

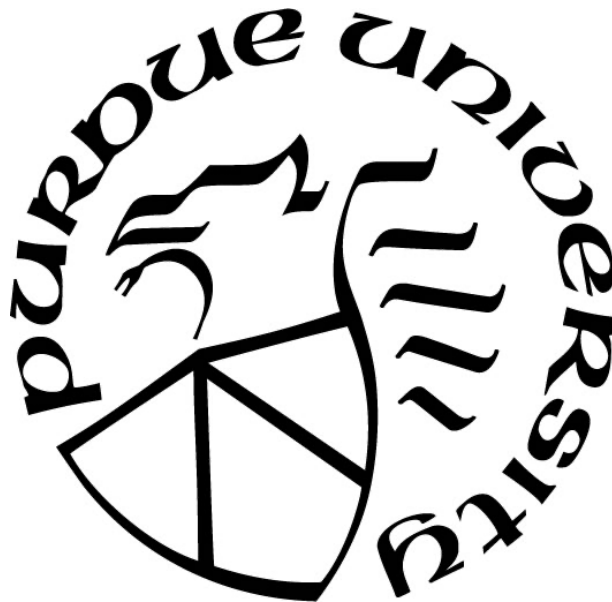
**BUCKHORN PLANTAIN (*PLANTAGO LANCEOLATA*) BIOLOGY AND  
2,4-D RESISTANCE IN TURF**

by  
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**A Dissertation**

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*Dedicated to the memory of my grandfathers, James R. Law and Richard I. Nickerson, both of whom were lost during my pursuit of graduate education. They were both lifelong farmers, proud stewards of the land, and WWII veterans.*

*“Harvest the crop, not the land.”*

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## ABSTRACT

Herbicide resistance poses a threat to sustainable vegetation management. Recently, the first report of 2,4-D resistance in buckhorn plantain (*Plantago lanceolata* L.) as well as the first report of 2,4-D resistance in turf was published. Additional 2,4-D resistant buckhorn plantain ecotypes have been reported in Indiana, Ohio, Pennsylvania, Georgia, and Virginia in the short time since. Thus, the aims of this research were to investigate the mechanism(s) of 2,4-D resistance in a resistant ecotype of buckhorn plantain, screen other potentially resistant ecotypes and measure them for fitness penalties, and identify effective turfgrass cultural control practices for managing buckhorn plantain.

A radiolabeled 2,4-D experiment was conducted to investigate absorption and translocation, and a 2,4-D dose-response experiment was conducted using malathion as a cytochrome P450 inhibitor to assess the potential mechanism of 2,4-D resistance in buckhorn plantain. The clearest difference between the resistant (IN-GW) and susceptible ecotype (IN-WL) was the interaction between ecotype and harvest period for [ $^{14}\text{C}$ ]2,4-D in the non-treated shoots. After 192 hr, the susceptible ecotype had a higher amount of [ $^{14}\text{C}$ ]2,4-D in the non-treated shoots (16.1%) than the resistant ecotype at any of the harvest periods (5.5-7.3%); the amount of [ $^{14}\text{C}$ ]2,4-D in the non-treated shoots was similar across all three harvest periods for the resistant ecotype. Thus, reduced translocation plays an apparent role in 2,4-D resistance in buckhorn plantain. Malathion pre-treatment did not fully revert the resistant ecotype back to susceptible. Thus, if cytochrome P450 metabolism is part of the 2,4-D resistance mechanism of this buckhorn plantain ecotype, it is likely a contributor and not the sole mechanism of resistance.

In total, this research identified four 2,4-D resistant buckhorn plantain ecotypes from Indiana and one from Ohio. Only one report of a failure to control buckhorn plantain was confirmed to be a susceptible ecotype. When compared to susceptible ecotypes in a garden study, no major fitness penalties were identified in resistant ecotypes.

Given that no specific cultural or biological control methods of buckhorn plantain have been recognized to date, two field trials were conducted to investigate the influence of 1) mowing height and nitrogen rate on buckhorn plantain coverage and 2) mowing frequency on buckhorn plantain coverage and seed production. Nitrogen fertilization and low mowing reduced buckhorn plantain coverage after 3 yr, but low mowing also increased crabgrass and dandelion as well as

reduced turf quality. Frequent mowing reduced viable seed production, but that did not translate into a reduction in buckhorn plantain coverage after 2 yr.

This research demonstrates the complex mechanism of action of 2,4-D, as the resistance mechanism for buckhorn plantain was not fully elucidated. It also highlights the importance of utilizing best management practices for managing weeds in turf, including rotating herbicide chemistries, high and frequent mowing, and nitrogen fertilization.

## CHAPTER 1. LITERATURE REVIEW

### 1.1 Buckhorn plantain

Buckhorn plantain (*Plantago lanceolata* L.), also known as English plantain, narrow-leaved plantain, ribwort plantain, and lamb's tongue, is a cool-season perennial, seldom annual or biennial, broadleaf that is a common weed in turfgrass systems (Christians et al., 2017) as well as in hayfields, pastures, farmyards, waste places, and roadsides (Cavers et al., 1980). Buckhorn plantain is a noxious, invasive, or restricted weed species in many U.S. states. The species originates from Eurasia (Cravers et al., 1980), but it has since established throughout the world save for subarctic and low-lying tropical areas (Tessene, 1968).

Despite being a dicotyledonous plant, the cotyledons of a germinating buckhorn plantain seedling appear grass-like. Mature leaves are lanceolate-shaped [3-40 cm long (including the petiole) by 0.5 to 3.5 cm wide (Cavers et al., 1980)], which is why the species name (*lanceolata*) was chosen. Leaves have prominent parallel veins, though closer inspection reveals reticulated veins that dicots are notorious for. Leaf margins vary from entire to serrated, and leaf blades, though generally glabrous, can be pubescent. Buckhorn plantain forms and grows from a central, basal rosette. Long, silky hairs are often present at the growing point. There is no visible aboveground stem, but a semi-woody caudex and/or undeveloped taproot is often present. Plants develop a few deep roots (1 m or more) as well as a mass of short (5-10 cm) fibrous roots near the soil surface (Sagar and Harper, 1964).

Buckhorn plantain has  $2n = 12$  chromosomes (Sagar and Harper, 1964) and reproduces by both seeds and basal shoots. Buckhorn plantain is a long day species (Snyder, 1948), flowering from mid-spring through early fall in the Midwest. Buckhorn plantain is a wind-pollinated, self-incompatible, obligate outcrosser (Sharma et al., 1992) with a relatively high level of gene flow (Van Dijk et al., 1988). It is a gynodioecious species (Ross, 1969) in which flowers may be pistillate, staminate, or hermaphroditic (Tessene, 1968). Also a protogynous species, the stigma protrudes 4-5 d prior to flower opening (Sharma et al., 1992). Inflorescences are long, leafless stalks (up to 45 cm in length) that bear dense, cylinder-shaped spikes (1-8 cm in length); single flowers are inconspicuous (Cavers et al., 1980; Warwick and Briggs, 1979).

