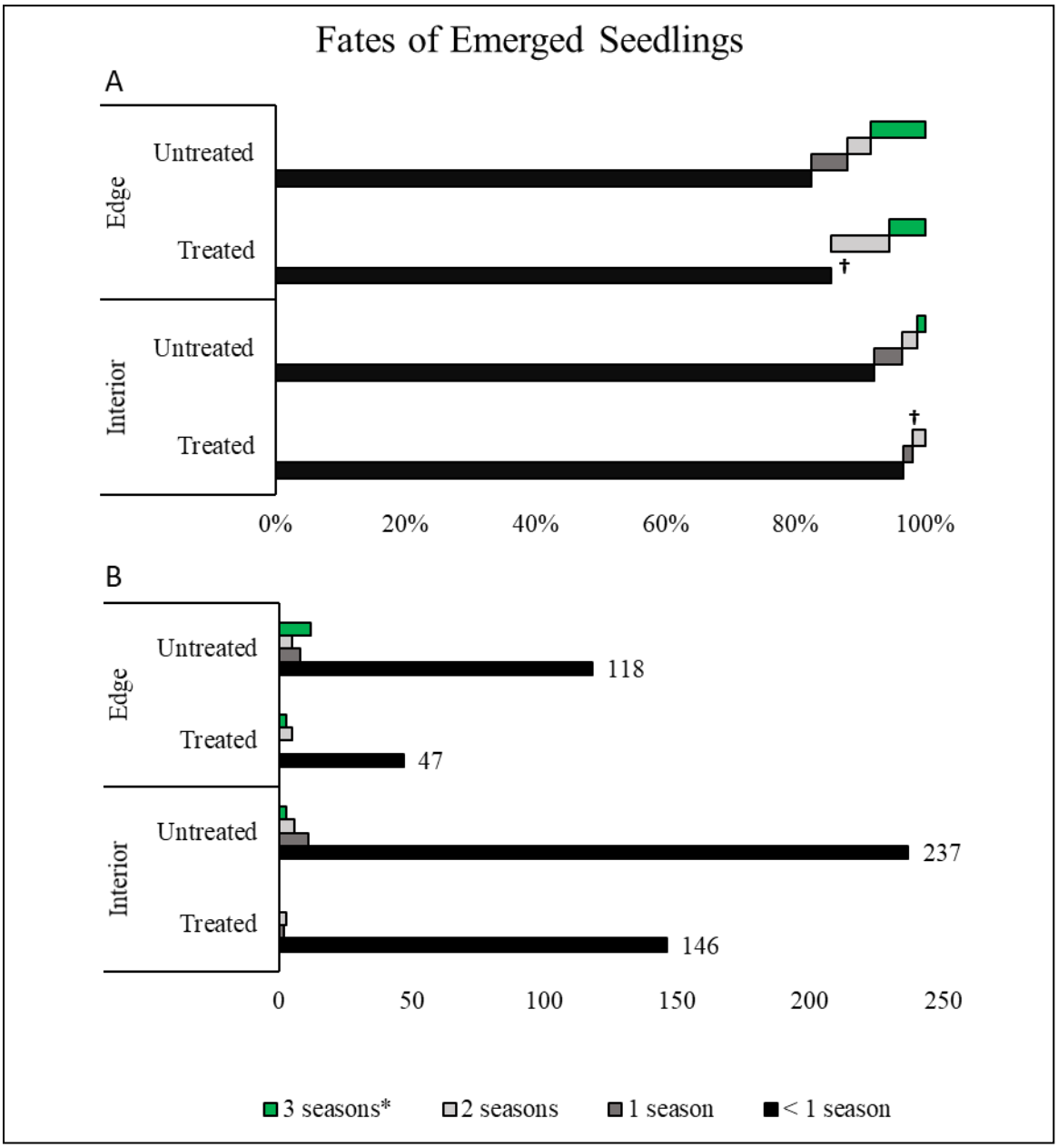

Figure 3.1: Distribution of seedling fates represented as (A) percentage within treatment and (B) raw counts. Drupes that did not produced seedlings were excluded. Plants that survived to the third season (*) were collected and height, biomass, and leaves produced were recorded. A (†) indicates that there were no plants whose fate placed them within that category.

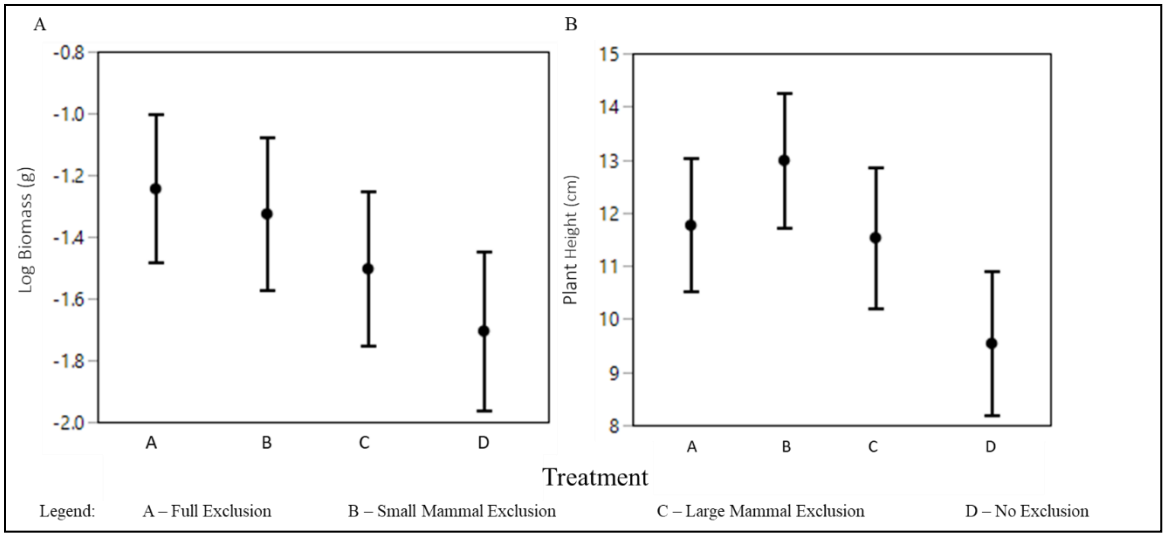

Figure 3.2: A) Log biomass LS means across exclusion treatments with 95% CI's. B) LS mean plant height across exclusion treatments with 95% CI's. In both growth metrics, performance of plants receiving no protection from herbivores was hindered.

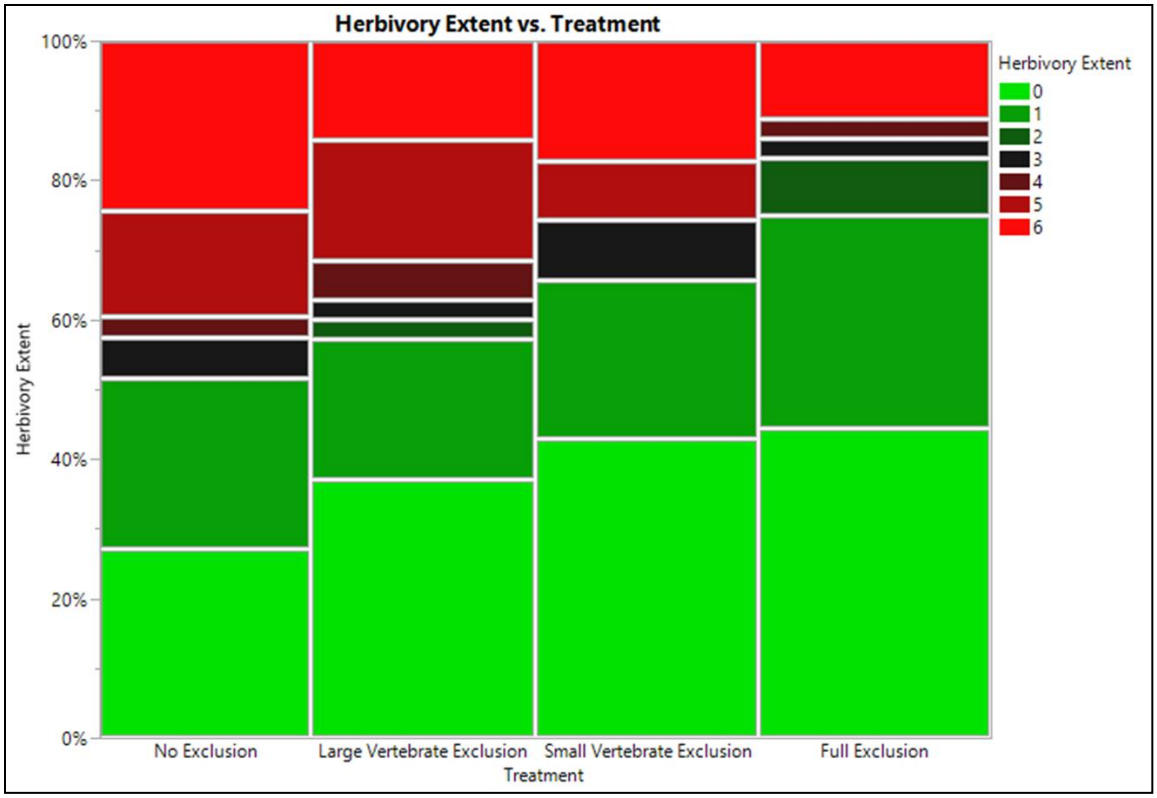

Figure 3.3: Distribution of herbivory extent classification for each event recorded for our four herbivore exclusion treatments. Herbivory severity was classified according to the scale: 0) no herbivory, 1) very low, classified as 1-25% of a leaf removed; 2) low, as 25-50% of a leaf removed; 3) moderate, as 50-99% of a leaf removed, 4) high, as 1 full leaf removed, 5) very high, as 2 or more full leaves removed, or 6) death of the plant. Increased protection led to a reduction in the most severe classifications.

Chapter 4. Chemical Diversity of Poison Ivy Leaf and Floral Volatiles as well as Urushiol Congeners

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Abstract:

Poison ivy *(Toxicodendron radicans* (L.) Kuntze) is a North American dioecious perennial liana best known for its capacity to cause contact allergenic dermatitis in humans due to the natural product urushiol. Poison ivy seems to respond favorably to several patterns of global change indicting that it will become both more abundant and more noxious in the future. As global change continues to augment both medical and ecological impacts of poison ivy, further investigations into its chemical ecology are warranted. To gain a better understanding of the chemical ecology of poison ivy, a series of investigations into poison ivy leaf and flower volatile and urushiol profiles were conducted. Through GC-MS analysis, we surveyed poison ivy floral and leaf volatiles as well as urushiols. In contrast to previous reports, the relative composition of C15 vs. C17 urushiols in leaves was quite varied, with some plants showing dramatically higher C17 accumulation levels relative to C15 urushiols in leaves, and these differences were stable over a two-month period. Female flowers produced higher C15:C17 urushiol ratio compared to leaves. Female plants showed wider variation of floral volatile composition compared to male counterparts. Ultimately this work demonstrates a more complex composition of urushiols within poison ivy, suggesting the necessity for more thorough investigations of other members in the *Toxicodendron* genus.

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Introduction:

Poison ivy is a dioecious, North American plant best known for its capacity to cause allergenic contact dermatitis on humans (aka poison ivy rash). Upwards of 50 million people annually come into contact with poison ivy and display the dreaded rash symptoms that can last for two to six weeks (Gayer and Burnett 1988, Epstein 1994, Pariser et al. 2003). The natural product responsible for causing the allergenic dermatitis is generically called urushiol. Urushiol is composed of alk(en)yl-catechol congeners that can have either a C15 or C17 carbon alkyl chain containing either zero to three double bonds (Majima and Cho 1907, Majima 1922, Hill et al. 1934b, Symes and Dawson 1953, Markiewitz and Dawson 1965b). Increasing degrees of unsaturation corresponds with increasing severity of poison ivy rash symptoms (Johnson et al. 1972). Urushiol is produced by a small number of *Toxicodendron* (Latin for "poison tree") species in North America, such as poison ivy (*T. radicans* (L.)), western poison oak (*T. diversilobum*), and poison sumac (*T. vernix*) (Gillis 1971). Poison ivy and poison sumac are reported to produce mostly C15 urushiols, whereas poison oak is reported to produce predominately C17 urushiols (Gross et al. 1975). Poison ivy urushiol congener composition is altered by elevated atmospheric CO_2 levels resulting in increased proportions of more unsaturated urushiol congeners (Mohan 2006, Ziska 2007) and thus is expected to become more allergenic with current patterns of global change.

While urushiol is an effective deterrent for human contact with poison ivy, the chemical ecology of poison ivy in natural habitats with resident animal species is poorly understood. There are a substantial number of vertebrate and arthropod herbivores or pollinators reported to associate with poison ivy leaves, drupes, and flowers (Martin et al. 1961, Popay and Field 1996, Penner et al. 1999, Senchina 2008), none of which show any demonstrable adverse reactions to touching or

ingesting plant material. There is a notable dearth of specific reports about any negative impacts (aversion to future feeding, allergenic dermatitis, etc.) on the herbivores after consuming the poison ivy plant material. Some work has been done to develop animal models for human urushiol allergenic dermatitis (i.e. Guinea pigs, Baer and Bowser 1963), though the applicability of this knowledge to natural settings remains lacking.

One approach to gain insight into possible herbivores is to evaluate whether there is differential defensive chemical accumulation in particular plant organs or tissues. To this end, most published sampling of urushiol in poison ivy is haphazard and typically lacked replication (Markiewitz and Dawson 1965b, Corbett and Billets 1975, Gross et al. 1975, Billets et al. 1976, Craig et al. 1978, Baer et al. 1980, Ma et al. 1980, ElSohly et al. 1982). Thus, the degree of urushiol variability in any given organ is poorly characterized. The goal of the current study was to evaluate this variability in poison ivy leaves and flowers more systematically by evaluating twenty individuals at two time points. Further, while urushiols have been found in both leaves and flowers previously, other metabolites, namely volatile compounds have not been evaluated. Poison ivy is a dioecious plant, and thus an obligate out-crosser. Identification of any flower specific volatiles may help lead further investigations in identifying which insect associates of poison ivy may be pollinators or possible herbivores.

Methods:

Tissue sample collection and preparation:

Seedlings were germinated from drupes collected in fall 2015 from the VARoaCo-1 liana in Catawba, Virginia (Benhase and Jelesko, 2014). Germinated seedlings were grown on 0.5X Murashige and Skoog media at 28°C and a 16h light cycle days. Seedlings were grown to the first true leaf stage, approximately two weeks post drupe emergence, and leaves were assessed for

volatiles. These seedlings were not sexed as flower morphology or the presence of seed panicles is necessary (Gillis 1971).

Inflorescence and leaf material from mature wild lianas were collected in the summer of 2017. These plants were predominately located on the Virginia Tech Campus in Montgomery County Virginia (37.2284° N, 80.4234° W), with VARoaCo-1 included as well (Benhase and Jelesko, 2014). Flowers were used to identify the sex of individual plants (Gillis 1971). A total of eight plants, four of each sex, were chosen for inflorescence volatile analysis. Two inflorescences were collected from each plant. To evaluate leaf urushiols, 10 representatives of each sex were sampled, resulting in 12 additional plants. Inflorescences and leaves were collected from May 24th to June 2nd 2017. This duration ensured that male and females flowers were open, as male plants tended to flower one week prior to females. A second leaf sampling, taken from the immediate proximal leaf from the first sampling, from each of these plants was performed on August 3rd, except for three plants that died prior to this date. Inflorescence and leaf materials were transported on ice from the collection site to the lab where inflorescences were immediately weighed and the open flowers counted prior to volatile analysis. Leaves and replicate inflorescences were stored at -80°C until processed for urushiol GC-MS analysis. Herbarium leaf presses were prepared for each individual plant and stored with the Virginia Tech Massey Herbarium (Table 4.2).

Volatile collection:

Leaf volatiles were measured from single compound leaves (14-66 mg fresh weight) in a 20 ml glass vial (Supelco, Bellefonte, PA) with screw cap and incubating in the presence of a 100 μm polydimethylsiloxane (PDMS) solid phase microextraction (SPME) fiber (Supelco, Bellefonte, PA) for 1 hour at room temperature. Inflorescence volatiles were similarly measured as leaf volatiles using a single panicle per sample, comprising 22-110 flowers per inflorescence.

Gas Chromatography-Mass Spectrometry Analysis:

GC-MS analysis of floral and leaf volatiles was performed by thermal desorption from the SPME fiber at 240°C in splitless mode and separated on a GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) using a 30 m x 0.25 mm i.d. x 0.25 µm film Zebron ZB-XLB column coupled with a QP2010S mass spectrometer (Shimadzu). The GC conditions were as follows: 40°C with 2 min hold, then raised to 220°C at 5°C/min, then raised to 240°C at 70°C/min followed by a 2 min hold time. Mass spectrometry was performed with an ion source temperature of 240°C, interface temperature of 280°C, electron ionization (EI) potential of 70 eV and scan range of 50 to 400 atomic mass units. Helium was used as a carrier gas at 1.9 mL/min. GC-MS analysis of inflorescence and leaf urushiols was preformed according to the methods described in Aziz et al. (2017).