Fruiting capsules yield one or two narrowly oval, brown to black, shiny seeds indented on one side (2 mm in length)(Cavers et al., 1980). Buckhorn plantain seed yield can be multiplicatively determined by the number of inflorescences per plant, capsule number per inflorescence, seed number per capsule, and seed mass (Primack and Antonovics, 1981). Seed weight ranges from 0.7 to 2.0 mg, with an average of up to 470 seeds per flowering plant (Mook et al., 1992). Approximately 20% of seedlings survive to adulthood, and the average half-life for adults is 2.7 yr (Mook et al., 1992).

There are several ecological traits of buckhorn plantain that contribute to its successful colonization of low maintenance turf. First, the species is able to germinate across an array of environmental conditions. Not only can buckhorn plantain germinate without light, light does not favor germination (Kuiper and Bos, 1992). Thus, buckhorn plantain is able to germinate when buried and can establish in tall, dense layers of vegetation (Blom, 1978). However, seedling emergence is inhibited by burial depths of 6 cm or more (Benvenuti et al., 2001).

Buckhorn plantain germination is seemingly less affected by temperature than other *Plantago* species. Buckhorn plantain germination is temperature-independent between 15 and 25°C, unlike the increase in germination for *P. major* and *P. media* between those temperatures (Sagar and Harper, 1960). Further, a 5°C prechilling treatment results in smaller differences in germinability for buckhorn plantain compared to *P. major* (Sagar and Harper, 1960). Fresh buckhorn plantain seeds have a lower germination capacity than 1- to 5-year-old seeds, which is also true for *P. coronopus*, *P. major*, and *P. media* (Blom, 1992). While *P. coronopus*, *P. major*, and *P. media* germination decreased after four or five years, buckhorn plantain germination did not decrease after six years (Kuiper and Bos, 1992). Buckhorn plantain can also germinate across a range of soil moistures, with approximately 70% germination at soil moistures as low as 8% (water by volume) in a dune sand (Blom, 1979; Kuiper and Bos, 1992). In that same study, maximum germination, approximately 90%, was achieved at a soil moisture of 21% (water by volume)(Blom, 1978; Kuiper and Bos, 1992).

Compared to other *Plantago* species (i.e., *P. coronopus*, *P. major*, and *P. media*), buckhorn plantain has the widest ecological amplitude (Mook et al., 1992). Buckhorn plantain tolerates adverse growing conditions, including compaction, low fertility (Hoveland et al., 1976), a wide range of soil pH values (Buchanan et al., 1975), and an adaptive lead tolerance that is transmitted to seed progeny (Wu and Antonovics, 1976). Fertilization can have mixed effects on

buckhorn plantain populations. In a hayfield, fertilization reduced buckhorn plantain biomass in undisturbed vegetation but increased biomass in a buckhorn plantain monoculture (Berendse, 1983).

Morphological differences between buckhorn plantain ecotypes can vary based on habitat and management. Left unmown, buckhorn plantain grows long, erect leaves and appears bush-like. As mowing height decreases and frequency increases, smaller, more prostrate leaves and plants result. Warwick and Briggs (1979) measured field samples of various ecotypes and found that leaf length, leaf width, scape length, and spike length were shorter/narrower for buckhorn plantain collected from a lawn compared to roadsides. However, when plants from these same ecotypes were cultivated, the between-habitat differences in ecotypes disappeared (Warwick and Briggs, 1979).

There may be genetic variation in buckhorn plantain plasticity (Schmitt et al., 1992). Environmental effects on life-history are large relative to genetic differences (Antonovics and Primack, 1982), and such environmental maternal effects may affect offspring fitness as well as contribute to differences among populations in seed and seedling demography (Schmitt et al., 1992). Gaspar et al. (2019) found that, “Epigenetic differences [of buckhorn plantain] were consistently related to genetic and environmental variation, and to a lesser degree to phenotypic variation and land use, with more grazed populations harboring greater epigenetic diversity.” In a related study, increased mowing intensity of the seed source was related to larger plant sizes, lower specific leaf areas, and lower levels of intrapopulation phenotypic variation in the offspring (Gaspar et al., 2020).

No specific cultural or biological control methods of buckhorn plantain have been identified to date. Chemical control options of buckhorn plantain in turf are primarily limited to synthetic auxin herbicides. The phenoxyacetic acid herbicides (2,4-D, dichlorprop, MCPA, and mecoprop) are generally efficacious on buckhorn plantain; pyridinecarboxylic acid herbicides (clopyralid, fluroxypyr, and triclopyr) provide fair to excellent control, with clopyralid being the most effective; quinclorac (a quinolinecarboxylic acid) yields poor to good control, and dicamba (a benzoic acid herbicide) does not control buckhorn plantain (Patton and Elmore, 2021). A new chemotype of synthetic auxins, aryloxyacetic acids, provides excellent control (Patton et al., 2018).

Recently, the first report of 2,4-D resistance in buckhorn plantain as well as the first report of 2,4-D resistance in turf was published (Patton et al., 2018). This resistant ecotype was identified after an application of 2,4-D + mecoprop + dicamba at  $1.3 + 0.4 + 0.1 \text{ kg ae ha}^{-1}$  failed to control buckhorn plantain. Pesticide spray records revealed 30 yr of 2,4-D-containing herbicide applications, and the site offered a few other signs of herbicide resistance. There was little to no synthetic auxin herbicide symptomology present on the buckhorn plantain, 10 to 40% buckhorn plantain ground coverage, and no other perennial broadleaf weeds present (Patton et al., 2018).

Upon communicating this confirmation of 2,4-D resistance in buckhorn plantain to turfgrass practitioners, other potentially resistant populations were brought to the attention of the Purdue Turfgrass Extension team. In a preliminary bioassay, a total of five ecotypes survived 2,4-D rates of  $16.8 \text{ kg ae ha}^{-1}$ , which is  $10\times$  the standard application rate of  $1.68 \text{ kg ae ha}^{-1}$ . Four of these ecotypes, including the ecotype confirmed to be 2,4-D resistant by Patton et al. (2018), were from Indiana, and the fifth ecotype was from Ohio (data not shown). Additionally, 2,4-D resistant buckhorn plantain ecotypes have been identified in Pennsylvania (Russell et al., 2020), Georgia (P.E. McCullough, personal communication), and Virginia (S.D. Askew, personal communication). These resistant buckhorn plantain ecotypes were located in multiple turfgrass systems, including a cemetery, home lawn, university campus, sports field, and golf course. Glyphosate resistance has also been confirmed in buckhorn plantain in a vineyard (Heap, 2021).

Few options exist for herbicides that provide excellent postemergence buckhorn plantain control in turf, especially those labeled for use in cool-season residential lawns (Patton and Elmore, 2021). Most herbicides that provide excellent postemergence control of buckhorn plantain in turf contain 2,4-D (Patton and Elmore, 2021), and the herbicides offering excellent control that do not contain 2,4-D are more expensive (Patton et al., 2018), not labeled for home lawns, and/or not labeled for cool-season turf (Patton and Elmore, 2021). As a likely result, 2,4-D was routinely applied in lieu of herbicide rotation, selecting for herbicide resistant individuals and eventually ecotypes (i.e., populations). Although the incidence of weed resistance is reportedly lower for synthetic auxins than other herbicide modes of action (Busi et al., 2017), the recent identification of numerous 2,4-D resistant buckhorn plantain ecotypes is alarming. Furthermore, the release and adoption of 2,4-D tolerant (Enlist<sup>TM</sup>) soybean (*Glycine max*) is

predicted to increase 2,4-D use (Peterson et al., 2016). Thus, the investigating 2,4-D resistance in buckhorn plantain is justified.

## 1.2 Cultural control of turfgrass weeds

It is often stated that a dense, healthy turf is the best defense against weed encroachment. Thus, turfgrass management practices that improve turfgrass health and vigor usually result in fewer weeds. Busey (2003) defined cultural management of turfgrass weeds as “the use of mowing, fertilization, irrigation, cultivation, planting, and turfgrass selection to affect weed populations.” For brevity and relevancy, only mowing and nitrogen fertilization will be discussed in this review.

There are three main components to mowing: height, frequency, and grass clippings management. All three components can affect weed populations; most markedly, though, is mowing height (Busey, 2003). Low mowing heights are associated with more weeds in turf (Busey, 2003). However, many of the mowing height experiments have focused on crabgrass (*Digitaria* spp.). Broadleaf weeds [i.e. mockstrawberry (*Duchesnea indica*) and wild violet (*Viola sororia*)] can also be reduced with a higher mowing height (Gray and Call, 1993), though mowing height research with broadleaf weeds is limited. It is important to note that frequent, low mowing favors genetically shorter ecotypes (Warwick and Briggs, 1978, 1979).

Turfgrasses have specialized growing points that allow them to survive frequent mowing (Christians et al., 2017). Thus, timely mowing is able to eventually kill most dicotyledon species lacking a prostrate growth habit and provides a competitive advantage for the desired turf. Mowing frequently enough such that weed inflorescences are cut prior to seed maturation can help to prevent seed rain and seed bank deposition. For example, delayed mowing of a cool-season pasture increased the weed seed bank (Williams, 1984). The influence of mowing frequency is not steadfast and can be weed species-specific. Fortnightly mowing removed field bindweed (*Convolvulus arvensis*) from warm-season turf but did not prevent dandelion (*Taraxacum officinale*) colonization (Timmons, 1950).

The influence of grass clippings management on turfgrass weeds is largely situation-specific (Busey, 2003). Collecting grass clippings can remove weed seed (Gaussoin and Branham, 1989). However, returning grass clippings can also reduce weed encroachment (Haley et al., 1985), as it recycles nutrient-rich organic matter (Law et al., 2016). Related to mowing, a



higher grazing intensity has been shown to decrease buckhorn plantain abundance (Verrier and Kirkpatrick, 2008).

In general, nitrogen fertilization reduces weeds (Busey, 2003). Nitrogen is often the growth-limiting nutrient for turf (Christians et al., 2016), and stimulating turfgrass growth via nitrogen fertilization increases density, helps the turf to fill in voids in the canopy, and improves its competitiveness with encroaching weeds. Similar to mowing height, most of the nitrogen-related research has focused on crabgrass (Busey, 2003). However, nitrogen fertilization reduces the coverage of dandelion (Callahan and Overton, 1978), white clover (*Trifolium repens*)(Templeton and Taylor, 1966), and ground ivy (*Glechoma hederacea*)(Kohler et al., 2004).

The Park Grass Experiment at the Rothamsted Experiment Station outside of London, England highlights the importance and demonstrates the effects of long-term fertilization practices: annual spring applications of 49 kg N ha<sup>-1</sup> as ammonium sulfate each year since 1856 has resulted in almost full turfgrass coverage (Woods and Rossi, 2011). However, the regular application of crushed limestone, phosphorus, or potassium increased broadleaf species abundance (Woods and Rossi, 2011). For example, potassium fertilization increased dandelion (*Taraxacum officinale*) abundance, and potassium plus crushed limestone further increased dandelion abundance (Woods and Rossi, 2011).

### 1.3 2,4-D and 2,4-D resistance

Commercialized in 1945, 2,4-D revolutionized chemical weed control (Peterson et al., 2016). The synthetic auxin herbicide provides selective control of broadleaf weeds and is relatively safe on grasses. The herbicide has been used worldwide on small grains, fruits, nuts, vegetables, pastures, rangelands, turfgrasses, rights-of-way, aquatic habitats, and forestry sites (Peterson et al., 2016). After glyphosate and acetolactate synthase inhibitors, synthetic auxin use ranks third globally; dicamba and 2,4-D are the two most used synthetic auxins by global treated area (Todd et al., 2020). An estimated 65% of the 2,4-D used annually between 1992 and 2000 in the United States was for agriculture and another 25% was used on home lawns (Borges et al., 2004). Thus, the turfgrass industry is a large market for 2,4-D use, and an estimated 5.2 million kg of active ingredient are applied to lawns annually (Borges et al., 2004).

Amine salts and esters are the two most commonly used formulation types of 2,4-D, although a choline salt of 2,4-D was recently developed and released (Sosnoskie et al., 2015). Amine formulations are soluble in water, able to form a solution, and are insoluble in petroleum oils, whereas ester formulations are insoluble in water and soluble in petroleum oils (Peterson et al., 2016). Amine salts can be formulated as liquid or dry preparations, and esters are formulated as emulsifiable concentrates for application in either water or oils (Peterson et al., 2016). Ester formulations are more efficacious in certain situations, including cold temperatures and dry conditions. However, esters are more volatile and pose a greater threat for off target injury. Compared to other forms of 2,4-D, the choline formulation has greater stability and lower opportunity for volatilization (Sosnoskie et al., 2015). Amines disassociate in water, which can cause hard water antagonism and reduce efficacy (Patton et al., 2016). Esters do not disassociate in water and therefore do not react with cations in hard water (Peterson et al., 2016).