Compound Identification:

All volatile compounds were identified by comparison of retention times and mass spectra to reference standards or by mass spectral comparison to entries in the NIST/Wiley libraries. (*E*)-β-Caryophyllene, α-humulene, ocimene, *δ*-cadinene, and germacrene D were identified with authentic standards. Alpha (*Z*,*E*)-Farnesene and (*E*,*E*)-farnesene were identified by referring to a farnescene mix sourced from green apple (*Malus domestica* "Granny Smith"). Copaene was identified by library suggestion (Wiley 7N). The distinct isomer of ocimene could not be identified. Urushiols were identified through diagnostic parent and fragmentation ions along with expected retention times as described in Aziz et al. (2017).

Data Analysis:

Leaf volatiles were measured in triplicate, and inflorescence volatiles were measured without replication from separate plants. Volatile abundance was measured as normalized peak

area in the total ion chromatogram (TIC) mode. High abundance of some compounds (peak area $1x10^8$, see Table 4.1) in certain inflorescence samples caused temporary saturation of the gas chromatogram detector resulting in an underestimation of total peak area. The peak area for such incomplete peaks was estimated by dividing the left inflection point height (TIC) of the incomplete peak by the left inflection point height of an intact peak (a sample from the same sex and similar gas chromatogram profile) then multiplying by the area under the curve of the intact peak. Fresh weight (mg) was used to normalize peak area.

The effects of sex of the plant and collection time on total urushiols were estimated using multifactorial ANOVA. Similarly, the same treatment effects were estimated for the ratio of C15:C17 urushiols. In both models, plant ID was run as a random effect to account for non-independence of multiple collections from the same plant and a natural log transformation was necessary for C15:C17 response variables. As floral samples and leaf samples were normalized to separate alkylresorcinol curves, total urushiols were not compared between tissue types. Further, inflorescences were only collected at the beginning of the season as by the second collection point inflorescences had senesced in males or developed into fruits in females. However, as the extraction efficiency of C15 to C17 urushiols should not change, the ratio of these two species can be compared between the two tissue types. This was accomplished with a two way ANOVA testing the treatment effects of sex and tissue type as well as their interaction. Similar to the previous models, individual was run as a random factor to maintain independence of the residuals. All statistical analysis was performed in JMP 13 (SAS Institute Inc., Cary NC).

Results:

Urushiol Abundance in Male and Female Plants:

For the urushiols present in the leaves, sex of the individual was not a strong predictor of total urushiol accumulation (*P*-value = 0.74) in expanded poison ivy leaves. Further, total urushiols were not significantly different between samples from the same plants collected two months apart (*P*-value=0.92), and the interaction term between sex and time was not significant (*P*-value = 0.26, Figure 4.3a). The ratio of total C15 urushiols to total C17 (C15:C17) urushiols did not show a strong response to either sex (*P*-value = 0.52), collection time (*P*-value = 0.61), or their interaction (*P*-value = 0.63, Figure 4.3b).

While the mean C15:C17 ratio was 3.3, the median value was 1.1, indicating that C15 urushiols reached greater abundance in only 11 of 20 individuals. Notably, 9 of 20 individuals accumulated much higher C17 urushiols, with the highest being 5.5x greater than the C15 urushiol levels. In the most extreme two cases, this ratio differed by 64-fold (max, 11.52; min, 0.18). Urushiol congeners varied across individuals but some patterns were found. Generally, our leaf samples contained very little C15:3 urushiols (Figure 4.4). Other congeners, in particular C15:2, were more varied, but interestingly the makeup of congeners within an individual was consistent within the two-month span.

Comparing urushiol levels in leaves to inflorescences, a significant interaction between tissue and sex was observed (*P*-value = 0.031, Figure 4.5), and the main effect of sex was significant (P-value = 0.032). Female inflorescences were biased towards accumulating C15 (mean C15:C17 ratio of 13.9) urushiols compared to male inflorescences (mean C15:C17 ratio of 0.283).

Survey of Floral and Leaf Volatiles:

Flowers from four male and four female poison ivy lianas were characterized for their volatile profiles and yielded 119 unique volatile compounds. However, just eight terpenoid compounds comprised 83% of total peak area across all samples. These compounds were 1,

copaene; 2, (*E*)-β-caryophyllene; 3, germacrene D; 4, α-humulene; 5, (*Z*,*E*)-farnesene; 6, (*E*,*E*)-farnesene; 7, azulene; and 8, *δ*-cadinene. The abundance of these eight compounds in each sample is reported in Table 4.1. Other identifiable compounds were primarily terpenes followed by a limited number of fatty acid derivatives (<1% total peak area). While male and female panicles did not differ greatly in the compounds that comprised their profiles, when normalized to panicle biomass, female tissues showed overall higher abundances of volatile compounds with average headspace levels being 1.4-fold higher than those in males.

Volatile profiles from leaves were quite different from those of inflorescences. Leaves showed only 66 unique compounds. In some replicates, as few as five compounds were identified. Across all samples, the top eight most abundant compounds were 1, copaene; 2, (*E*)-β-caryophyllene; 3, α-humulene; 5, azulene; 6, an unidentified hydrocarbon; α-longipinene; 8, α-cubebene. These eight compounds comprised 85% of total volatiles collected across all leaf samples (Table 4.2). However, while these were the most abundant compounds, some samples did not contain the full set. Leaves had much lower abundances of volatiles than inflorescence with 14-fold lower average headspace levels. Representative spectra for male and female inflorescence and leaf samples are shown in Figure 4.1.

Discussion:

As sessile organisms, plants have developed an incredible diversity of secondary metabolites that aid them in responding to both abiotic and biotic stress. These secondary metabolites can function to deter herbivores, prevent and respond to microbial infections, induce the production of other secondary metabolites, and attract pollinators (Savatin et al. 2014). There is an innate need for plants to balance the production of these chemicals in response to their metabolic costs (e.g., allocation of resources from primary metabolites associated with growth and

development) and ecological costs (e.g., decrease in fitness as a result of lowered herbivory defense) (Neilson et al. 2013, Kessler 2015). Therefore, the selection pressures for maintaining the production of secondary metabolites are presumed to be high (Agrawal 2011).

The poison ivy inflorescences predominately released volatile terpenes, with sesquiterpenes being the major compounds. Other chemical classes represent less than 1% of the total volatile fraction. Among the most predominant volatiles were alpha- (*Z*,*E*)-farnesene and (*E*,*E*)-farnesene, notable for their very low levels in the leaf volatile profiles. Germacrene D was also absent in the leaf volatile profile, and has been shown to attract both wasps and moths (Bengtsson et al. 2001, Cornille et al. 2011). Specificity of these two chemicals in the inflorescence may suggest their role in the attraction of certain insect pollinators for poison ivy, and the repulsion of those that would be herbivores. However, insect pollinators of poison ivy are numerous and broadly distributed across taxa, including coleopterans, dipterans, hemipterans, hymenopterans, and lepidopterans (Senchina and Summerville 2018). Thus, whether inflorescence specific volatiles play any role in attraction of specific pollinator types may be unlikely and would require extensive testing, though other roles such as antimicrobial activity of beta-caryophyllene have been demonstrated (Huang et al. 2012).

The ecological selection pressures responsible for shaping urushiol levels and congener composition in the Toxicodendron genus have not been elucidated. Little work has been shown to demonstrate the potential anti-microbial effects of urushiol in reducing sporulation rates of *Cladisporium herbarum* (Kim 1997) , and inhibition of *Helicobacter pylori* (Suk 2011). Other bacterial species such as *E. coli, staphylococcus aureusa,* and *Bacillus subtilis* show no sensitivity to urushiols (Kim 1997). Ultimately, more thorough investigations into fungal and bacterial inhibition by urushiol is warranted.

Herbivory could be another such selection pressure, though studies identifying herbivores of poison ivy have been largely limited to observational reports of particular animal species consuming poison ivy leaves (Martin et al. 1951). Attempts to develop animal models for human urushiol allergenic dermatitis were largely restricted to Guinea pig and mouse models. Guinea pigs can be sensitized to both poison ivy extract (Rackemann and Simon 1934) and synthetic pentadecyl-catechol (PDC, i.e. C15 urushiol (Baer and Bowser 1963) and show repeated allergenic dermatitis symptoms with subsequent skin exposure that approximate human poison ivy rash symptoms. However, these responses were restricted to Guinea pigs less than six months old (Baer and Bowser 1963). Mice are rapidly sensitized by urushiol components placed on the skin; however, instead of subsequent dermal challenge the murine system comprised subsequent treatment of the ears with PDC, resulting in ear thickening that is used as an index of dermatitis severity (Dunn et al. 1982). Rabbits can also be sensitized to urushiol, though the intensity of subsequent allergenic dermatitis was not quantified (Murphy et al. 1983). Relatively few Rhesus macaques showed sensitivity to urushiol-induced allergenic dermatitis, and these atypical responses were 50 – 100 times less severe than either humans or Guinea pigs (Bowser et al. 1964).

Most germane to the possibility that herbivores may serve as selection pressures for urushiol production, Guinea pigs previously sensitized to PDC were hyposensitized after oral ingestion of urushiol from poison ivy or poison oak (Watson et al. 1983). However, overall the potential incidence and relevance of oral hyposensitization of wild fauna feeding on poison ivy is currently unknown. Goats can be used to reduce poison ivy infestations, and these animals do not show any evidence of either allergenic dermatitis or gastric distress (Popay and Field 1996). While many insects have also been observed feeding on poison ivy, whether these insects are simply

unaffected by urushiol or have some mechanism to deactivate the urushiol molecule (by esterifying the hydroxyl groups) has not been explored.

While these examples demonstrate that small mammals can show urushiol-induced allergenic dermatitis, the presumed native fauna targets of a hypothesized urushiol chemical defense are currently an enigma. One approach to evaluating a chemical defense role is assessing preferential accumulation of the plant chemical defense. While limited in sample size, this study suggests that intraspecific variation in both total urushiol and the relative abundance of C15 and C17 urushiol congeners' accumulation, was quite high. In addition, variation in both total urushiol accumulation levels and the ratio of C15:C17 congeners were similar across a two-month time interval. Thus, variation in urushiol levels were not likely an individual plant's response to an immediate biotic or abiotic stressor. More experimentation is needed in order to definitively rule out various stresses such as herbivory. Another possibility is that urushiol accumulation and/or the ratio of C15:C17 urushiol congeners is a stable genetic trait in this population. This is consistent with work being done in the genus *Populus*, where sexual homomorphism is observed in non-reproductive traits (McKown et al. 2017).

The present study identified poison ivy plant leaves with C17 urushiols that were equal or greater than C15 urushiol levels. Early reports of urushiols in poison ivy suggested that the species only accumulated C15 urushiols (Markiewitz and Dawson 1965). However, soon after work in poison ivy and poison oak showed the presence of both C15 and C17 urushiols in both species, with poison ivy predominately accumulating C15 urushiols and poison oak accumulating C17 urushiols (Corbett and Billets 1975, Gross et al. 1975, Billets et al. 1976, Craig et al. 1978). These reports lacked clear description of what comprised their samples, leading to uncertainty of how many individual plants were used to prepare extracts, a trend that continued in reports that followed

(Ma et al. 1980, ElSohly et al. 1982). While more care has been taken to address this concern in reports focused on poison oak (Gartner et al. 1993), to our knowledge only one study (Baer et al. 1980) was specific in the preparation of extracts from singular poison ivy plants, and was limited to only two individuals which accumulated no C17 urushiols. This is especially pertinent to the current study, which shows the high variation of urushiol constituents between individuals. Thus, whether previous reports concluded that poison ivy predominately produces C15 urushiols, rather than a variable mixture of C15 and C17 urushiols as we show, was due to poor sample size or another reason is unclear. Further, at least in hypocotyl and internode tissues, C15 and C17 urushiols accumulate in different cell types (Aziz, et al. 2017) that are perhaps responding to different signals of plant stress. Identification of the cues that drive the accumulation of one set of congeners over another may lead to the elucidation for the role, or perhaps the lack thereof, urushiols play in plant defense.

References:

Agrawal, A. A. 2011. Current trends in the evolutionary ecology of plant defence. Functional Ecology **25**:420-432.

Baer, H., and R. T. Bowser. 1963. Antibody production and development of contact skin sensitivity in guinea pigs of various ages. Science **140**:1211-1212.

Baer, H., M. Hooton, H. Fales, A. Wu, and F. Schaub. 1980. Catecholic and other constituents of the leaves of Toxicodendron radicans and variation of urushiol concentrations within one plant. Phytochemistry **19**:799-802.

Bengtsson, M., A.-C. Bäckman, I. Liblikas, M. I. Ramirez, A.-K. Borg-Karlson, L. Ansebo, P. Anderson, J. Löfqvist, and P. Witzgall. 2001. Plant Odor Analysis of Apple: Antennal Response of Codling Moth Females to Apple Volatiles during Phenological Development. Journal of Agricultural and Food Chemistry **49**:3736-3741.

Billets, S., J. C. Craig, M. D. Corbett, and J. F. Vickery. 1976. Component analysis of the urushiol content of poison ivy and poison oak. Phytochemistry **15**:533-535.

Bowser, R. T., R. L. Kirschstein, and H. Baer. 1964. Contact sensitivity in Rhesus monkeys to poison ivy extracts and fluorodinitrobenzene. Pro Soc Exp Biol Med **117**:763-766.

Corbett, M. D., and S. Billets. 1975. Characterization of poison oak urushiol. Journal of pharmaceutical sciences **64**:1715-1718.

Cornille, A., J. G Underhill, A. Cruaud, M. Hossaert-McKey, S. D Johnson, K. Tolley, F. Kjellberg, S. Noort, and M. Proffit. 2011. Floral volatiles, pollinator sharing and diversification in the fig-wasp mutualism: Insights from Ficus natalensis, and its two wasp pollinators (South Africa).

Craig, J. C., C. W. Waller, S. Billets, and M. A. Elsohly. 1978. New GLC analysis of urushiol congeners in different plant parts of poison ivy, Toxicodendron radicans. Journal of pharmaceutical sciences **67**:483-485.

Dunn, I. S., D. J. Liberato, R. G. Dennick, N. Castagnoli, and V. S. Byers. 1982. A murine model system for contact sensitization to poison oak or ivy urushiol components. Cellular Immunology **68**:377-388.

ElSohly, M. A., P. D. Adawadkar, C.-Y. Ma, and C. E. Turner. 1982. Separation and characterization of poison ivy and poison oak urushiol components. Journal of Natural Products **45**:532-538.

Epstein, W. L. 1994. Occupational poison ivy and oak dermatitis. Dermatologic Clinics **3**:511-516.

Gartner, B. L., C. Wasser, E. Rodriguez, and W. L. Epstein. 1993. Seasonal variation of urushiol content in poison oak leaves. Dermatitis **4**:33-36.

Gayer, K. D., and J. W. Burnett. 1988. Toxicodendron dermatitis. Cutis **42**:99-100.

Gillis, W. T. 1971. The systematics and ecology of poison-ivy and the poison-oaks (Toxicodendron, Anacardiaceae). Rhodora **73**:72-159.

Gross, M., H. Baer, and H. M. Fales. 1975. Urushiols of poisonous Anacardiaceae. Phytochemistry **14**:2263-2266.

Hill, G. A., V. Mattacotti, and W. Graham. 1934. The toxic principle of the poison ivy. Journal of the American Chemical Society **56**:2736-2738.

Johnson, R. A., H. Baer, C. H. Kirkpatrick, C. R. Dawson, and R. G. Khurana. 1972. Comparison of the contact allergenicity of the four pentadecylcatechols derived from poison ivy urushiol in human subjects. Journal of Allergy and Clinical Immunology **49**:27-35.

Kessler, A. 2015. The information landscape of plant constitutive and induced secondary metabolite production. Current Opinion in Insect Science **8**:47-53.

Kim, M.-J., C.-J. Kim, and S.-S. Kwak. 1997. Antifungal activity of urushiol components in the sap of Korean lacquer tree (Rhus vernicifera Stokes). Korean Journal of Plant Resources **10**:231-234.

Ma, C.-Y., M. A. Elsohly, and J. K. Baker. 1980. High-performance liquid chromatographic separation of urushiol congeners in poison ivy and poison oak. Journal of Chromatography A **200**:163-169.

Majima, R. 1922. Uber den hauptbestandteil des Japan-lacs, VIII. Mitteilung: stellung der doppelbindungen in der seitenkette des urushiols und beweisfuhrung, dab das urushiol eine mischung ist. Berichte der Deutschen Chemischen Gesellschaft **55(b)**:172-191.

Majima, R., and S. Cho. 1907. Ueber einen hauptbestandteil des japanischen lackes. Berichte der Deutschen Chemischen Gesellschaft **40**:632.

Markiewitz, K. H., and C. R. Dawson. 1965. On the Isolation of the Allegenically Active Components of the Toxic Principle of Poison Ivy. The Journal of Organic Chemistry **30**:1610-1613.

Martin, A. C., H. S. Zim, and A. L. Nelson. 1951. American wildlife & plants. Dover Publications, Inc., New York, New York, USA.