Absorption of 2,4-D is influenced by a number of factors. High temperatures and humidity increase 2,4-D absorption, as can light, adjuvants, lower pH, and ester formulations (Peterson et al., 2016). Translocation is affected by fewer factors [e.g. water stress reduces 2,4-D translocation but not absorption (Basler et al., 1961)], though 2,4-D tolerance has been linked to reduced translocation (Pillmoor and Gaunt, 1981). After absorption, 2,4-D is translocated along the phloem-loading pathway (Devine and Hall, 1990). Moving in the direction of carbohydrate source to sink, a concentration gradient drives the transport of 2,4-D (Devine and Hall, 1990). Ion trapping keeps the ionic form of 2,4-D within the phloem for transport (Riederer, 2005), and 2,4-D is able to concentrate in the cell due to a greater number of 2,4-D influx carriers relative to efflux carriers (Delbarre et al., 1996).

In addition to reduced translocation, 2,4-D metabolism also contributes to differences in susceptibility to the herbicide. Direct conjugation and ring hydroxylation are the primary methods of 2,4-D plant metabolism, though side-chain cleavage also occurs (Peterson et al., 2016). The 2,4-D metabolic pathway used by a particular plant determines its susceptibility to the herbicide. Sensitive dicots usually perform direct conjugation, resulting in reversible compounds that can be converted back into an active form (Davidonis et al., 1980). Conversely, tolerant monocots usually metabolize 2,4-D into irreversible, nontoxic compounds via ring hydroxylation (Pillmoor and Gaunt, 1981).

Auxin signaling and perception is complex and not fully understood. Auxin binding, the first of two steps in auxin perception, is of no exception. Given that 2,4-D is a synthetic auxin, identifying auxin binding sites may provide insight about how 2,4-D works or fails to work, in the case of herbicide resistance. There are three known classes of auxin receptors: auxin-binding protein 1 (ABP1)(Shi and Yang, 2011), Transport Inhibitor Resistant 1/auxin-signaling F-box (TIR1/AFB1-5)(Wang and Estelle, 2014), and S-phase kinase-associated protein 2 (SKP2)(Jurado et al., 2010). These three auxin receptor classes differ both in their subcellular localizations as well as their functional roles (Zazimalova et al., 2014).

TIR1 and its paralogues, the AFBs, are thought to be the principal auxin receptors (Peterson et al., 2016). In Arabidopsis, TIR1 and AFB1-5 make up a family of six auxin receptors. Based on genomic duplication, the family is comprised of three pairs: TIR1 and AFB1, AFB2 and AFB3, and AFB4 and AFB5. Selectivity for synthetic auxin herbicides exists within the TIR1 family. For example, AFB5 receptors are the primary site of action for picolinate herbicides (Prigge et al., 2016). Furthermore, an Arabidopsis AFB5 mutant was insensitive to picloram but sensitive to 2,4-D and IAA (Walsh et al., 2006). The differential binding affinities of auxin transporters and nuclear auxin receptors for synthetic auxins may contribute to plant sensitivity and/or potential cross-resistance to herbicides. An additional F-Box protein (SKP2), distinct from TIR1, is also associated with auxin binding (Jurado et al., 2010). While SKP2 is seemingly involved in auxin signaling, its role in auxin perception is not clear (Napier, 2014). Additionally, ABP1 is considered to be another auxin-binding site, although its mechanism of action is currently unknown. ABP1 may play a functional role, such as a more rapid auxin response (Napier, 2014).

Grossmann (2010) classified three phases of the physiological response of sensitive dicots to synthetic auxin herbicides: stimulation, inhibition, and decay. Stimulation occurs within minutes of synthetic auxin herbicide application, resulting in increased ethylene production, abscisic acid accumulation, and metabolic activation of ion channels and ATPases (Grossmann, 2010). Abnormal, unregulated growth occurs during the stimulation phase, causing epinasty, tissue swelling, stem twisting, and leaf strapping (Grossmann, 2010). During the inhibition phase, primary metabolite assembly decreases due to the production of reactive oxygen species, stomatal closure, and the plant's defense response (Grossmann, 2010). Within 3 days of 2,4-D

application, plant growth halts and the decay phase begins; the destruction of chloroplasts, membranes, and the vascular system leads to plant death (Grossmann, 2010).

The first reported cases of 2,4-D resistance occurred in 1957 on climbing dayflower (*Commelina diffusa*) and wild carrot (*Daucus carota*) (Heap, 2021). Since then, a total of at least 45 cases of 2,4-D resistance across 25 weeds have been described (Table 1.1) (Heap, 2021). While over half of the reported cases of 2,4-D resistance have been identified in the last 20 years (Heap, 2021), the prevalence of herbicide resistance is lower for synthetic auxins than other modes of action, especially acetyl Co-A carboxylase (ACCase)- and acetolactate synthase (ALS)-inhibiting herbicides (Busi et al., 2017). It may be that the initial frequencies of auxinic herbicide resistance is low, the resistance mechanisms do not result in a high level of herbicide resistance (Walsh et al., 2009), and/or fitness penalties are associated with auxinic herbicide resistance traits due to pleiotropic effects (Roux et al., 2004).

Investigating the mechanism(s) of 2,4-D resistance in buckhorn plantain may help to inform management practices and preserve herbicidal efficacy (Busi et al., 2017). Just as the mechanism of herbicidal action is complex, so are the apparent mechanisms of 2,4-D resistance (Goggin et al., 2018). Neither are fully understood. Herbicide resistance mechanisms fall into two broad categories: target-site and non-target-site (Murphy and Tranel, 2019). While there is at least one report of at least partial target-site 2,4-D resistance (LeClere et al., 2018) (Table 1.2), others hypothesize that potential fitness penalties, the recessive nature of certain auxin herbicide resistance genes, functional redundancy of auxin receptors, and/or the complex nature of synthetic auxin herbicide mode of action may contribute to the lack of a target site resistance mechanism (Peterson et al., 2016).

Mutations to the TIR1 coreceptor alter binding affinity in *Arabidopsis* (Yu et al., 2013), which could confer 2,4-D or other synthetic auxin herbicide resistance. Mutations to the Aux/IAA transcriptional repressor proteins could also result in synthetic auxin herbicide resistance, but those mutations may incur a fitness cost (Mithila et al., 2011). Although many of the reported cases of 2,4-D resistance do not describe cross-resistance to other synthetic auxins, cross-resistance within phenoxyacetic acids (e.g. MCPA and mecoprop) but not to other chemical classes (e.g. benzoates and picolines) has been demonstrated (Heap, 2021). This may be due to either a lack of screening or a true absence of cross-resistance (Peterson et al., 2016). Enhanced metabolism of synthetic auxins at multiple binding sites and/or for multiple chemical

families is another potential cross-resistance mechanism (Yu and Powles, 2014), which may cause resistance issues for herbicides that have yet to be released or discovered (Ma et al., 2013).

Table 1.1. List of weeds resistant to 2,4-D as reported by Heap (2021) and others.

<b>Species</b>	<b>Common name</b>	<b>Cotyledon</b>	<b>Life cycle*</b>
<i>Amaranthus hybridus</i>	Smooth pigweed	Dicot	Annual
<i>Amaranthus palmeri</i>	Palmer amaranth	Dicot	Annual
<i>Amaranthus tuberculatus</i>	Tall waterhemp	Dicot	Annual
<i>Arctotheca calendula</i>	Capeweed	Dicot	Annual
<i>Brassica kaber</i>	Wild mustard	Dicot	Annual
<i>Brassica rapa</i>	Birdsrape mustard	Dicot	Annual or biennial
<i>Carduus acanthoides</i>	Plumeless thistle	Dicot	Biennial
<i>Carduus nutans</i>	Musk thistle	Dicot	Biennial or perennial
<i>Carduus pycnocephalus</i>	Italian thistle	Dicot	Annual
<i>Cirsium arvense</i>	Canada thistle	Dicot	Perennial
<i>Commelina diffusa</i>	Spreading dayflower	Monocot	Annual or perennial
<i>Conyza canadensis</i>	Horseweed	Dicot	Annual
<i>Conyza sumatrensis</i>	Sumatran fleabane	Dicot	Annual
<i>Daucus carota</i>	Wild carrot	Dicot	Biennial
<i>Fimbristylis miliacea</i>	Globe fringerush	Monocot	Annual or perennial
<i>Galium aparine</i>	Catchweed bedstraw	Dicot	Annual
<i>Hirschfeldia incana</i>	Shortpod mustard	Dicot	Annual, biennial, or perennial
<i>Lactuca serriola</i>	Prickly lettuce	Dicot	Annual or biennial
<i>Limnocharis flava</i>	Sawah flowering rush	Monocot	Annual or perennial
<i>Limnophila erecta</i>	Marshweed	Dicot	Annual
<i>Papaver rhoeas</i>	Corn poppy	Dicot	Annual
<i>Parthenium hysterophorus</i>	Santa Maria feverfew	Dicot	Annual
<i>Plantago lanceolata</i>	Buckhorn plantain	Dicot	Annual, biennial, or perennial
<i>Raphanus raphanistrum</i>	Wild radish	Dicot	Annual or biennial
<i>Sinapis arvensis</i>	Wild mustard	Dicot	Annual
<i>Sisymbrium orientale</i>	Oriental mustard	Dicot	Annual
<i>Sonchus oleraceus</i>	Annual sowthistle	Dicot	Annual
<i>Sphenoclea zeylanica</i>	Gooseweed	Dicot	Annual

\* Life cycles courtesy of the United States Department of Agriculture.

Table 1.2. Mechanisms of 2,4-D resistance reported in the literature.

Species	Resistance mechanism(s)	Citation(s)
<i>Papaver rhoeas</i>	Differential metabolism, ethylene response, and translocation	Rey-Caballero et al., 2016; Torra et al., 2017; Palma-Bautista et al., 2020
<i>Lactuca serriola</i>	Reduced absorption and translocation	Burke et al., 2009; Riar et al., 2011
<i>Raphanus raphanistrum</i>	Reduced translocation	Goggin et al., 2016
<i>Raphanus raphanistrum</i>	Upregulation of the Aux/IAA repressors <i>IAA29</i> and <i>IAA30</i> in population R1 and increased constitutive MAPK phosphorylation across the 11 R populations studied	Goggin et al., 2018
<i>Amaranthus tuberculatus</i>	Rapid metabolism	Figueiredo et al., 2018
<i>Amaranthus tuberculatus</i>	Enhanced metabolism	Shergill et al., 2018
<i>Sisymbrium orientale</i>	Reduced translocation	Dang et al., 2018
<i>Carduus nutans</i>	Reduced translocation and enhanced metabolism	Harrington and Woolley, 2010
<i>Amaranthus hybridus</i>	Reduced translocation and enhanced metabolism	Palma-Bautista et al., 2020
<i>Conyza canadensis</i>	Enhanced metabolism	Palma-Bautista et al., 2020
<i>Conyza sumatrensis</i>	Enhanced metabolism	Palma-Bautista et al., 2020
<i>Hirschfeldia incana</i>	Reduced translocation	Palma-Bautista et al., 2020
<i>Parthenium hysterophorus</i>	Reduced translocation and enhanced metabolism	Palma-Bautista et al., 2020
<i>Kochia scoparia</i>	KsIAA16 auxin co-receptor	LeClere et al., 2018

Mechanisms of synthetic auxin resistance in select, well-characterized weed species have been described by Busi et al. (2017). Corn poppy (*Papaver rhoeas*) and wild radish (*Raphanus raphanistrum*) are two of the described species, both of which have 2,4-D resistant ecotypes. Certain corn poppy ecotypes display cross-resistance to other phenoxycarboxylic acid, pyridinecarboxylic acid, or benzoic acid herbicides (Busi et al., 2017). Compared to susceptible plants, reductions in 2,4-D translocation and ethylene production were observed in 2,4-D resistant corn poppy (Rey-Caballero et al., 2016). Additionally, 2,4-D metabolites were detected in the shoots and roots 48 h after application in resistant corn poppy with reduced translocation (Torra et al., 2017). When treated with malathion, a cytochrome P450 inhibitor, these resistant ecotypes were rendered susceptible, suggesting that enhanced cytochrome P450 2,4-D metabolism plays a role in the herbicide resistance (Torra et al., 2017). Both reduced translocation and enhanced metabolism may contribute to 2,4-D resistance, but the primary mechanism remains unknown.

Wild radish ecotypes resistant to 2,4-D were first discovered in 1999 in Australia (Walsh et al., 2004). Since then, ecotypes displaying an approximate 20-fold increase in resistance have been identified (Busi and Powles, 2017). Within these 2,4-D resistant ecotypes, two separate resistance mechanisms have been recognized. Similar to corn poppy, reduced translocation of 2,4-D is one mechanism of resistance (Goggin et al., 2016). In fact, Busi et al. (2017) indicate that reduced translocation is the primary mechanism of resistance in wild radish. However, a differential perception of auxin and/or signal transduction among resistant ecotypes was also observed (Jugulam et al., 2013). An unpublished genome-wide transcriptomics study of a resistant ecotype revealed an upregulation of auxin-induced transcriptional repressors and defense genes that was not observed in a susceptible ecotype (Busi et al., 2017). Given that select resistant ecotypes translocated [ $^{14}\text{C}$ ]2,4-D in a similar manner to susceptible ecotypes, this is likely a second resistance mechanism (Busi et al., 2017).