Martin, A. C., H. S. Zim, and A. L. Nelson. 1961. American wildlife & plants: a guide to wildlife food habits: the use of trees, shrubs, weeds, and herbs by birds and mammals of the United States. Courier Corporation.

McKown, A. D., J. Klápště, R. D. Guy, R. Y. Soolanayakanahally, J. La Mantia, I. Porth, O. Skyba, F. Unda, C. J. Douglas, Y. A. El-Kassaby, R. C. Hamelin, S. D. Mansfield, and Q. C. B.

Cronk. 2017. Sexual homomorphism in dioecious trees: extensive tests fail to detect sexual dimorphism in Populus. Scientific Reports **7**:1831.

Mohan, J. E. Z., L. H.; Schlesinger, W. H. ; Thomas R. B.; Sicher, R. C.; George, K.; and Clark, J. S. 2006. Biomass and toxicity responses of poison ivy(Toxicodendron radicans) to elevated atmospheric CO2. PNAS **103**.

Murphy, J. C., E. S. Watson, and E. C. Harland. 1983. Toxicological evaluation of poison oak urushiol and its esterified derivative. Toxicology **26**:135-142.

Neilson, E. H., J. Q. D. Goodger, I. E. Woodrow, and B. L. Møller. 2013. Plant chemical defense: at what cost? Trends in plant science **18**:250-258.

Pariser, D. M., R. I. Ceilley, A. M. Lefkovits, B. E. Katz, and A. S. Paller. 2003. Poison ivy, oak and sumac. Dermatology Insights **4**:26-28.

Penner, R., G. E. E. Moodie, and R. J. Staniforth. 1999. The dispersal of fruits and seeds of Poison-ivy, *Toxicodendron radicans*, by Ruffed Grouse, *Bonasa umbellus*, and squirrels, *Tamiasciurus hudsonicus* and *Sciurus carolinensis*. Canadian Field-Naturalist **113**:616-620.

Popay, I., and R. Field. 1996. Grazing animals as weed control agents. Weed Technology **10**:217-231.

Rackemann, F. M., and F. A. Simon. 1934. The sensitization of guinea pigs to poison ivy. Science **79**:399.

Savatin, D. V., G. Gramegna, V. Modesti, and F. Cervone. 2014. Wounding in the plant tissue: the defense of a dangerous passage. Frontiers in Plant Science **5**:470.

Senchina, D. S. 2008. Fungal and animal associates of Toxicodendron spp.(Anacardiaceae) in North America. Perspectives in Plant Ecology, Evolution and Systematics **10**:197-216.

Senchina, D. S., and K. S. Summerville. 2018. Great diversity of insect floral associates may partially explain ecological success of poison ivy (Toxicodendron radicans subsp. negundo [Greene] Gillis, Anacardiaceae). The Great Lakes Entomologist **40**:3.

Suk, K. T., S. K. Baik, H. S. Kim, S. M. Park, K. J. Paeng, Y. Uh, I. H. Jang, M. Y. Cho, E. H. Choi, M. J. Kim, and Y. L. Ham. 2011. Antibacterial Effects of the Urushiol Component in the Sap of the Lacquer Tree (*Rhus verniciflua* Stokes) on *Helicobacter pylori*. Helicobacter **16**:434-443.

Symes, W. F., and C. R. Dawson. 1953. Separation and structural determination of the olefinic components of poison ivy urushiol, cardanol and cardol. Nature **171**:841-842.

Watson, E. S., J. C. Murphy, and M. A. ElSohly. 1983. Immunologic studies of poisonous Anacardiaceae: oral desensitization to poison ivy and oak urushiols in guinea pigs. J Invest Dermatol **80**:149-155.

Ziska, L. H. S., R. C.; George, K.; and Mohan, J. E. . 2007. Rising Atmospheric Carbon Dioxide and Potential Impacts on the Growth and Toxicity of Poison Ivy (Toxicodendron radicans). Weed Science **55**.

Tables:

Table 4.1: Peak areas for eight most abundant floral compounds by sample. Values are based on relative abundances calculated from total ion chromatograms (TIC) normalized to sample weight.

Sample	(*E*)-β-Caryophyllene	(*Z*,*E*)-Farnesene	Copaene	α-Humulene	(*E*,*E*)-Farnesene	Ocimene	δ-Cadinene	Germacrene D	Other
Rt (min)	23.39	24.99	22.16	24.35	25.36	12.35	25.95	23.68	
F1	5.18E+06	1.56E+06	1.36E+06	3.99E+05	1.31E+06	2.47E+05	3.26E+05	2.95E+05	2.95E+05
F2	1.10E+07*	2.00E+06	6.07E+05	1.52E+06*	2.92E+06	1.03E+06	4.02E+05	7.56E+05	7.56E+05
F3	1.41E+07	2.34E+06	3.80E+-06*	1.01E+06	2.73E+06	8.92E+04	4.28E+05	3.03E+04	3.03E+04
F4	1.28E+06	3.69E+05	3.09E+05	7.14E+04	2.62E+05	1.87E+05	6.94E+04	9.81E+03	9.81E+03
M1	3.61E+06	5.31E+05	8.91E+04	2.19E+05	5.85E+05	3.93E+04	3.36E+05	6.13E+04	6.13E+04
M2	4.06E+06*	5.01E+05	1.88E+06	9.23E+05	3.62E+05	3.42E+05	1.61E+05	3.03E+04	3.03E+04
M3	5.85E+06	2.05E+06	1.00E+06	2.69E+05	2.91E+06	7.51E+04	3.70E+04	6.11E+04	6.11E+04
M4	8.46E+06	2.05E+06	1.91E+05	5.45E+05	2.79E+06	2.60E+05	4.81E+05	1.12E+05	1.12E+05

* Area estimated by peak comparison (see Methods)

Table 4.2: Peak area for eight most abundant leaf compounds by sample. Values are based on total ion chromatograms (TIC) normalized to sample weight.

Sample	(*E*)-β-caryophyllene	Copaene	α-Humulene	Azulene	Unidentified Hydrocarbon	α-Longipinene	α-Cubebene	Other
Rt(min)	23.99	22.16	24.35	24.54, 23.61	23.56	21.37	21.27	
L1-1	7.80E+05	1.74E+05	4.36E+04	-	-	-	7.96E+03	2.42E+04
L1-2	1.34E+06	-	7.06E+04	4.16E+04	3.16E+04	2.40E+05	4.48E+05	1.81E+05
L1-3	7.31E+05	4.59E+04	3.68E+04	-	2.02E+04	-	-	1.52E+05
L2-1	5.03E+05	1.58E+04	3.17E+04	-	1.53E+04	1.22E+04	-	1.08E+05
L2-2	3.19E+05	7.08E+03	1.93E+04	-	1.04E+04	-	-	7.05E+04
L2-3	9.81E+04	9.49E+03	5.72E+03	4.05E+04	3.48E+03	-	-	3.97E+04
L3-1	1.92E+05	-	1.10E+04	4.92E+04	2.32E+04	-	-	1.82E+05
L3-2	3.17E+05	1.69E+04	1.47E+04	3.66E+04	-	-	8.29E+03	1.19E+05
L3-3	2.50E+05	4.27E+04	1.37E+04	5.99E+04	-	-	3.07E+04	1.51E+05

Table 4.3: Mature poison ivy sample documentation. (*) Denotes plants that died between the first and second collections. (**) Indicates unsuccessful extraction of urushiols from the later time point leaf sample.

Sample ID	VPI catalog number	Sex	Fresh Biomass of Pannicle (mg)	Open Flowers Count	GPS Coordinates
F1	109542	F	363	62	37.2231898, -80.4115705
M2	109543	M	1175	75	37.2231898, -80.4115705
F3	109544	F	219	28	37.2263526, -80.4287581
M4*	109545	M	1533	65	37.2268724, -80.4323747
M5	109546	M	437	30	37.2282405, -80.4323747
F6	109547	F	124	22	37.2283908, -80.4328012
M7**	109548	M	281	31	37.2287995, -80.4317467
F8	109549	F	876	110	37.3813384, -80.1109415
M9	109550	M	NA	NA	37.2245153, -80.4164836
M10*	109551	M	NA	NA	37.2268724, -80.4281648
F11	109552	F	NA	NA	37.2260653, -80.4394088
M12	109553	M	NA	NA	37.2262912, -80.4286749
F13	109554	F	NA	NA	37.2282405, -80.4323747
M14	109555	M	NA	NA	37.2283874, -80.4325893
M15	109556	M	NA	NA	37.2283972, -80.4326610
F16	109557	F	NA	NA	37.2248125, -80.4163341
F17	109558	F	NA	NA	37.2248594, -80.4164719
M18	109559	M	NA	NA	37.2244598, -80.4162905
F19	109560	F	NA	NA	37.2284861, -80.4328957
F20	109561	F	NA	NA	27.2288975, -80.4320927

Figures:

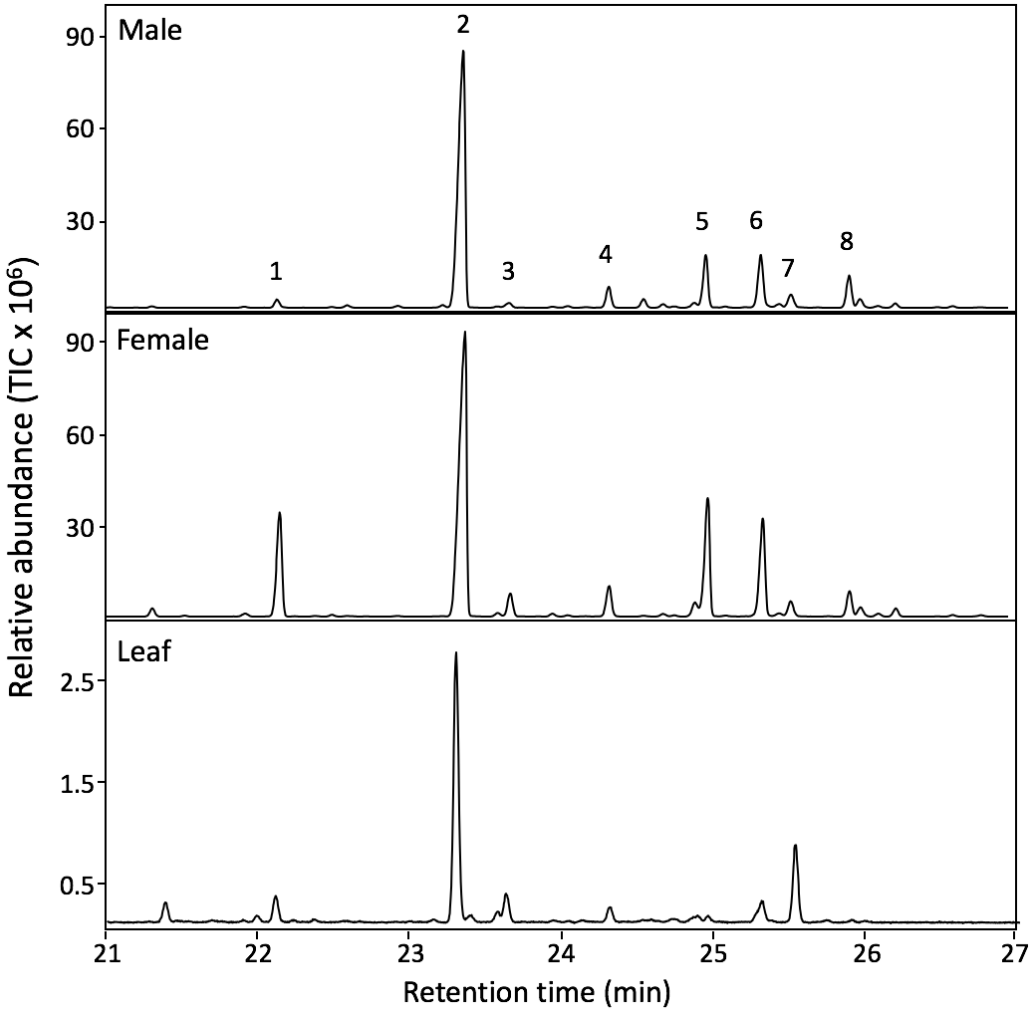

Figure 4.1. Volatile organic compounds from inflorescence and leaf of *T. radicans*. Representative GC-MS chromatograms of volatile compounds collected by SPME from male and female inflorescences and leaf tissue. 'Male' is male sample 1 and 'female' is female sample 1. 1, copaene; 2, (*E*)-β-caryophyllene; 3, germacrene D; 4, α-humulene; 5, (*Z*,*E*)-farnesene; 6, (*E*,*E*)-farnesene; 7, azulene; 8, *δ*-cadinene.

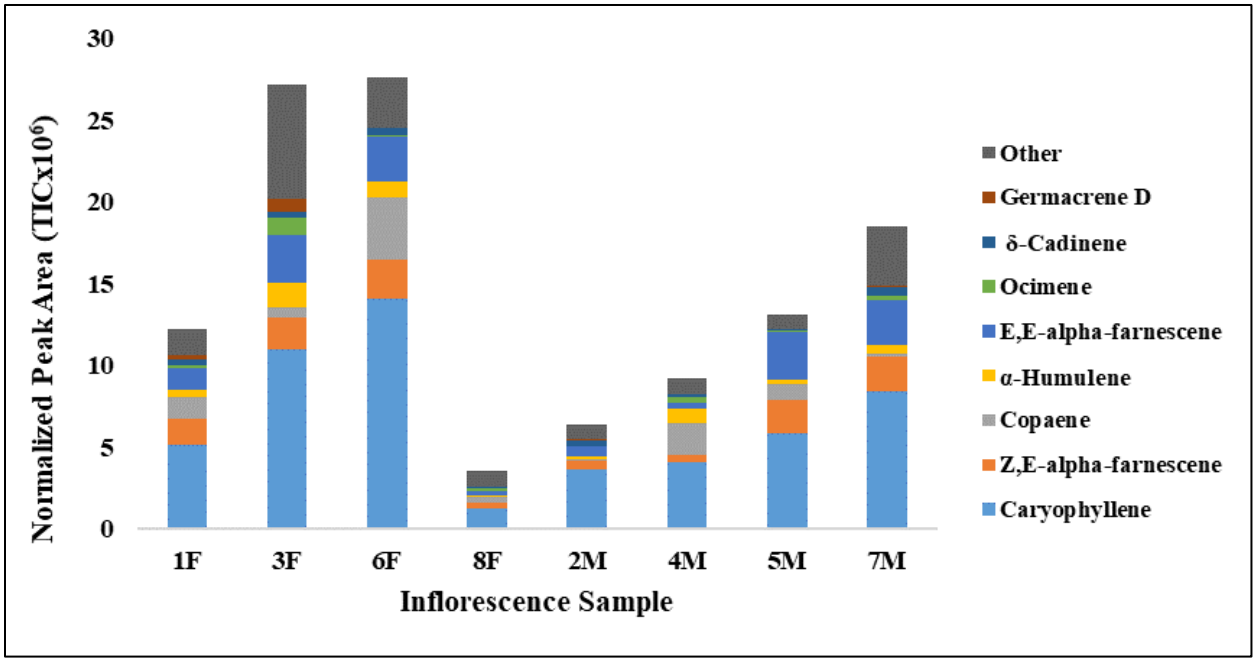

Figure 4.2: Floral volatile abundance by sex. Abundance of the eight predominant floral volatiles for each sample after adjusting for detector saturation (see Methods and Table 4.1). F/M 1-4 = female or male inflorescence samples 1-4.

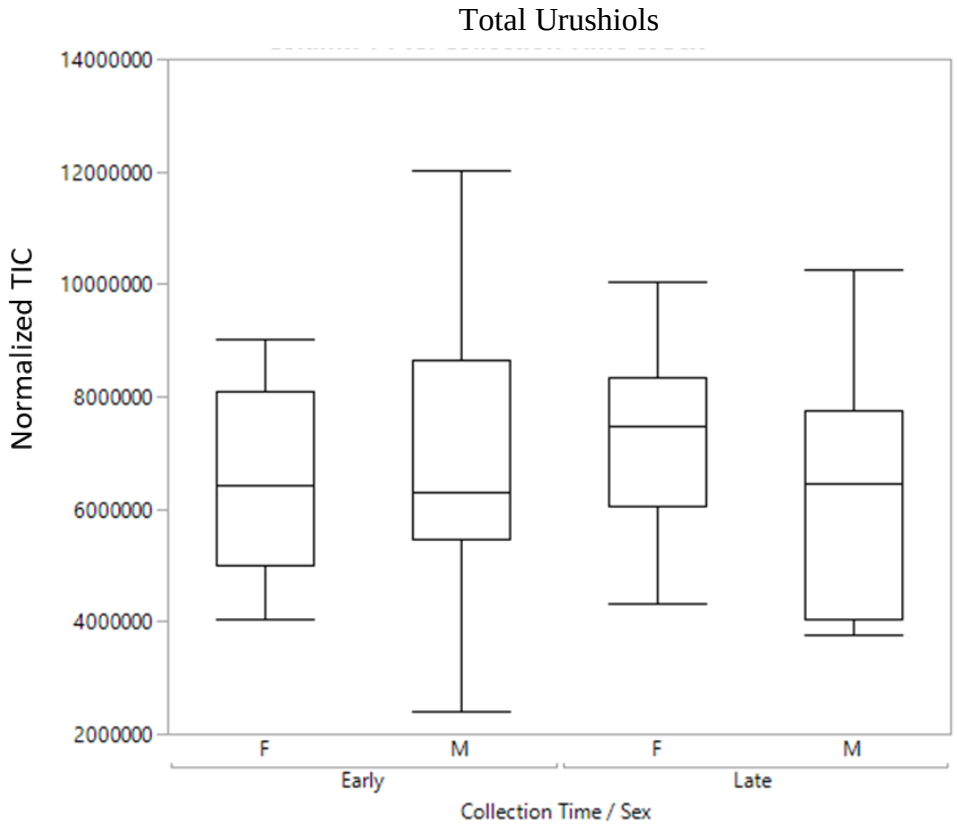

Figure 4.3: GC-MS quantification of total urushiols in mature poison ivy leaves. Mean total urushiols are selected ion counts normalized to an internal standard of alkylresorcinol. While variation between individual plants was high, neither sex of the plants nor the course of the growing season explained this variance.