Thus far, nearly all of the reported mechanisms of 2,4-D resistance have been non-target-site in nature (Table 1.2). A majority of these non-target-site resistance mechanisms involve reduced translocation, enhanced metabolism, or both. Cytochrome P450 likely mediates the enhanced 2,4-D metabolism in some of these resistant ecotypes, as pretreatment with malathion, a cytochrome P450 inhibitor, reversed the phenotype from resistant to at least partially susceptible in *Amaranthus hybridus*, *Amaranthus tuberculatus*, *Conyza canadensis*, *Conyza sumatrensis*, *Papaver rhoeas*, and *Parthenium hysterophorus*.

Evolved resistance to 2,4-D in *Brassica kaber* (Jugulam et al., 2005), *Sisymbrium orientale* (Preston and Malone, 2015), and *Raphanus raphanistrum* (Busi and Powles, 2017) were shown to be single dominant alleles. A semi-dominant single resistance genetic trait endowing reduced 2,4-D translocation was reported in *Lactuca serriola* (Riar et al., 2011). Thus far, only one molecular 2,4-D resistance mechanism in a weed species has been functionally validated: LeClere et al. (2018) identified a 2-nt base change in *Kochia scoparia*, resulting in a glycine to asparagine amino acid change within a highly conserved region of an AUX/indole-3-acetic acid (IAA) protein, KsIAA16.

## 1.4 Objectives

The three main objectives of this research are to: 1) determine the influence of absorption, translocation, and cytochrome P450 metabolism in a 2,4-D resistant buckhorn plantain ecotype, 2) conduct a 2,4-D bioassay of 12 buckhorn plantain ecotypes and screen for fitness penalties associated with 2,4-D resistance, and 3) determine how mowing and fertilization can affect buckhorn plantain persistence and viable seed production in cool-season turf.

## 1.5 References

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## CHAPTER 2. REDUCED TRANSLOCATION OF 2,4-D CONTRIBUTES TO 2,4-D RESISTANCE IN *PLANTAGO LANCEOLATA*

### 2.1 Abstract

Investigating the mechanism(s) of 2,4-D resistance in buckhorn plantain may help to inform management practices and preserve herbicidal efficacy. Given that the 2,4-D resistance is likely a non-target mutation, altered absorption and/or translocation as mechanisms of resistance were tested using  $^{14}\text{C}$  radiolabeled 2,4-D and comparing resistant buckhorn plantain with a susceptible ecotype. The objective of this experiment was to determine if absorption or translocation play a role in 2,4-D resistance in a resistant buckhorn plantain ecotype. Plants were oversprayed with 2,4-D at a rate of  $1.68 \text{ kg ae ha}^{-1}$  with the youngest fully-expanded leaf excluded. The isolated leaf was then treated with four  $0.5\text{-}\mu\text{l}$  droplets of radiolabeled  $[^{14}\text{C}]2,4\text{-D}$  on the adaxial leaf surface, avoiding prominent veins, for a total of  $5.32 \text{ kBq plant}^{-1}$ . Plants were harvested at 24, 96, and 192 HAT and separated into treated leaf, treated leaf wash, nontreated shoots, and the caudex + roots. The clearest difference between the resistant and susceptible ecotype was the interaction between ecotype and harvest period for  $[^{14}\text{C}]$  from 2,4-D in the non-treated shoots. After 192 hr, the susceptible ecotype had a higher amount of  $[^{14}\text{C}]$  from 2,4-D in the non-treated shoots (16.1%) than the resistant ecotype at any of the harvest periods (5.4-7.3%); the amount of  $[^{14}\text{C}]$  from 2,4-D in the non-treated shoots was similar across all three harvest periods for the resistant ecotype. Thus, reduced translocation plays an apparent role in 2,4-D resistance in buckhorn plantain.

## 2.2 Introduction

Commercialized in 1945, 2,4-D revolutionized chemical weed control (Peterson et al., 2016). The synthetic auxin herbicide provides selective control of broadleaf weeds and is relatively safe on grasses. The herbicide has been used worldwide on small grains, fruits, nuts, vegetables, pastures, rangelands, turfgrasses, rights-of-way, aquatic habitats, and forestry sites (Peterson et al., 2016). After glyphosate and acetolactate synthase inhibitors, synthetic auxin use ranks third globally; dicamba and 2,4-D are the two most used synthetic auxins by global treated area (Todd et al., 2020). An estimated 65% of the 2,4-D used annually between 1992 and 2000 in the United States was for agriculture and another 25% was used on home lawns (Borges et al., 2004). Thus, the turfgrass industry is a large market for 2,4-D use, and an estimated 5.2 million kg of active ingredient are applied to lawns annually (Borges et al., 2004).

Absorption of 2,4-D is influenced by a number of factors. High temperatures and humidity increase 2,4-D absorption, as can light, adjuvants, lower pH, and ester formulations (Peterson et al., 2016). Translocation is affected by fewer factors [e.g. water stress reduces 2,4-D translocation but not absorption (Basler et al., 1961)], though 2,4-D tolerance has been linked to reduced translocation (Pillmoor and Gaunt, 1981). After absorption, 2,4-D is translocated along the phloem-loading pathway (Devine and Hall, 1990). Moving in the direction of carbohydrate source to sink, a concentration gradient drives the transport of 2,4-D (Devine and Hall, 1990). Ion trapping keeps the ionic form of 2,4-D within the phloem for transport (Riederer, 2005), and 2,4-D is able to concentrate in the cell due to a greater number of 2,4-D influx carriers relative to efflux carriers (Delbarre et al., 1996).

In addition to reduced translocation, 2,4-D metabolism also contributes to differences in susceptibility to the herbicide. Direct conjugation and ring hydroxylation are the primary methods of 2,4-D plant metabolism, though side-chain cleavage also occurs (Peterson et al., 2016). The 2,4-D metabolic pathway used by a particular plant determines its susceptibility to the herbicide. Sensitive dicots usually perform direct conjugation, resulting in reversible compounds that can be converted back into an active form (Davidonis et al., 1980). Conversely, tolerant monocots usually metabolize 2,4-D into irreversible, nontoxic compounds via ring hydroxylation (Pillmoor and Gaunt, 1981).

The first reported cases of 2,4-D resistance occurred in 1957 on climbing dayflower (*Commelina diffusa*) and wild carrot (*Daucus carota*) (Heap, 2020). Since then, a total of at least



45 cases of 2,4-D resistance across 25 weeds have been described (Heap, 2021). While over half of the reported cases of 2,4-D resistance have been identified in the last 20 years (Heap, 2021), the prevalence of herbicide resistance is lower for synthetic auxins than other modes of action, especially acetyl Co-A carboxylase (ACCase)- and acetolactate synthase (ALS)-inhibiting herbicides (Busi et al., 2017). The slower development of resistance instances may be that the initial frequencies of auxinic herbicide resistance is low, the resistance mechanisms do not result in a high level of herbicide resistance (Walsh et al., 2009), and/or fitness penalties are associated with auxinic herbicide resistance traits due to pleiotropic effects (Roux et al., 2004).

Investigating the mechanism(s) of 2,4-D resistance in buckhorn plantain may help to inform management practices and preserve herbicidal efficacy (Busi et al., 2017). Just as the mechanism of herbicidal action is complex, so are the apparent mechanisms of 2,4-D resistance (Goggin et al., 2018). Neither are fully understood. Herbicide resistance mechanisms fall into two broad categories: target-site and non-target-site (Murphy and Tranel, 2019). While there is at least one report of at least partial target-site 2,4-D resistance (LeClere et al., 2018), others hypothesize that potential fitness penalties, the recessive nature of certain auxin herbicide resistance genes, functional redundancy of auxin receptors, and/or the complex nature of synthetic auxin herbicide mode of action may contribute to the lack of a target site resistance mechanism (Peterson et al., 2016). Thus far, nearly all of the reported mechanisms of 2,4-D resistance have been non-target-site in nature (Busi et al., 2017). A majority of these non-target-site resistance mechanisms involve reduced translocation, enhanced metabolism, or both. Cytochrome P450 likely mediates the enhanced 2,4-D metabolism in some of these resistant ecotypes, as pretreatment with malathion, a cytochrome P450 inhibitor, reversed the phenotype from resistant to at least partially susceptible in *Amaranthus hybridus*, *Amaranthus tuberculatus*, *Conyza canadensis*, *Conyza sumatrensis*, *Papaver rhoeas*, and *Parthenium hysterophorus* (Palma-Bautista et al., 2020).

Recently, the first report of 2,4-D resistance in buckhorn plantain as well as the first report of 2,4-D resistance in turf was published (Patton et al., 2018). This resistant ecotype was identified after an application of 2,4-D + mecoprop + dicamba at  $1.3 + 0.4 + 0.1 \text{ kg ae ha}^{-1}$  failed to control buckhorn plantain. Pesticide spray records revealed 30 yr of 2,4-D-containing herbicide applications, and the site offered a few other signs of herbicide resistance. There was little to no synthetic auxin herbicide symptomology present on the buckhorn plantain, 10 to 40%

buckhorn plantain ground coverage, and no other perennial broadleaf weeds present (Patton et al., 2018).

Given that the 2,4-D resistance is likely a non-target mutation, altered absorption and/or translocation as mechanisms of resistance were tested using  $^{14}\text{C}$  radiolabeled 2,4-D and comparing resistant buckhorn plantain with a susceptible ecotype. The objective of this experiment was to determine if absorption or translocation play a role in 2,4-D resistance in a resistant buckhorn plantain ecotype.

## **2.3 Materials and Methods**

### **2.3.1 Plant material**

Individual plants from the resistant and susceptible ecotypes described by Patton et al. (2018) were harvested from their respective field locations and transplanted into 7.6 cm square pots with a sandy loam soil. The buckhorn plantain transplants were then grown in a greenhouse with 27/21 C day/night temperatures and a 14 hr photoperiod for 4 wk in the Purdue University Horticulture Plant Growth Facility in West Lafayette, IN (40.421°N, 86.914°W). Plants were irrigated with municipal tap water as needed (approximately daily) and fertigated weekly with two water-soluble fertilizers (3:1 mixture of 15-2.2-12.5 and 21-2.2-16.6 [N-P-K], respectively; The Scotts Co., Marysville, OH 43040) to provide the following (in mg L<sup>-1</sup>): 200 N, 26 P, 163 K, 50 Ca, 20 Mg, 1.0 Fe, 0.5 each Mn and Zn, 0.24 each Cu and B, and 0.1 Mo. Seventy-six percent of the nitrogen provided was in the nitrate form. Plants were transported directly from West Lafayette, IN to Raleigh, NC and then acclimated to a laboratory growth chamber for 3 d before experiment initiation.

### **2.3.2 Radiolabeled experiment**

Radiolabeled 2,4-D research was then conducted at North Carolina State University in Raleigh, NC (35.787°N, 78.672°W). Following acclimation, the youngest fully expanded leaf was marked and then covered with aluminum foil to exclude overspray. Plants had 5-9 leaves, and leaves were < 12 cm in length at application. Plants were then moved outdoors and oversprayed with commercially formulated 2,4-D (Weedar® 64, Nufarm Americas Inc., Alsip, IL) at a rate of 1.68 kg ae ha<sup>-1</sup> plus nonionic surfactant (Induce surfactant, Helena Chemical Co., Memphis, TN)

at 0.25% v/v using a CO<sub>2</sub> pressurized sprayer boom calibrated to deliver 814 L ha<sup>-1</sup>. Following foliar overspray, plants were immediately returned to the laboratory and four 0.5-μl droplets of radiolabeled [<sup>14</sup>C]2,4-D plus nonionic surfactant at 0.25% v/v were applied to the foil-covered leaf on the adaxial leaf surface, avoiding prominent veins, for a total of 5.32 kBq plant<sup>-1</sup>.

Plants were harvested at 24, 96, and 192 HAT and separated into treated leaf, treated leaf wash, nontreated shoots, and the caudex + roots. Treated leaf washes were also performed at 0 hr to confirm recovery, which was 99.5% of applied. At each harvest timing, the treated leaf was excised and slowly swirled for 5 s in a 20-ml scintillation vial containing 20 ml of 50:50 v/v methanol:deionized water to remove any unabsorbed [<sup>14</sup>C]2,4-D from the leaf surface. Radioactive recovery from the leaf wash was determined by diluting a 1-ml aliquot of leaf-wash solution to 20 ml of scintillation fluid (Ultima Gold LLT 6013371; PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA), which was subjected to liquid scintillation spectroscopy (LSS) using a PerkinElmer Tri-Carb 2800TR Liquid Scintillation Analyzer (PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA) to calculate total absorption relative to the percentage of total-applied radioactivity. Following the leaf wash, the treated leaf, nontreated shoots, and the caudex + roots were further dissected, wrapped in ashless filter paper, placed in a coin envelope, dried at 60 C for 48 hr, weighed, and combusted using a biological oxidizer (OX-500 Biological Material Oxidizer, R.J. Harvey Instrument Co., Tappan, NY) and quantified using LSS.