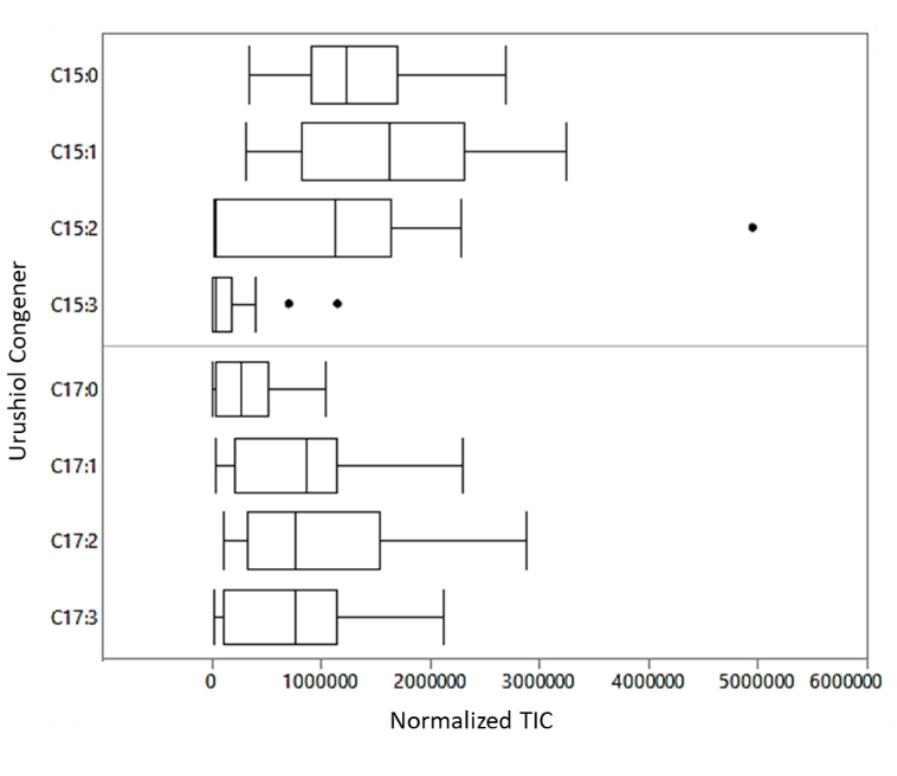

Figure 4.4: GC-MS quantification of urushiol congeners in mature poison ivy leaves. Mean totals of urushiol congeners are selected ion counts normalized to an internal standard of alkylresorcinol.

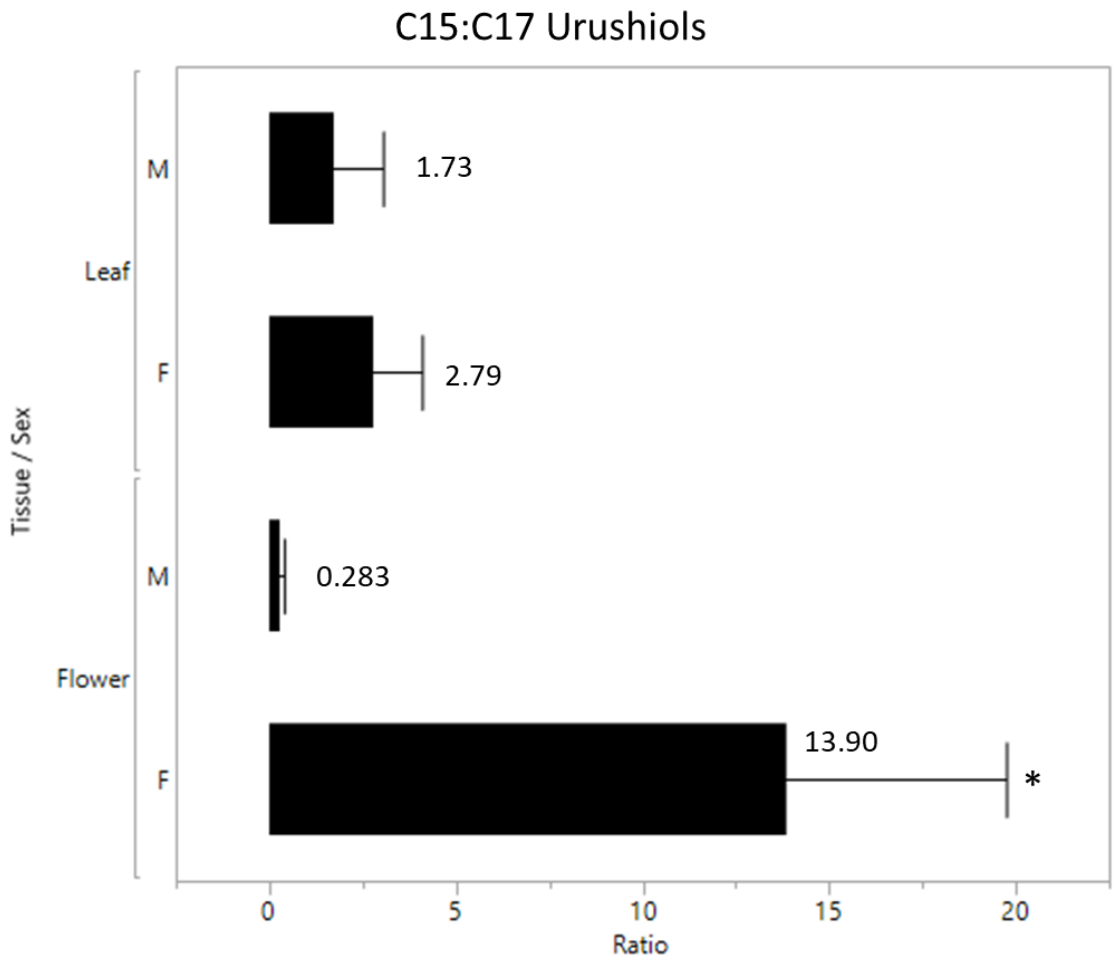

Figure 4.5: GC-MS quantification of urushiol congener ratio in mature poison ivy leaves and flowers. Ratios of urushiol congeners are selected ion counts normalized to an internal standard of alkylresorcinol. (*) Denotes a statistically significant (P-value = <0.05) treatment.

Chapter 5: Transient Heterologous Gene Expression Methods for Poison Ivy Leaf and Cotyledon Tissues.*

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Abstract:

Poison ivy (*Toxicodendron radicans* (L.) Kuntze) is a widely recognized native plant species because of its production of urushiol which is responsible for delayed contact dermatitis symptoms in humans. Poison ivy is predicted to become both more prevalent and more noxious in response to projected patterns of climate change. Future studies on poison ivy chemical ecology will require reverse genetics to investigate urushiol metabolism. A prerequisite for reverse genetic procedures is the introduction and expression of recombinant DNA into poison ivy tissues. Poison ivy leaves and cotyledons were marginally susceptible to vacuum- and syringe-Agroinfiltration and expression of two firefly luciferase (*LUC*)-based reporter genes. The efficacy of Agroinfiltration and transient *LUC* expression was dependent upon leaf age and plant growth environmental conditions, with young leaves grown in magenta boxes showing highest transient *LUC* expression levels. Agroinfiltrated leaves showed an Agrobacterium-dependent accumulation of brown-colored pigments. Biolistic transformation of a *LUC* reporter gene did not show brown pigment accumulation and readily displayed transient luciferase bioluminescence in both leaves and cotyledon tissues. These studies establish best practices for introducing and transiently expressing recombinant DNA into poison ivy leaf and cotyledon tissues, upon which future reverse genetic procedures can be developed.

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Introduction:

Poison ivy (*Toxicodendron radicans* (L.) Kuntz) is a native North American species best known for its capacity to induce allergenic dermatitis symptoms after contact with human skin. The natural product responsible for the dreaded skin rashes are alk(en)yl-catechol congeners generically called urushiol, which is found in all members of the *Toxicodendron* genus (Majima 1922, Hill et al. 1934a, Symes and Dawson 1953, 1954, Markiewitz and Dawson 1965a, Kurtz and Dawson 1971, Gross et al. 1975). Urushiol chemical ecology is largely enigmatic because humans appear to be uniquely sensitive to the allergenic effects of urushiol, whereas wild and domesticated animals appear to be largely unaffected upon contact with poison ivy. In fact, deer (Martin et al. 1951) and goats (Popay and Field 1996) eat poison ivy foliage with no apparent ill effects. There are few studies focused on poison ivy physiology and/or ecology. Two studies are noteworthy as they establish that poison ivy responds to elevated atmospheric CO_2 levels by growing faster, accumulating more biomass, and shifting urushiol congener levels toward more allergenic forms of urushiol (Mohan et al. 2006, Ziska et al. 2007). Given the current pattern of increasing anthropogenic CO_2 emissions, poison ivy is poised to become more prevalent and more noxious, yet most aspects of urushiol metabolism and ecology are largely unexplored. For example, Dewick (1997) postulated that urushiol is derived from fatty acid biosynthesis beginning with a C16 fatty acid-CoA starter molecule that is extended by a presumed polyketide synthase activity into a tetraketide, that is subsequently modified to form alk(en)yl-catechol (urushiol) congeners. With that said, none of these proposed urushiol metabolites, enzyme activities, enzymes, or genes have been empirically validated to date. Dewick's hypothesized formation of a fatty acid tetraketide that is cyclized into an alkyl-phenol by a polyketide synthase activity is likely because a number of plant polyketide synthases use fatty acid-CoA starting molecules in the production of alkyl-

phenols (Abe et al. 2004a, Taura et al. 2009, Kim et al. 2010, Matsuzawa et al. 2010, Kim et al. 2013, Taura et al. 2016). The capacity to investigate this hypothesis was recently advanced with the publication of the poison ivy (*Toxicodendron radicans*) leaf and root transcriptome (Weisberg et al. 2017).

The capacity to express recombinant DNA molecules in plants enables detailed investigations of the role of both endogenous and foreign genes. Recombinant genes in plants can be expressed either as transient unintegrated transgenes or as transgenes that are stably integrated into the plant nuclear or plastid genomes. Understanding the molecular basis for urushiol biosynthesis and urushiol chemical ecology will require a variety of molecular genetic methods, all of which require the introduction and expression of recombinant DNA in poison ivy plant cells and tissues. The principle methods of introducing recombinant DNA constructs into plant cells are biolistic and Agrobacterium-based transformation procedures.

Agrobacterium tumefaciens was initially investigated as a soil-borne phytopathogen responsible for crown gall tumor formation. The etiology of pathology requires segments of bacterial tumor inducing (Ti) plasmid DNA transfer, stable integration, and expression of these bacterial DNA sequences within the plant cell nucleus (Drummond et al. 1977). This inherent plant gene transformation capability of *Agrobacterium tumefaciens* was re-engineered to replace the tumor formation regions on the Ti plasmid (i.e. disarmed binary Ti-plasmids) with recombinant "genes of interest". The potential utility of Agrobacterium-mediated transient transformation was first demonstrated by expressing high levels of transient *GUS* reporter gene activity in bean, tobacco, and poplar leaves by simply infiltrating *Agrobacterium tumefaciens* harboring a *GUS* reporter gene on a binary T-DNA plasmid directly into the leaf mesophyll interstitial space (Kapila et al. 1997). The general applicability of the technique is shown by transient reporter gene

expression in the leaves of diverse plant species such as switchgrass (VanderGheynst et al. 2008), habanero pepper (Arcos-Ortega et al. 2010), cowpea epicotyl (Bakshi et al. 2011), rice (Andrieu et al. 2012), soybean (King et al. 2015), and persimmon (Mo et al. 2015), to name a few.

Biolistic transformation is often used when Agrobacterium-mediated transformation proves problematic. Biolistic transformation is the acceleration of nucleic acid-coated micro particles to high velocity (using a so-called gene gun) resulting in entry into the plant cell cytoplasm, where the nucleic acid subsequently uncoats from the micro particle, and is either transiently expressed or stably integrates into the plant nuclear genome (Klein et al. 1987). Alternatively, if combined with appropriate plastid DNA sequences the transgene can integrate into, and be expressed from, the plastid genome (Daniell et al. 1990). Biolistic transformation is the method of choice for stable transformation of major cereal crops (Klein et al. 1988, Wang et al. 1988) that are otherwise recalcitrant to Agrobacterium-mediated stable transformation.

Development of reverse genetic methods in poison ivy will enable molecular-genetic investigations of poison ivy ecophysiology and chemical ecology, as well as provide foundational molecular genetic approaches in other members of the *Toxicodendron* genus (e.g. poison oak and poison sumac). As an initial step toward the implementation of such molecular genetic studies, the present report describes the introduction and transient expression of recombinant firefly luciferase (*LUC*) reporter gene constructs in poison ivy cells and tissues through Agroinfiltration and biolistic transformation.

Methods:

Plant Material:

Poison ivy (*Toxicodendron radicans subsp. radicans*) drupes were sourced from the RoaCo-1 liana located in Catawba VA (Benhase and Jelesko 2013). Drupes were mechanically

and chemically scarified (Benhase and Jelesko 2013). Axenic seedlings were germinated on Petri plates of 0.5X MS basal salts media (Plant Natural, Bozeman, MT), in the dark for four nights. Germinating seedlings were then transferred to either sterile 0.5X MS media in magenta boxes or pots containing non-sterile Sunshine Mix 1 (Plant Natural, Bozeman, MT). The plants were grown at 28°C under 16 hour light/day cycle. Poison ivy leaves were typically Agroinfiltrated with a syringe at the 3-4 true leaf stage. *Nicotiana benthamiana* plants were directly germinated and grown in pots containing Sunshine Mix, under the same environmental conditions as the poison ivy seedlings. *N. benthamiana* plants were syringe Agroinfiltrated four weeks post germination.

Agroinfiltration Transformation:

Three luciferase containing plasmids were used in this study. Plasmid pJGJ204 contains an Arabidopsis *RBCS1B-LUC* gene fusion comprised of the *RBCS1B* promoter-exon 3, resulting in an in-frame *RBCS1B-LUC* chimeric fusion protein (Jelesko et al. 1999).The Firefly luciferase *LUC* was PCR amplified from pJGJ102 (Jelesko et al. 1999) using oligonucleotide primers (oJGJ234 5’-GGGGAGAAGTTTGTACAAAAAAGCAGGCTATGGAAGACGCCAAAAACATA-3’; and oJGJ235 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTATTTTACAATTTGGACTTTC-3’) that incorporated 5’-*attB1* and 3’-*attB2* sequences. The resulting *LUC* PCR fragment was BP subcloned into pDONR221 using BP ClonaseII (Thermofisher, Waltham, MA) to yield plasmid pJGJ404. The *LUC* gene from pJGJ404 was subcloned into pMCD32 (Curtis and Grossniklaus 2003) using LR ClonaseII (Invitrogen) to yield plasmid pJGJ410 expressing the *LUC* gene from a double CaMV35S promoter. Plasmid pJGJ411 containing a *LUC-INT* gene was similarly subcloned, except using pLUK07 (Mankin et al. 1997) as the template in the initial PCR reaction. Plasmid p19 (Voinnet et al. 2003) was utilized for enhancing transient expression levels of the

transgenes in plant cells. *Agrobacterium tumefaciens* strain GV3101 containing either p19 (Voinnet et al. 2003), pJGJ204, pJGJ410, or pJGJ411 were grown overnight (16 hrs) in 5 mL LB medium supplemented with 50 µg/mL kanamycin and 50 µg/mL gentamycin. The culture OD_{600} was estimated from a 1:10 culture dilution prior to initial centrifugation (15mins. at 4°C, 3000Xg). Cultures were resuspended in MMA buffer (10mM MES, 10 mM $MgCl_2$, 20 µM acetosyringone) by vortexing for either final OD_{600} of 4 or 0.4. The *Agrobacterium* strains containing a *LUC*-containing plasmid (pJGJ204, pJGJ410, or pJGJ411) were mixed with GV3101/p19 at equivalent concentration and incubated at 22°C for 1 hr prior to Agroinfiltration (Kapila et al. 1997). Leaf tissues were either vacuum-Agroinfiltrated or syringe-Agroinfiltrated using a 3mL Luer Lok syringe (Becton Dickinson, Franklin Lakes, NJ), on the leaf underside (Kapila et al. 1997). Plants were returned to a 28°C growth chamber for 24 hrs before assay of luciferase activity.

Biolistic-Mediated Transformation:

Plasmid pJGJ411 was isolated from *Escherichia coli* OmniMax strain first by large scale alkaline lysis (Ausubel et al. 2006), followed by purification using a QIAprep Miniprep kit (Qiagen, Valencia, CA). Purified pJGJ411 plasmid DNA was coated on (0.6 µm) gold particles, accelerated using a 1100 psi rupture disk (Analytical Scientific Instruments US Inc., Richmond, CA) in a BIO-RAD (Richmond, CA) Biolistic PDS-1000 He Particle Delivery System. Poison ivy cotyledon/1st true leaf-stage seedlings in magenta boxes were subjected to particle bombardment at a distance of approximately 13cm. After bombardment, plants were returned to a 28°C growth chamber for 24hrs before assay of luciferase activity.

Luciferase Assay

Prior to luciferase imaging, transformed poison ivy tissues were placed onto water agar plates and then sprayed with luciferin and subjected to *in vivo* luciferase imaging, as previously

described (Jelesko et al. 1999). Qualitative photon emission imaging was obtained from "slice" images. Quantification of vacuum-Agroinfiltration and biolistic transformed poison ivy cotyledon and 1st true leaf *in vivo* luciferase activity (photon emission) was estimated using "gravity" images. A fixed area defined by the area that encompassed either the largest cotyledon (6,358 pixels^2) or largest 1st true leaf (30,458 pixels^2) was used to measure the number of emitted photons for each subsequent cotyledon, leaf, and background. The background heat photon emission was estimated for each specified tissue by randomly measuring the apparent gravity photon emission in ten regions corresponding to the absence of poison ivy tissue in the reflected light images, and the upper 95% confidence interval value was calculated. The upper 95% confidence interval value established the upper boundary of background heat photon emission levels corresponding to cotyledon and leaves, respectively. The respective upper background photon emission value was subtracted from the value of the respective tissue's highest photon emission level, and the resulting difference was equally divided into five bins, thereby providing a rough distribution of photon emission levels for each transformed tissue.

Results:

Transient LUC-INT gene expression in poison ivy leaves using syringe Agroinfiltration:

Poison ivy leaves were subjected to syringe-Agroinfiltration using *Agrobacterium tumefaciens* strain GV3101 harboring plasmid pJGJ411 containing a double CaMV promoter driving the expression of firefly luciferase gene containing an artificial intron (*LUC-INT*). The intron in the *LUC-INT* gene abolished low expression levels in Agrobacterium cells relative to the continuous *LUC* open reading frame in pJGJ410 (Figure 5.4), and thus all luciferase activity was derived from transient luciferase expression in poison ivy cells. Poison ivy leaves from potting soil grown plants subjected to syringe-Agroinfiltration generally did not display luciferase activity

above background heat photon emission levels in qualitative pseudo-color superimposed slice photon emission images (Figure 5.1 A). On very few occasions weak photon emission above background levels of syringe-Agroinfiltrated leaves was observed. In contrast, syringe-Agroinfiltrated leaves on poison ivy plants grown axenically in magenta boxes displayed small patches of consistent photon emission over regions that were syringe-Agroinfiltrated (Figure 5.1 A). Thus, magenta box grown poison ivy leaves were more susceptible to syringe-Agroinfiltration-mediated transient DNA transformation. However, not all leaves from magenta box grown poison ivy plants were equally susceptible to syringe-Agroinfiltration. Older leaves of magenta box grown plants were less susceptible to syringe-Agroinfiltration transient *LUC-INT* expression compared to younger leaves (Figure 5.1 B). Younger leaves more readily took up the Agrobacterium-infiltration solution delivered from the blunt syringe. Consequently, young poison ivy leaves in magenta box grown plants consistently showed higher transient luciferase expression levels than older leaves from the same plants, whereas changing the bacterial concentration ten-fold had little effect on transient luciferase expression levels (Figure 5.7). With that said, even the best poison ivy transient luciferase expression levels were dramatically lower than *Nicotiana benthamiana* leaves syringe-Agroinfiltrated with pJGJ411 (Fig. 5.1 C). The poison ivy leaves did not take up as much of the Agrobacterium solution as the *N. benthamiana* leaves with each syringe-Agroinfiltration attempt. However, these differences in relative infiltration volumes were far less than the difference in magnitude of photon emission between poison ivy and *N. benthamiana* (Figure 5.1 A and 5.1 C).

Syringe-Agroinfiltrated poison ivy leaves displayed brown discoloration within the regions that were effectively infiltrated with bacteria (Figure 5.1 D). This brown discoloration was dependent upon the presence of the Agrobacterium, because syringe infiltration with just the MMA

media lacking *Agrobacterium tumefaciens* did not acquire the brown pigmentation. When plants are infiltrated with bacteria, many plant species induce the synthesis and accumulation of phytoalexin compounds as part of a plant defense response (Hammerschmidt 1999). Thus, the overall low transient luciferase expression levels may have been a consequence of reduced Agrobacterium vigor in response to a biotic stress response, however this possibility was not further investigated in this study.

Transient AtRBCS1B-LUC gene expression in poison ivy leaves using vacuum-Agroinfiltration:

Vacuum-Agroinfiltration was investigated as alternative method for transient heterologous plant gene expression in poison ivy. An Arabidopsis chimeric *AtRBCS1B-LUC* chimeric gene fusion (pJGJ204 comprised of the *AtRBCS1B* promoter, exons I, intron I, exon II, intron II, and partial exon III fused in-frame to the Firefly *LUC* open reading frame), was used to vacuum-Agroinfiltrate excised poison ivy leaves and cotyledons from plants grown in magenta boxes. Vacuum-Agroinfiltrated leaves were placed on 0.5 X MS media plates, sprayed with luciferin at 24 hours post infiltration, and then imaged for three sequential one hour imaging sessions beginning at approximately 48 hrs post Agroinfiltration. The *AtRBCS1B-LUC* chimeric gene produces a low rate of photon emission in transgenic Arabidopsis plants. Superimposed "slice" images with complete subtraction of background photons displayed a few blue spots over leaves/cotyledons (Figure 5.5 A), whereas superimposed "slice" imaging that retained some background photons displayed higher qualitative photon accumulation over leaves vacuum-Agroinfiltrated with the plasmid containing the *AtRBCS1B-LUC* chimeric gene, relative to vacuum-Agroinfiltrations with the vector control plasmid pSLK7292 (Figure 5.5 B) indicating low but demonstrable *AtRBCS1B-LUC* recombinant protein expression over background heat photons. Photon emission levels were significantly higher in pJGJ204 (*AtRBCS1B-LUC*) vacuum-

Agroinfiltrated leaves and cotyledons compared to pSLK7292 vacuum-Agroinfiltrated leaves and cotyledons (P-value < 0.05), during the first two of three sequential one hour imaging sessions quantified using "gravity" imaging (Figure 5.2). There was a consistent trend of gradually lower total photon emission levels over the course of three hours suggesting that luciferin substrate levels were a limiting factor during the imaging session. The vacuum-Agroinfiltrated leaves and cotyledons also showed browning of infiltrated tissues similar to the syringe Agroinfiltrated leaves. This was particularly apparent in cases where the cotyledon or leaf segments were not fully vacuum-Agroinfiltrated, and showed a lighter green color (see arrows in Figure 5.5 C) typical of uninfiltrated leaves. These results demonstrate that a heterologous Arabidopsis genomic *RBCS1B* promoter and intron containing *RBCS1B-LUC* gene fusion was expressed in poison ivy leaves and cotyledons.

Biolistic transient LUC *gene expression in poison ivy leaves and cotyledon:*

The induction of an apparent biotic stress response (i.e. accumulation of brown pigmentation) in Agroinfiltrated poison ivy leaf tissue might limit the efficiency of Agroinfiltration transient DNA transformation. To avoid such a possibility, biolistic transformation of isolated pJGJ411 (*LUC-INT*) plasmid DNA directly into poison ivy leaves and cotyledons was investigated. Using superimposed slice photon imaging, punctuate foci of photon emission were observed on both cotyledons and leaves (Figure 5.6). The distribution of luciferase expression was not uniform, both across tissue type and between replicated tissues on the same targeting field. Likewise, quantification of gravity images demonstrated that the amount of photon emission from any given leaf or cotyledon was variable, ranging from background heat photons to quite intense photon emission (Figure 5.3). This variability is likely a consequence of the uneven and unpredictable spreading of plasmid-coated gold particles during particle acceleration. Unlike all

poison ivy tissues transformed by Agroinfiltration, the biolistic transformed poison ivy cotyledons and leaf tissues did not produce demonstrable brown pigmentation.

Discussion:

These studies lay the foundation for future transgenic reverse genetic approaches to investigate various poison ivy physiologies by demonstrating that exogenous DNA can be effectively introduced and expressed in poison ivy cotyledon and leaf tissues. Because the CaMV35S promoter is weakly active in *Agrobacterium tumefaciens* (Figure 5.4, and (Mankin et al. 1997) it was essential to use intron-containing firefly luciferase gene constructs (*CaMV35S-LUC-INT*, and *AtRBCS1B-LUC*) to validate poison ivy-specific transient luciferase activity. This was particularly germane in the case of the *AtRBCS1B-LUC* construct used in the vacuum-Agroinfiltrated leaves and cotyledons because this reporter gene construct is expressed at relatively low levels in plant cells (Jelesko et al. 1999, Jelesko et al. 2004). The two introns in the *AtRBCS1B-LUC* gene ensured that all luciferase activity was due to expression in transiently transformed poison ivy cells and not within the *Agrobacterium tumefaciens* strain GV3101.

The age of poison ivy leaves was an important factor for the relative susceptibility of syringe Agroinfiltration transient transformation. Leaves from potting soil-grown poison ivy plants were mostly recalcitrant to syringe-Agroinfiltration transient transformation. On the other hand, young poison ivy leaves from plants grown in magenta boxes showed consistent syringe-Agroinfiltration transient *LUC-INT* expression. Moreover, overall plant age was not the critical determinant, but rather the relative age of target leaves. As shown in Figure 5.1 B, the fifth emerged leaf (young leaf) showed greater syringe-Agroinfiltration transient luciferase expression levels than that of the second emerged leaf (older leaf) on the same plant. The qualitative levels of *LUC-INT* expression levels in poison ivy leaves were dramatically lower than the levels

observed in *Nicotiana benthamiana* leaves syringe-Agroinfiltrated with the same reporter gene construct. Poison ivy cotyledons and leaves were also susceptible to vacuum-Agroinfiltration transient transformation using a chimeric *AtRBCS1B-LUC* transgene. Leaves infiltrated with *Agrobacterium tumefaciens* strain GV3101 displayed discoloration consistent with an inducible plant biotic stress response. Both syringe- and vacuum-Agroinfiltrated leaves demonstrated the accumulation of uncharacterized brown pigments (Figure 5.1 D, Figure 5.5 C). This pigment accumulation was not observed for leaves infiltrated with MMA buffer lacking Agrobacteria (Figure 5.1 D). The relative *LUC* expression levels in the brightest poison ivy leaves was dramatically less that that observed in control *N. benthamiana* leaves syringe Agroinfiltrated with the same construct. This difference could have been due to a variety of parameters including the quantity of Agrobacteria penetrating into the apoplastic space of leaves, and/or a biotic stress response that inhibited bacterial vigor or T-DNA transfer into the plant cells. Nevertheless, poison ivy leaves and cotyledons showed significant transient *LUC-INT* and *AtRBCS1B-LUC* luciferase expression levels over controls indicating the feasibility of using Agroinfiltration transient poison ivy transformation.

As expected, poison ivy cotyledons and leaves were readily transiently transformed using a biolistic method. Biolistically transformed poison ivy leaves and cotyledons did not produce brown pigmented regions and thus averted a poison ivy biotic stress response against bacterial pathogen associated molecular patterns (PAMPs). As is typical of biolistic transformation of plant tissues, the transient *LUC-INT* expression levels were highly variable amongst both leaf and cotyledon tissues. This is in large part to previously recognized inconsistencies in both plasmid DNA coating of gold microparticles (Sanford et al. 1993) and heterogeneous particle spread on the

macro carrier disk, resulting in heterogeneous distribution during particle acceleration towards the target tissue.

These results demonstrate the feasibility of poison ivy leaf and cotyledon transient transformation with recombinant reporter gene constructs using either Agroinfiltration or biolistic methods. Both poison ivy DNA transformation methods are important technical advancements enabling the introduction of recombinant DNA constructs designed for a variety of reverse genetic (e.g. RNA interference or viral induced gene silencing) and genome editing methods (e.g. zinc finger, TALEN, or CRISPR-CAS9) to enable molecular genetic investigations of poison ivy ecophysiology and metabolism.

References:

Abe, I., Utsumi, Y., Oguro, S., and Noguchi, H., 2004. The first plant type III polyketide synthase that catalyzes formation of aromatic heptaketide. *FEBS Lett.* 562:171-176.

Andrieu, A., Breitler, J.C., Sire, C., Meynard, D., Gantet, P., and Guiderdoni, E., 2012. An in planta, Agrobacterium-mediated transient gene expression method for inducing gene silencing in rice (Oryza sativa L.) leaves, Rice.

Arcos-Ortega, G.F., Chan-Kuuk, R.A., Gonzalez-Kantun, W.A., Souza-Perera, R., Nakazawa-Ueji, Y.E., Aviles-Berzunza, E., Godoy-Hernandez, G., Lawton, M.A., and Aguilar, J.J.Z., 2010. Agrobacterium tumefaciens-transient genetic transformation of Habanero pepper (*Capsicum chinense* Jacq.) leaf explants, Electron J Biotechn.

Ausubel, F.A., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., 2006. Current Protocols in Molecular Biology. John Wiley & Sons, New York.

Bakshi, S., Sadhukhan, A., Mishra, S., and Sahoo, L., 2011. Improved Agrobacterium-mediated transformation of cowpea via sonication and vacuum infiltration. *Plant Cell Rep* 30:2281-2292.

Benhase, E.B. and Jelesko, J.G., 2013. Germinating and culturing axenic poison ivy seedlings. *HortScience* 48:1525-1529.

Curtis, M.D. and Grossniklaus, U., 2003. A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant physiology* 133:462-469.

Daniell, H., Vivekananda, J., Nielsen, B.L., Ye, G.N., Tewari, K.K., and Sanford, J.C., 1990. Transient foreign gene expression in chloroplasts of cultured tobacco cells after biolistic delivery of chloroplast vectors. *Proc Natl Acad Sci U S A* 87:88-92.

Dewick, P.M., 1997. *Medicinal natural products: a biosynthetic approach*. John Wiley & Sons, Chichester, UK.

Drummond, M.H., Gordon, M.P., Nester, E.W., and Chilton, M.D., 1977. Foreign DNA of bacterial plasmid origin is transcribed in crown gall tumours [*Agrobacterium tumefaciens*]. *Nature* 269:535-536.

Gross, M., Baer, H., and Fales, H.M., 1975. Urushiols of poisonous Anacardiaceae. *Phytochemistry (Oxf.)* 14:2263-2266.

Hammerschmidt, R., 1999. Phytoalexins: What have we learned after 60 years? *Annu Rev Phytopathol* 37:285-306.

Hill, G.A., Mattacotti, V., and Graha, W.D., 1934. The toxic principle of the poison ivy. *J. Am. Chem. Soc.* 56:2736-2738.

Jelesko, J.G., Carter, K., Thompson, W., Kinoshita, K., and Gruissem, W., 2004. Meiotic recombination between paralogous *RBCSB* genes on sister chromatids of *Arabidopsis thaliana*. *Genetics* 166:947-957.

Jelesko, J.G., Harper, R., Furuya, M., and Gruissem, W., 1999. Rare germinal unequal crossing-over leading to recombinant gene formation and gene duplication in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA.* 96:10302-10307.

Kapila, J., DeRycke, R., VanMontagu, M., and Angenon, G., 1997. An Agrobacterium-mediated transient gene expression system for intact leaves. *Plant Sci* 122:101-108.

Kim, S.S., Grienenberger, E., Lallemand, B., Colpitts, C.C., Kim, S.Y., Souza Cde, A., Geoffroy, P., Heintz, D., Krahn, D., Kaiser, M., Kombrink, E., Heitz, T., Suh, D.Y., Legrand, M., and Douglas, C.J., 2010. LAP6/POLYKETIDE SYNTHASE A and LAP5/POLYKETIDE SYNTHASE B encode hydroxyalkyl alpha-pyrone synthases required for pollen

development and sporopollenin biosynthesis in *Arabidopsis thaliana*. *Plant Cell* 22:4045-4066.

Kim, S.Y., Colpitts, C.C., Wiedemann, G., Jepson, C., Rahimi, M., Rothwell, J.R., McInnes, A.D., Hasebe, M., Reski, R., Sterenberg, B.T., and Suh, D.Y., 2013. Physcomitrella PpORS, basal to plant type III polyketide synthases in phylogenetic trees, is a very long chain 2'-oxoalkylresorcinol synthase. *J. Biol. Chem.* 288:2767-2777.

King, J.L., Finer, J.J., and McHale, L.K., 2015. Development and optimization of agroinfiltration for soybean. *Plant Cell Rep* 34:133-140.

Klein, T.M., Fromm, M., Weissinger, A., Tomes, D., Schaaf, S., Sletten, M., and Sanford, J.C., 1988. Transfer of foreign genes into intact maize cells with high-velocity microprojectiles. *Proc Natl Acad Sci U S A* 85:4305-4309.

Klein, T.M., Wolf, E.D., Wu, R., and Sanford, J.C., 1987. High-Velocity Microprojectiles for Delivering Nucleic-Acids into Living Cells. *Nature* 327:70-73.

Kurtz, A.P. and Dawson, C.R., 1971. Synthesis of compounds structurally related to poison ivy urushiol. 4. 3-(1-Alkyl)alkylcatechols of varying side-chain shape and flexibility. *J. Med. Chem.* 14:733-737.

Majima, R., 1922. Uber den hauptbestandteil des Japan-lacs, VIII. Mitteilung: stellung der doppelbindungen in der seitenkette des urushiols und beweisfuhrung, dab das urushiol eine mischung ist. *Ber. Dtsch. Chem. Ges.* 55(b):172-191.

Mankin, S.L., Allen, G.C., and Thompson, W.F., 1997. Introduction of a plant intron into the luciferase gene of *Photinus pyralis*. *Plant Mol. Biol. Report.* 15:186-196.

Markiewitz, K.H. and Dawson, C.R., 1965. On the isolation of the allegenically active components of the toxic principle of poison ivy. *J. Org. Chem.* 30:1610-1613.

Martin, A.C., Zim, H.S., and Nelson, A.L., 1951. *American wildlife & plants*. Dover Publications, Inc., New York, New York, USA.

Matsuzawa, M., Katsuyama, Y., Funa, N., and Horinouchi, S., 2010. Alkylresorcylic acid synthesis by type III polyketide synthases from rice *Oryza sativa*. *Phytochemistry (Oxf.)* 71:1059-1067.

Mo, R.L., Huang, Y.M., Yang, S.C., Zhang, Q.L., and Luo, Z.R., 2015. Development of Agrobacterium-mediated transient transformation in persimmon (Diospyros kaki Thunb.). *Sci Hortic-Amsterdam* 192:29-37.

Mohan, J.E., Ziska, L.H., Schlesinger, W.H., Thomas, R.B., Sicher, R.C., George, K., and Clark, J.S., 2006. Biomass and toxicity responses of poison ivy (*Toxicodendron radicans*) to elevated atmospheric CO2. *Proc. Natl. Acad. Sci. U. S. A.* 103:9086-9089.

Popay, I. and Field, R., 1996. Grazing animals as weed control agents. *Weed Technol.* 10:217-231.

Sanford, J.C., Smith, F.D., and Russell, J.A., 1993. Optimizing the biolistic process for different biological applications. *Methods Enzymol.* 217:483-509.

Symes, W.F. and Dawson, C.R., 1953. Separation and structural determination of the olefinic components of poison ivy urushiol, cardanol and cardol. *Nature* 171:841-842.

Symes, W.F. and Dawson, C.R., 1954. Poison ivy “urushiol”. *J. Am. Chem. Soc.* 76:2959-2963.

Taura, F., Iijima, M., Yamanaka, E., Takahashi, H., Kenmoku, H., Saeki, H., Morimoto, S., Asakawa, Y., Kurosaki, F., and Morita, H., 2016. A novel class of plant Type III polyketide synthase involved in orsellinic acid biosynthesis from *Rhododendron dauricum*, *Front Plant Sci*. 7:1452.

Taura, F., Tanaka, S., Taguchi, C., Fukamizu, T., Tanaka, H., Shoyama, Y., and Morimoto, S., 2009. Characterization of olivetol synthase, a polyketide synthase putatively involved in cannabinoid biosynthetic pathway. *FEBS Lett.* 583:2061-2066.

VanderGheynst, J.S., Guo, H.Y., and Simmons, C.W., 2008. Response surface studies that elucidate the role of infiltration conditions on *Agrobacterium tumefaciens*-mediated transient transgene expression in harvested switchgrass (*Panicum virgatum*). *Biomass Bioenerg* 32:372-379.

Voinnet, O., Rivas, S., Mestre, P., and Baulcombe, D., 2003. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J.* 33:949-956.

Wang, Y.C., Klein, T.M., Fromm, M., Cao, J., Sanford, J.C., and Wu, R., 1988. Transient expression of foreign genes in rice, wheat and soybean cells following particle bombardment. *Plant Mol Biol* 11:433-439.

Weisberg, A.J., Kim, G., Westwood, J.H., and Jelesko, J.G., 2017. Sequencing and de novo assembly of the *Toxicodendron radicans* (poison ivy) transcriptome. *Genes (Basel)* 8:317

Ziska, L.H., Sicher, R.C., George, K., and Mohan, J.E., 2007. Rising atmospheric carbon dioxide and potential impacts on the growth and toxicity of poison ivy (*Toxicodendron radicans*). *Weed Sci.* 55:288-292.

Figures:

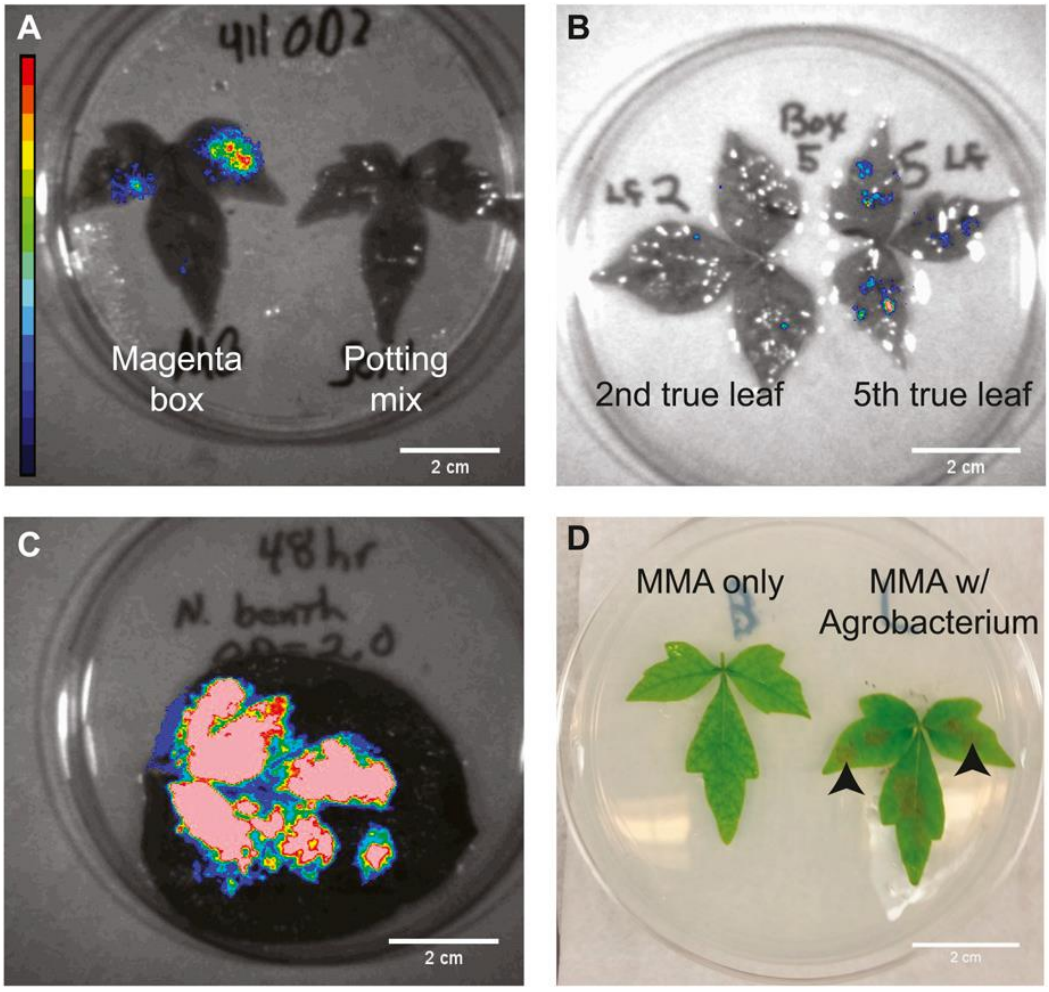

Figure 5.1: Imaging of Agroinfiltrated leaves. Panels A-C single photon pseudocolor images (panel A insert is pseudocolor step gradient with high photon emission shown as red to low photon emission as dark blue) superimposed on reflected light image (gray-scale). Panel D, digital color image of poison ivy leaves infiltrated with either MMA buffer or MMA buffer with Agrobacterium containing the Firefly luciferase construct. Arrows indicate browning at site of Agroinfiltration. A-B, composite pseudocolor single photon imaging on reflected light image of poison ivy leaves Agroinfiltrated with Firefly luciferase construct. C, *Nicotiana benthamiana* leaf similarly Agroinfiltrated and imaged.

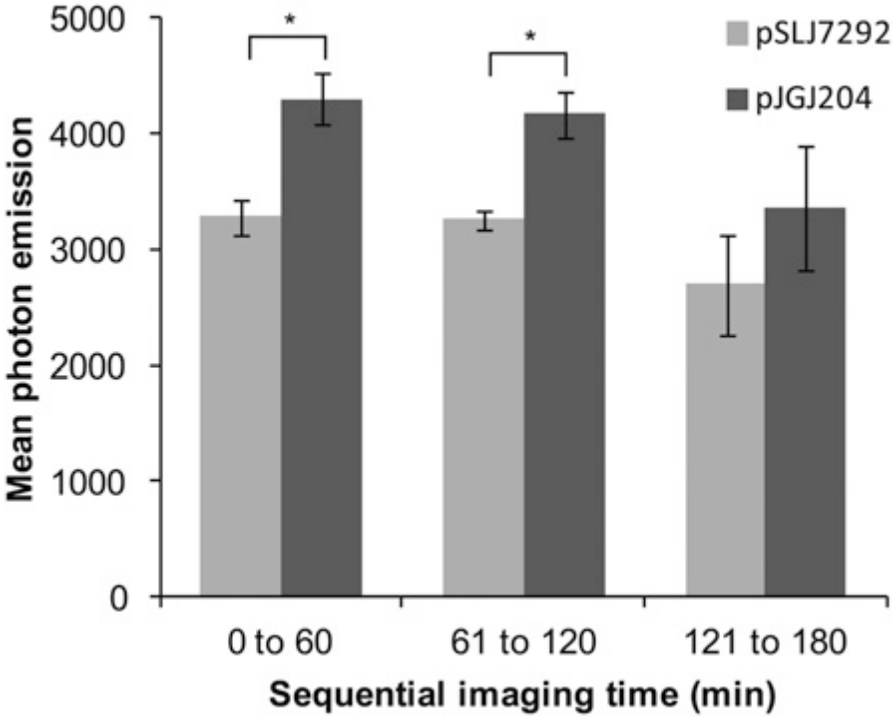

Figure 5.2: Transient *AtRBCS1B-LUC* expression in vacuum-infiltrated poison ivy cotyledons and leaves. Leaves were imaged at 48 hours post vacuum-Agroinfiltration for three consecutive one hour imaging sessions. The experiment was replicated two times. The mean photon emission values were plotted with standard error and asterisks indicated *P*-value ≤ 0.05 in two-sample *T*-test comparing vector control (pSLK7292) with *AtRBCS1B-LUC* binary plasmid pJGJ204.

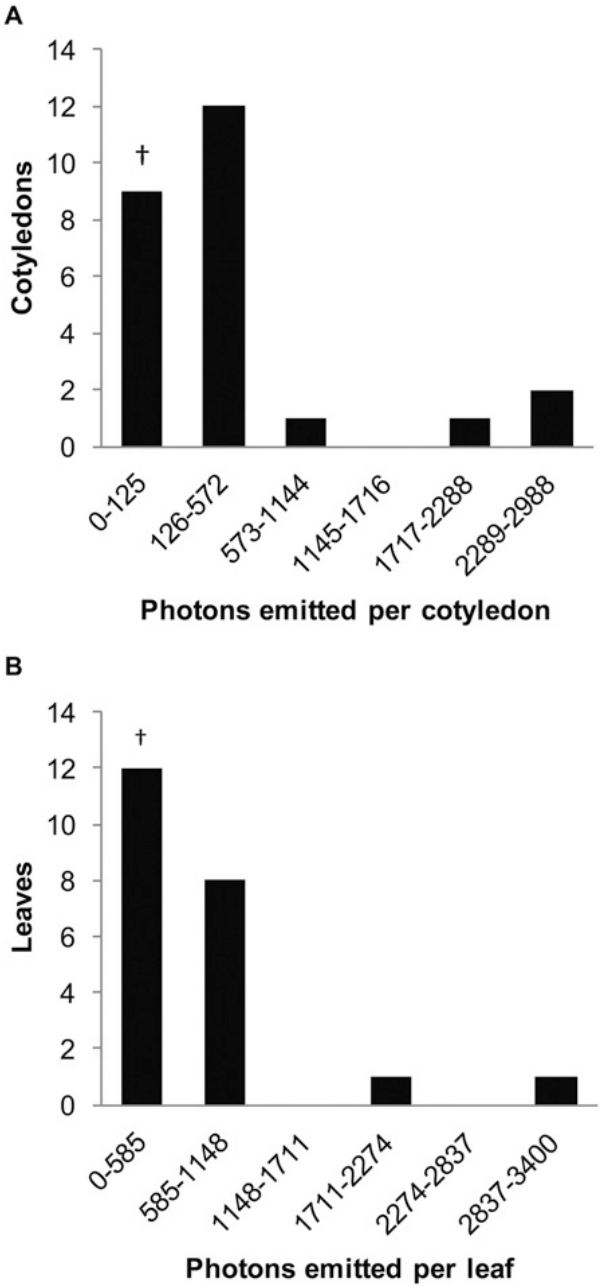

Figure 5.3: The number of cotyledons or leaves with a specified range of photons emitted on a per leaf or per cotyledon basis. The dagger (†) indicates the background photon emission bin for cotyledons (panel A) and leaves (panel B), see Materials and Methods section for calculation of background photon emission levels.

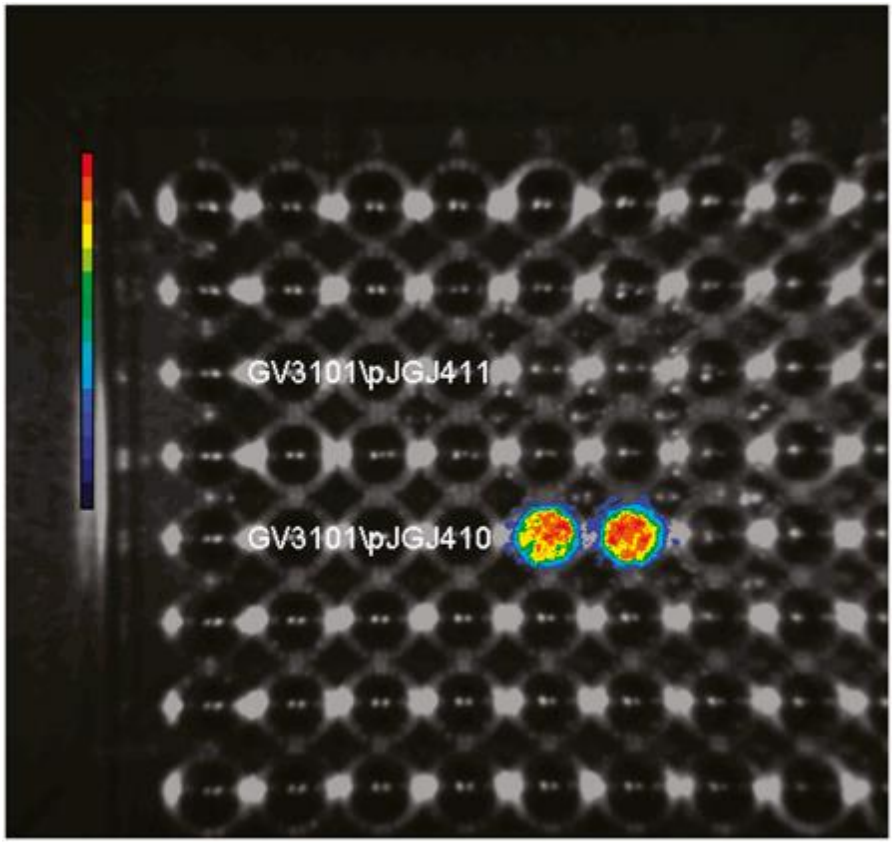

Figure 5.4: Superimposed photon emission images of *Agrobacterium tumefaciens* strains GV3103/pJGJ411 (*LUC-INT* reporter gene, wells C5-6) and GV3101/pJGJ410 (*LUC* reporter gene, wells E5-6) duplicate cultures placed in 96-well microtiter plate. Pseudocolor step gradient is the same as Fig. 1A.

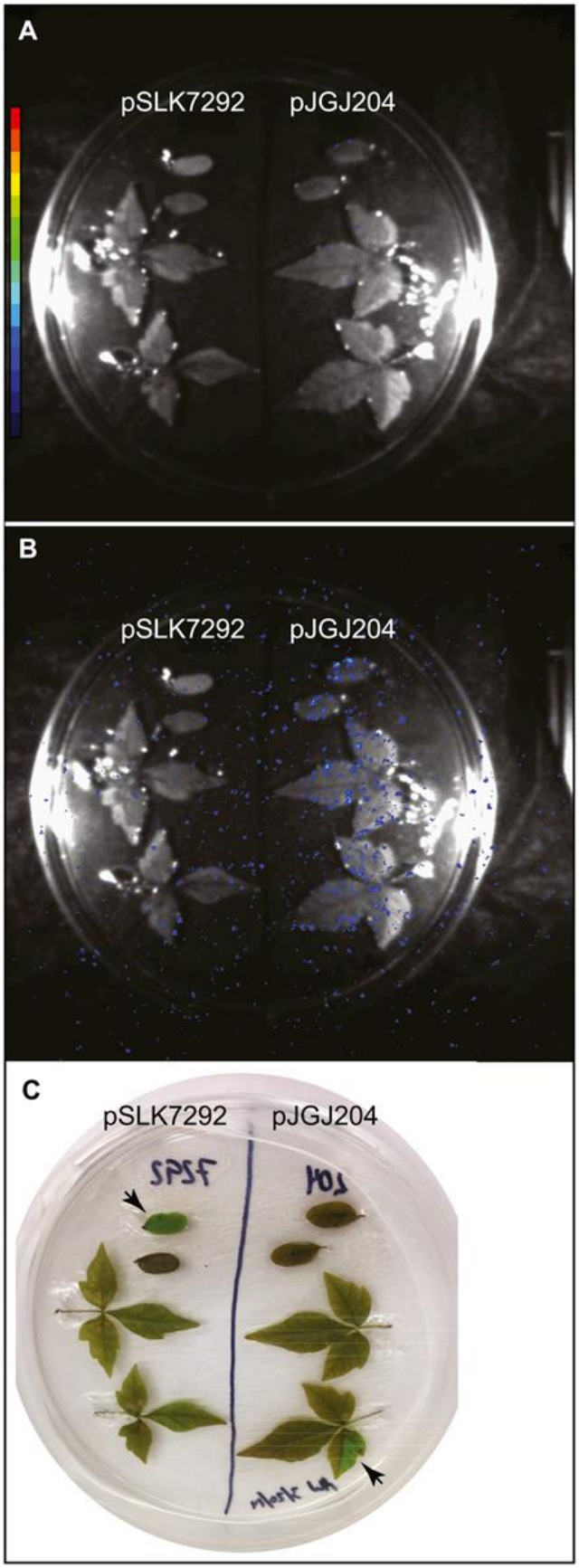

Figure 5.5: Poison ivy cotyledons and leaves vacuum-infiltrated with *A. tumefaciens* harboring either empty vector (pSLK7292) or *AtRBCS1B::LUC* (pJGJ204). A, composite pseudocolor photon image with background heat photon levels subtracted. Pseudocolor step gradient is the same as Fig. 1A. B, composite pseudocolor photon image with background heat photon levels displayed. C, digital color image displaying mostly water-soaked infiltrated tissues and non-infiltrated tissues (indicated by arrows pointing towards lighter-green non-infiltrated tissues).

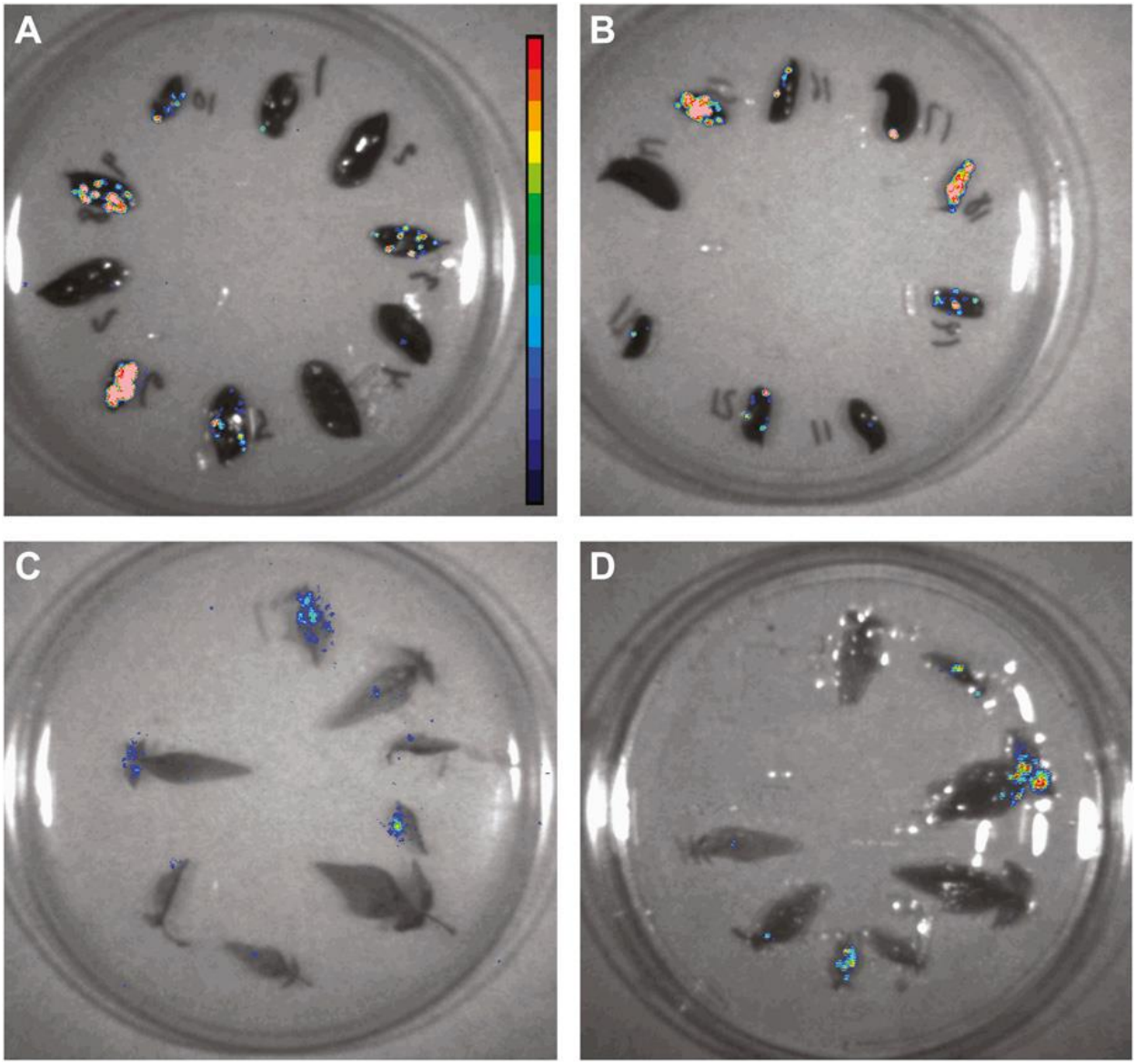

Figure 5.6: Superimposed photon emission of cotyledons (A-B) and true leaves (C-D). Pseudocolor step gradient same as Fig. 1A.

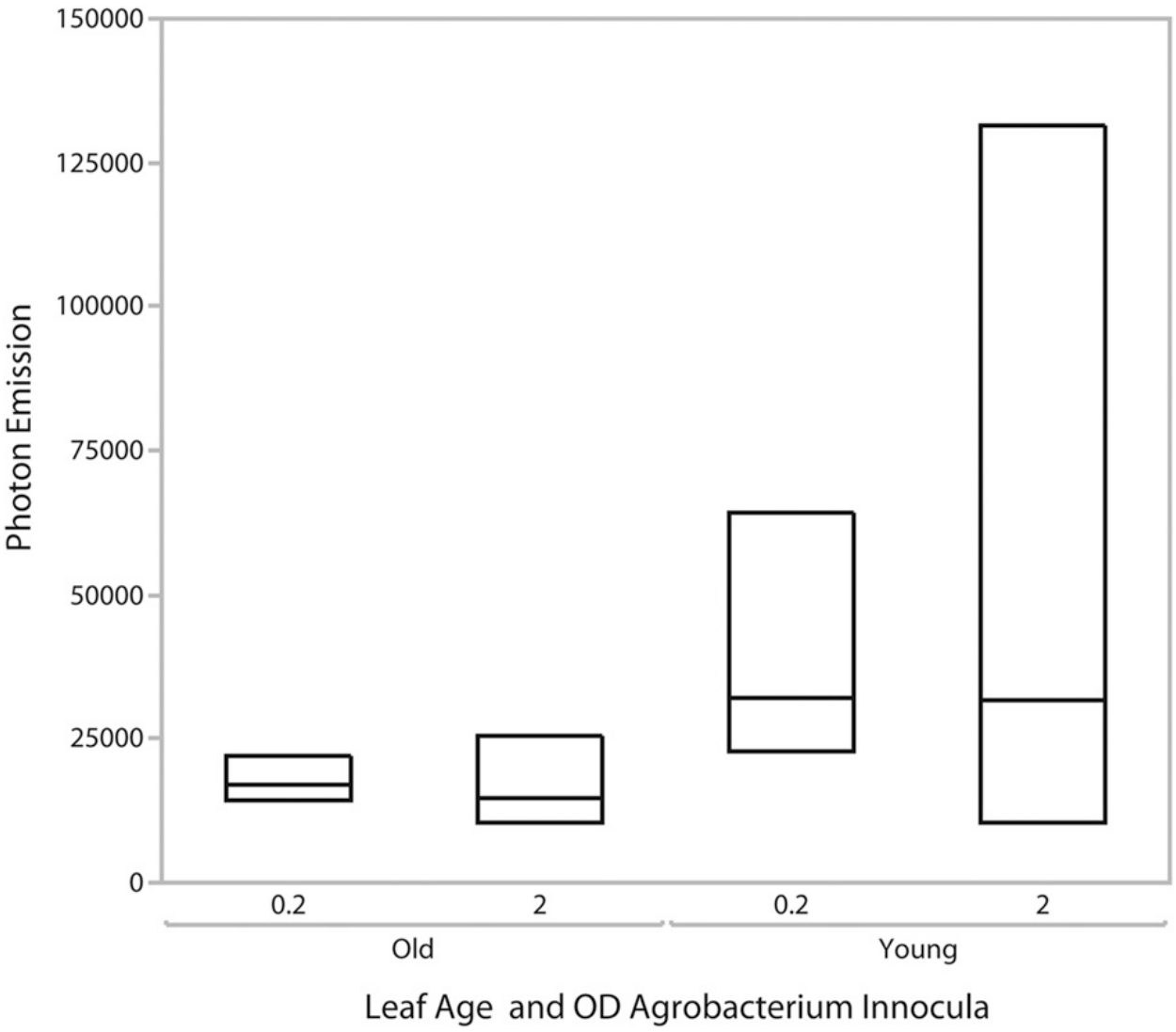

Figure 5.7: Box plots of photon emission levels from poison ivy leaves of different age and Agrobacterium inoculum concentrations. Leaves were from Magenta box grown poison ivy plants. Three leaves were independently imaged for each treatment combination. The line bisecting each box is the median value. Agrobacterium inoculum concentration was either 0.2 OD or 2.0 OD at 600nm.

Appendix: Investigation of the urushiol biosynthetic gene pathway in poison ivy using viral induced gene silencing.

Urushiol is a mixture of various alk(en)yl-catechol congeners, a class of alkylphenols (Symes and Dawson, 1953). Urushiols can exist either as 3-pentadecyl catechols or as 3-heptadecyl catechols. The hydrocarbon tail can be unsaturated or consist of one to three carbon-to-carbon double bonds (Markiewitz and Dawson, 1965). Urushiol is produced by the members of the *Toxicodendron* genus of plants which include poison ivy (*Toxicodendron radicans*), poison oak *(Toxicodendron. diversilobum*), and the Japanese lac tree (*Toxicodendron. vernicifluum*), among others. The relative abundance of the urushiol congeners differs between the members of *Toxicodendron* genus. For instance, in poison ivy, the 3-pentadecyl mono, di and triolefins are most common (though see Chapter 4) while in poison oak the 3-heptadecyl mono, di and triolefins are most common. The double bonds of the olefins are found in the same locations in both the 3-pentadecyl and 3-heptadecyl species at the 8, 11, 13 positions of the hydrocarbon tail (Sunthankar and Dawson, 1954, Markiewitz and Dawson, 1965).

Urushiol sourced from the sap of the Japanese lac tree (*Toxicodendron vernicifluum*) has a long history as a useful and highly valued natural product in Asian cultures (Lu et al., 2013). Lacquerware production is nearly entirely based upon the sap from the Japanese lac tree (Japanese word for this sap is “urushi”) that is spread over wooden objects to form a high luster waterproof polymeric coating. The sap is largely composed of urushiol and small amounts of the enzyme laccase that is responsible for oxidation activated urushiol polymerization through semi- and ortho-quinone intermediates that can undergo Michael addition reactions (Kumanotani, 1978). Research into lacquerware curing processes and the chemical structure of urushiol also shows promise of the natural product as an engineering material (Zheng et al., 2014, Xia et al., 2009, Zheng et al.,

2009). Moreover, the ortho-hydroxyls have the ability to chelate various metals, allowing for applications such as the potential for urushiol-copper chelate polymers as an anticorrosive coating (Xia et al., 2009). In other applications, urushiol can produce super-hydrophobic films fabricated through layer-by-layer assembly (Zheng et al., 2009). These inherent chemical properties hold enormous opportunities for a variety of high performance material science applications.

While research on urushiol as a high performance material continues to develop, there is a lack of research on how to source urushiol in a controlled and sustainable manner. Current cultivation of the Japanese lac tree relies on existing wild populations that are tapped by scoring the bark of the lac tree and collecting "urushi" on a drip-by-drip basis. The current reliance on sap harvesting from the Japanese lac tree will be unable to supply urushiol on an industrial scale. This presents a distinct need to either produce urushiol by chemical synthesis, or produce urushiol using a synthetic biology approach. Attempts to produce urushiol by organic synthesis show marginal success, largely because of the innate reactivity of the catechol moiety to oxidation, and thus currently impractical for large scale sourcing (ElSohly et al., 1986, Kurtz and Dawson, 1971, Miyakoshi et al., 1991a, Miyakoshi et al., 1991b, Byck and Dawson, 1967). A synthetic biology-oriented urushiol production platform utilizing an organic feedstock from industrial waste streams would be a key innovation before urushiol can be implemented in broader material science applications.

The early and intermediate steps in urushiol biosynthesis are based upon precedents in known plant biochemistry/enzymology. The initial biosynthetic step is likely a type III PKS-like enzyme activity that utilizes a fatty acid (FA) FA-CoA starter and elongates it to a FA-tetraketide (Dewick, 1997). As the initial step, the bulk of our investigations were focused on three type III *PKS-like* genes identified from the poison ivy transcriptome (Weisberg, 2014). Viral induced gene

silencing (VIGS) was the initial approach of choice taken to suppress the expression of the endogenous putative urushiol biosynthesis genes based on preliminary success with the technique. Compared to other transgenic methods, VIGS is an attractive alternative where forward genetics and/or the development of transgenic lines is difficult or not yet available (Liu et al., 2002). A suitable VIGS vector that can infect and replicate in poison ivy has not been previously reported. However, the tobacco rattle virus (TRV) is effective in many hosts such as tobacco, tomato, pepper, Arabidopsis, and strawberry (Burch-Smith et al., 2004, Liu et al., 2002, Tian et al., 2014), representing an initial candidate vector for use in poison ivy. The phytoene desaturase (*PDS*) gene is generally targeted as a proof-of-concept target gene, because suppression of *PDS* mRNA levels results in a leaf bleaching phenotype in various plant tissues as *PDS* is necessary for the production of carotenoids (Qin et al., 2007). This approach was used to optimize VIGS in a number of plant species with a high level of success (Fu et al., 2006, Liu et al., 2002, Dinesh-Kumar et al., 2003) and as such was the approach that we took.

Preliminary experiments of TRV-induced VIGS of the poison ivy *TrPDS* gene resulted in the expected leaf bleaching phenotype associated with silencing of the *PDS* gene (Figure A1) at low frequency. Thus, in principle VIGS in poison was possible, but it was necessary to increase the efficiency and frequency of VIGS in poison ivy to a level where reproducibility could be assured. Numerous attempts to optimize this procedure were unsuccessful (Table A1) at improving the efficiency or reproducibility of the *TrPDS* VIGS phenotype. No combination of changes in growth conditions, target tissues, nor method of viral introduction improved upon our baseline results. After a period of time, further attempts to replicate the conditions of our initial success in silencing *TrPDS* expression were entirely unsuccessful. We were not able to determine the exact cause of why we were unable to replicate our initial success. It is possible that poison ivy might

have effective disease resistance mechanisms that restrict TRV viral replication. If this were the case, our initial success with *TrPDS* VIGS could have been due to natural variation in our wild-collected poison ivy seed that were permissive to TRV replication and thus *TrPDS* VIGS. However, subsequent poison ivy seed collections did not contain TRV replication permissive genotypes. Alternative viruses have been developed for VIGS such as the Apple Latent Spherical Virus which has been shown to infect woody plant species (Igarashi et al., 2009), however our attempts to use this virus on 25 seedlings were also unsuccessful. Ultimately, we could not determine why we were not able to replicate our initial success with VIGS.

Thus, another approach was attempted. For this line of inquiry, *Agrobacterium* harboring *TrPKS* genes were infiltrated into the leaves of *Nicotiana benthamiana* (Wydro et al., 2006). Quick transient expression of heterologous proteins has been developed using the *pEAQ* vectors, leading to high expression of recombinant proteins in as few as three days (Sainsbury et al., 2009, Peyret and Lomonossoff, 2013). *TrPKS* cDNAs were subcloned into the appropriate pEAQ vector and Agroinfiltrated into *N. benthamiana* leaves. These presumed TrPKS protein expressing leaf extracts were assessed for accumulation of novel metabolites through GC-MS analysis (Aziz et al., 2017). This approach aimed to build additional support that the putative urushiol biosynthesis genes are capable of performing the proposed catalytic reactions in other plant species. Moreover, coinfiltration of Agrobacterium lines containing cDNAs encoding sequential steps in the biosynthesis of a secondary metabolic pathway have been used to reconstitute the entire biosynthetic pathway in *Nicotiana benthamiana* (Lau and Sattely, 2015). We limited this approach to the same three type III *PKS-like* genes. After 12 attempts, this approach was not successful in demonstrating reproducible novel metabolite production. Despite validating protein accumulation through SDS-PAGE gels and western blot analysis, we rarely saw any novel metabolites compared

to our mock infiltrated tobacco plants. In cases where a novel peak was found, typically occurring in tobacco plants expressing the gene we named *TrPKS1*, the fragmentation of the compound did not match any of our expected urushiol intermediates. Further, mass spectral comparison to entries in the NIST library did not match any submitted compounds. The metabolite analysis procedure had a polar metabolite extraction requirement (specifically it required the metabolite to be readily soluble in chloroform). However, if a *TrPKS*-produced metabolite was present as a Co-enzyme A conjugate or another amphiphilic metabolite, it is very likely that it would not readily fractionate into the polar extraction phase and thus elude detection by our standard urushiol extraction procedure.

One final approach was attempted, in which we identified poison ivy leaf tissues that accumulated relatively high and low amounts of urushiol and assess these tissue for differential gene expression of our type III *PKS-like* genes. This was accomplished through dissecting leaves into veins, the petiole, and the interveinal leaf tissues. We were able to demonstrate high accumulation of urushiols in the veins and petiole relative to the interveinal leaf tissue (Figure A2). With one of our type III *PKS-like* genes, so called *TrPKS1*, we were able to demonstrate a reproducible trend of higher steady state *TrPKS1* mRNA levels, assessed through qRT-PCR, in vein tissues (Figure A3). Similar trends were not observed for our other genes of interest, *TrPKS2* and *TrPKS3*. Thus, only steady state *TrPKS1* mRNA levels were positively correlated with tissues that accumulated higher steady state urushiol levels. These results suggest that the *TrPKS1* may be involved in urushiol biosynthesis. However, definitive proof that *TrPKS1* is an urushiol biosynthetic gene will require subsequent validation using either a biochemical approach or some as yet to be developed reverse genetic procedure in poison ivy.

References:

Aziz, M., Sturtevant, D., Winston, J., Collakova, E., Jelesko, J. & Chapman, K. 2017. MALDI-MS Imaging of Urushiols in Poison Ivy Stem. *Molecules,* 22**,** 711.

Burch-Smith, T. M., Anderson, J. C., Martin, G. B. & Dinesh-Kumar, S. P. 2004. Applications and advantages of virus-induced gene silencing for gene function studies in plants. *The Plant Journal,* 39**,** 734-746.

Byck, J. S. & Dawson, C. R. 1967. The synthesis of compounds structurally related to poison ivy Urushiol. 4,5-dimethyl-3-pentadecylcatechol. *Journal of Organic Chemistry,* 32**,** 1084-8.

Dewick, P. M. 1997. *Medicinal natural products: a biosynthetic approach,* Chichester, UK, John Wiley & Sons.

Dinesh-Kumar, S., Anandalakshmi, R., Marathe, R., Schiff, M. & Liu, Y. 2003. Virus-induced gene silencing. *Plant Functional Genomics***,** 287-293.

ElSohly, M. A., Adawadkar, P. D., Benigni, D. A., Watson, E. S. & Little Jr, T. L. 1986. Analogs of poison ivy urushiol. Synthesis and biological activity of disubstituted n-alkylbenzenes. *Journal of Medicinal Chemistry,* 29**,** 606-611.

Fu, D., Zhu, B., Zhu, H., Zhang, H., Xie, Y., Jiang, W., Zhao, X. & Luo, Y. 2006. Enhancement of virus-induced gene silencing in tomato by low temperature and low humidity. *Molecules and cells,* 21**,** 153.

Igarashi, A., Yamagata, K., Sugai, T., Takahashi, Y., Sugawara, E., Tamura, A., Yaegashi, H., Yamagishi, N., Takahashi, T. & Isogai, M. 2009. Apple latent spherical virus vectors for reliable and effective virus-induced gene silencing among a broad range of plants including tobacco, tomato, Arabidopsis thaliana, cucurbits, and legumes. *Virology,* 386**,** 407-416.

Kumanotani, J. 1978. Laccase-catalyzed polymerization of urushiol in precisely confined Japanese lacquer system. *Die Makromolekulare Chemie,* 179**,** 47-61.

Kurtz, A. P. & Dawson, C. R. 1971. Synthesis of compounds structurally related to poison ivy urushiol. 4. 3-(1-Alkyl)alkylcatechols of varying side-chain shape and flexibility. *J Med Chem,* 14**,** 733-7.

Lau, W. & Sattely, E. S. 2015. Six enzymes from mayapple that complete the biosynthetic pathway to the etoposide aglycone. *Science,* 349**,** 1224-8.

Liu, Y., Schiff, M. & Dinesh-Kumar, S. 2002. Virus-induced gene silencing in tomato. *The Plant Journal,* 31**,** 777-786.

Lu, R., Yoshida, T. & Miyakoshi, T. 2013. Oriental Lacquer: A Natural Polymer. *Polymer Reviews,* 53**,** 153-191.

Markiewitz, K. H. & Dawson, C. R. 1965. On the isolation of the allegenically active components of the toxic principle of poison ivy. *Journal of Organic Chemistry,* 30**,** 1610-1613.

Miyakoshi, T., Du, Y. & Kumanotani, J. 1991a. Synthesis of 3-(Omega-Phenylalkyl)Catechols, and 4-(Omega-Phenylalkyl)Catechols, the Sap Exuded from a Burmese Lac Tree, Melanorrhoea-Usitate. *Bulletin of the Chemical Society of Japan,* 64**,** 1054-1056.

Miyakoshi, T., Kobuchi, H., Niimura, N. & Yoshihiro, Y. 1991b. Synthesis of Urushiols with Pentadecatrienyl Side-Chain, 2 Constituents of the Sap of a Lac Tree, Rhus-Vernicifera. *Bulletin of the Chemical Society of Japan,* 64**,** 2560-2562.

Peyret, H. & Lomonossoff, G. P. 2013. The pEAQ vector series: the easy and quick way to produce recombinant proteins in plants. *Plant molecular biology,* 83**,** 51-58.

Qin, G., Gu, H., Ma, L., Peng, Y., Deng, X. W., Chen, Z. & Qu, L.-J. 2007. Disruption of phytoene desaturase gene results in albino and dwarf phenotypes in Arabidopsis by impairing chlorophyll, carotenoid, and gibberellin biosynthesis. *Cell Research,* 17**,** 471-482.

Sainsbury, F., Thuenemann, E. C. & Lomonossoff, G. P. 2009. pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. *Plant biotechnology journal,* 7**,** 682-693.

Sunthankar, S. V. & Dawson, C. R. 1954. The structural identification of the olefinic components of Japanese Lac urushiol. *Journal of the American Chemical Society,* 76**,** 5070-5074.

Symes, W. F. & Dawson, C. R. 1953. Separation and structural determination of the olefinic components of poison ivy urushiol, cardanol and cardol. *Nature,* 171**,** 841-2.

Tian, J., Pei, H., Zhang, S., Chen, J., Chen, W., Yang, R., Meng, Y., You, J., Gao, J. & Ma, N. 2014. TRV–GFP: a modified Tobacco rattle virus vector for efficient and visualizable analysis of gene function. *Journal of Experimental Botany,* 65**,** 311-322.

Weisberg, A. 2014. *Investigations into the Molecular Evolution of Plant Terpene, Alkaloid, and Urushiol Biosynthetic Enzymes.* Virginia Polytechnic Institute and State University.

Wydro, M., Kozubek, E. & Lehmann, P. 2006. Optimization of transient Agrobacterium-mediated gene expression system in leaves of *Nicotiana benthamiana*. *ACTA BIOCHIMICA POLONICA-ENGLISH EDITION-,* 53**,** 289.

Xia, J. R., Xu, Y. L., Hu, B. H. & Lin, J. H. 2009. A rapid approach to urushiol-copper(I) coordination polymer under UV irradiation. *Progress in Organic Coatings,* 65**,** 510-513.

Zheng, X. L., Weng, J. B., Huang, Q. M., Hu, B. H., Qiao, T. & Deng, P. 2009. Fabrication of a stable poly(vinylpyrrolidone)/poly(urushiol) multilayer ultrathin film through layer-by-

layer assembly and photo-induced polymerization. *Colloids and Surfaces a-Physicochemical and Engineering Aspects,* 337**,** 15-20.

Zheng, X. L., Weng, J. B., Li, S. Y., Liu, H. Q., Hu, B. H., Li, Y. H., Meng, X. F. & Ruan, H. R. 2014. Anticorrosive ultrathin film derived from bio-based urushiol-Ti by layer-by-layer self-assembly. *Chemical Engineering Journal,* 245**,** 265-275.

Tables:

Table A1: Attempts to optimize VIGS in poison ivy.

Method of Introduction	Temperature (°C)	Target Tissue	Growth Media	Replicates	Increased Efficiency?
Particle Bombardment	28	Cotyledons	Synthetic	43	NA (Baseline)
Particle Bombardment	28	True Leaves	Synthetic	37	No
Particle Bombardment	15	Cotyledons	Synthetic	11	No
Particle Bombardment	28	Cotyledons	Soil	4	No
Particle Bombardment	15	Cotyledons	Soil	4	No
Carborundum Inoculation	28	Cotyledons	Synthetic	6	No
Carborundum Inoculation	28	True Leaves	Synthetic	6	No
Surrogate Plant	28	True Leaves	Synthetic	37	No
Surrogate Plant	28	True Leaves	Soil	37	No

Figures:

Figure A1: Viral induced phytoene desaturase (PDS) suppression phenotypes. (Left) Plants that showed strong suppression phenotypes were quite rare and resulted in inhibiting plant growth. (Right) The spread of the virus though the plant tissues appeared to be limited. In most cases, symptoms were limited to the vasculature of only a single leaf with no subsequent spread to leaves that emerged after the initiation of these symptoms.

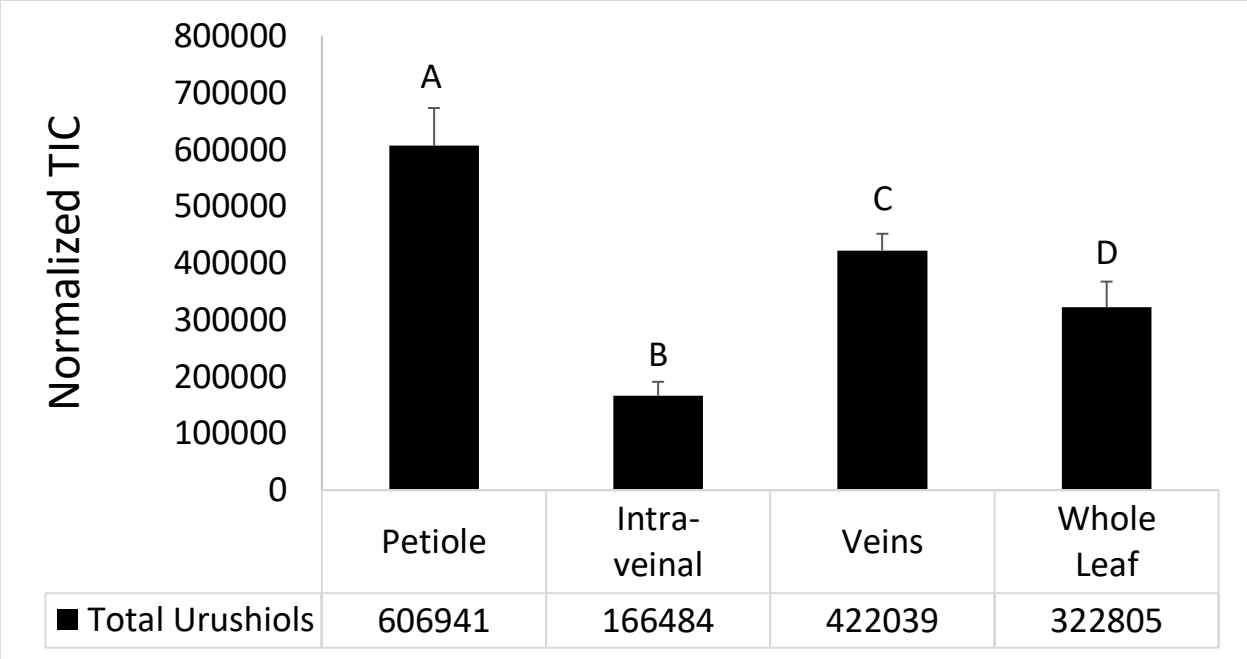

Figure A2: Accumulation of total urushiols in leaf tissues. Veins and petioles accumulated higher levels of total urushiols relative to intraveinal leaf tissue (P-value = <0.0001). Mean total urushiols are selected ion counts normalized to an internal standard of alkylresorcinol (Aziz et al., 2017).

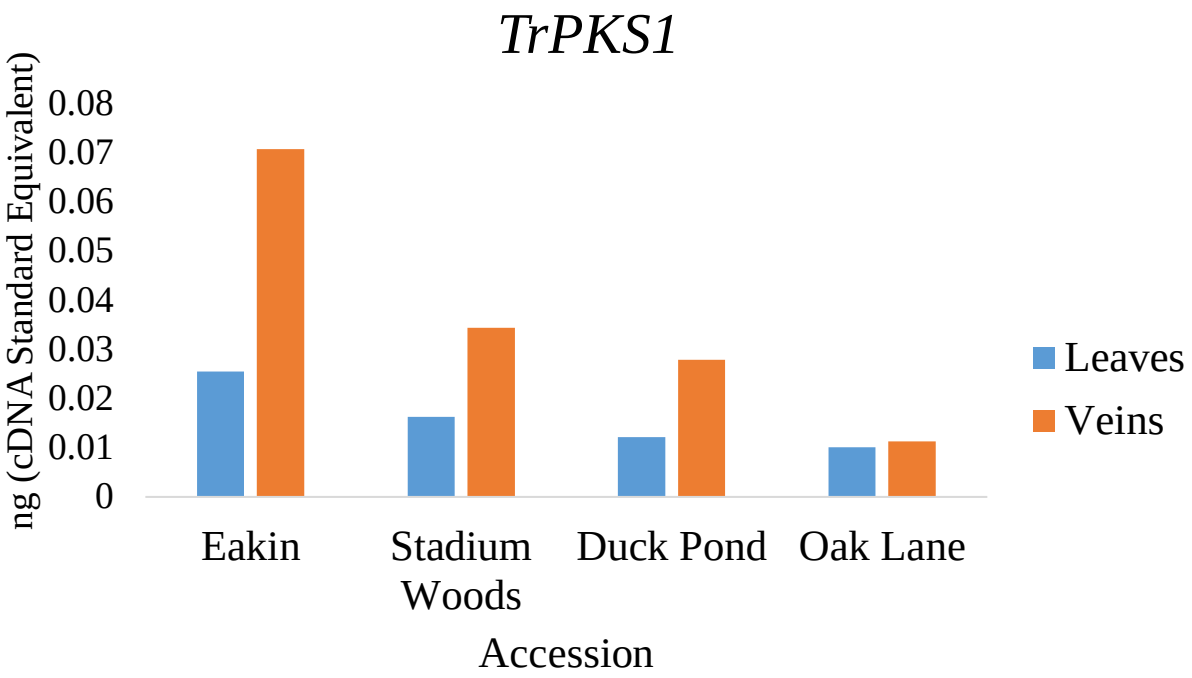

Figure A3: *TrPKS1* mRNA steady-state levels in dissected poison ivy intraveinal leaf and vein tissues. We saw a consistent trend where tissues high in urushiol accumulation as had high mRNA steady state levels of *TrPKS1*. No differential trend was observed for *TrPKS2* or *TrPKS3*.