The experimental design was a randomized complete block design arranged as a 2×4 factorial (two ecotypes by four harvest periods) with five blocks and two runs. Percentage of [<sup>14</sup>C] from 2,4-D absorption was calculated by total amount of radioactivity applied minus radioactivity recovered in the leaf wash. Translocation to non-treated plant parts (i.e., non-treated shoots and the caudex + roots) was calculated by measuring the total amount of radioactivity in previously mentioned plant samples (other than the treated leaf) and dividing by the total amount of radioactivity applied. Whole-plant [<sup>14</sup>C] from 2,4-D recovery was low (<80%) for four plants, which were analyzed as missing data. Recovery did not exceed 120% for any plants. After dropping the low recovery plants, the mean recovery was 96% of the [<sup>14</sup>C] from 2,4-D applied.

All data were analyzed using PROC GLIMMIX is SAS software (SAS Institute, Cary, NC). Where run by treatment interactions were not significant ( $P > 0.05$ ), data were pooled across runs. Means were separated using Tukey's honest significant difference test ( $\alpha = 0.05$ ). Data were

subject to nonlinear (i.e., quadratic) regression and analysis using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA 92037).

## 2.4 Results

There was a run by ecotype by harvest period interaction for absorbed [ $^{14}\text{C}$ ] from 2,4-D ( $P = 0.0414$ ) and [ $^{14}\text{C}$ ] from 2,4-D in the treated leaf ( $P = 0.0016$ ), so data are presented by run. The full interaction was not significant for [ $^{14}\text{C}$ ] from 2,4-D in the non-treated shoots ( $P = 0.6668$ ) nor [ $^{14}\text{C}$ ] from 2,4-D in the roots ( $P = 0.2883$ ), so runs were pooled for those data.

In run 1, there were no differences for ecotype ( $P = 0.6146$ ), harvest period ( $P = 0.2719$ ), or the ecotype by harvest period interaction ( $P = 0.7092$ ) for percent absorbed [ $^{14}\text{C}$ ] from 2,4-D in the plants. In run 2, there were differences in absorption for ecotype ( $P = 0.0017$ ) and the ecotype by harvest period interaction ( $P = 0.0015$ ), but not for harvest ( $P = 0.1779$ ). In the second run, the susceptible ecotype absorbed more [ $^{14}\text{C}$ ] from 2,4-D than the resistant ecotype (70.6 vs 58.7% of applied, respectively); for the interaction between ecotype and harvest period, the resistant ecotype absorbed less [ $^{14}\text{C}$ ] from 2,4-D after 24 hr than the susceptible ecotype after 24 hr (Figure 2.1; Table 2.1). The susceptible ecotype absorbed a similar amount of [ $^{14}\text{C}$ ] from 2,4-D across all three harvest periods in the second run. However, the resistant ecotype absorbed more [ $^{14}\text{C}$ ] from 2,4-D at 96 hr compared to 24 hr, both of which were similar to the 192 hr harvest.

Ecotype ( $P = 0.0087$ ) was significant for [ $^{14}\text{C}$ ] from 2,4-D recovered in the treated leaf, but neither harvest period ( $P = 0.3786$ ) nor the ecotype by harvest interaction ( $P = 0.9405$ ) were different in run 1. The resistant ecotype had a higher amount of [ $^{14}\text{C}$ ] from 2,4-D in the treated leaf compared to the susceptible ecotype (50.3 vs. 38.1%, respectively). In run 2, only the ecotype by harvest period interaction was significant ( $P = 0.0004$ ) for [ $^{14}\text{C}$ ] from 2,4-D in the treated leaf. For this interaction, the susceptible ecotype at 24 hr had a higher amount of [ $^{14}\text{C}$ ] from 2,4-D in the treated leaf than the resistant ecotype at 24 hr as well as the susceptible ecotype at 192 hr (Figure 2.2; Table 2.1).

For the non-treated shoots, ecotype ( $P < 0.0001$ ) and the ecotype by harvest period interaction ( $P = 0.0037$ ) were both significant across both runs. The susceptible ecotype translocated more [ $^{14}\text{C}$ ] from 2,4-D to the non-treated shoots than the resistant ecotype (11.9 vs. 6.6%, respectively) across harvest periods. The ecotype by harvest period interaction was the most telling: after 192 hr, the susceptible ecotype had a higher amount of [ $^{14}\text{C}$ ] from 2,4-D in the non-

treated shoots (16.1%) than the resistant ecotype at any of the harvest periods (5.5-7.3%); the amount of [ $^{14}\text{C}$ ] from 2,4-D in the non-treated shoots was statistically similar across all three harvest periods for the resistant ecotype (Figure 2.3; Table 2.1).

Across both runs, there were no differences for ecotype ( $P = 0.0558$ ), harvest period ( $P = 0.4073$ ), or the ecotype by harvest period interaction ( $P = 0.1826$ ) for [ $^{14}\text{C}$ ] from 2,4-D recovered in the caudex + roots with an average [ $^{14}\text{C}$ ] from 2,4-D recovery of  $< 4\%$  from these tissues (Table 2.1).

Table 2.1. Absorbance, treated leaf, non-treated shoots, and roots + caudex results from the radiolabeled experiment. Means followed by the same letter within the same column are not significantly different according to Tukey's honest significant difference test ( $\alpha = 0.05$ ).

Ecotype	Harvest period	Absorbance (run 1)	Absorbance (run 2)	Treated leaf (run 1)	Treated leaf (run 2)	Non-treated shoots	Roots + caudex
----- % of applied [ $^{14}\text{C}$ ] from 2,4-D -----							
Resistant	24	62.9	44.9 b	49.9	36.5 b	7.3 b	3.5
	96	71.7	69.0 a	53.4	56.1 ab	7.1 b	3.3
	192	61.3	62.3 ab	47.6	58.1 ab	5.4 b	3.1
Mean		65.3	58.8	50.3	50.2	6.5	3.3
Susceptible	24	56.8	75.6 a	38.7	67.4 a	7.3 b	2.4
	96	66.3	65.9 a	42.3	48.8 ab	12.4 ab	1.5
	192	65.2	70.4 a	33.2	41.2 b	15.7 a	3.3
Mean		62.8	70.6	38.1	52.4	11.7	2.4
P-value		0.7092	0.0015	0.9405	0.0004	0.0037	0.1826

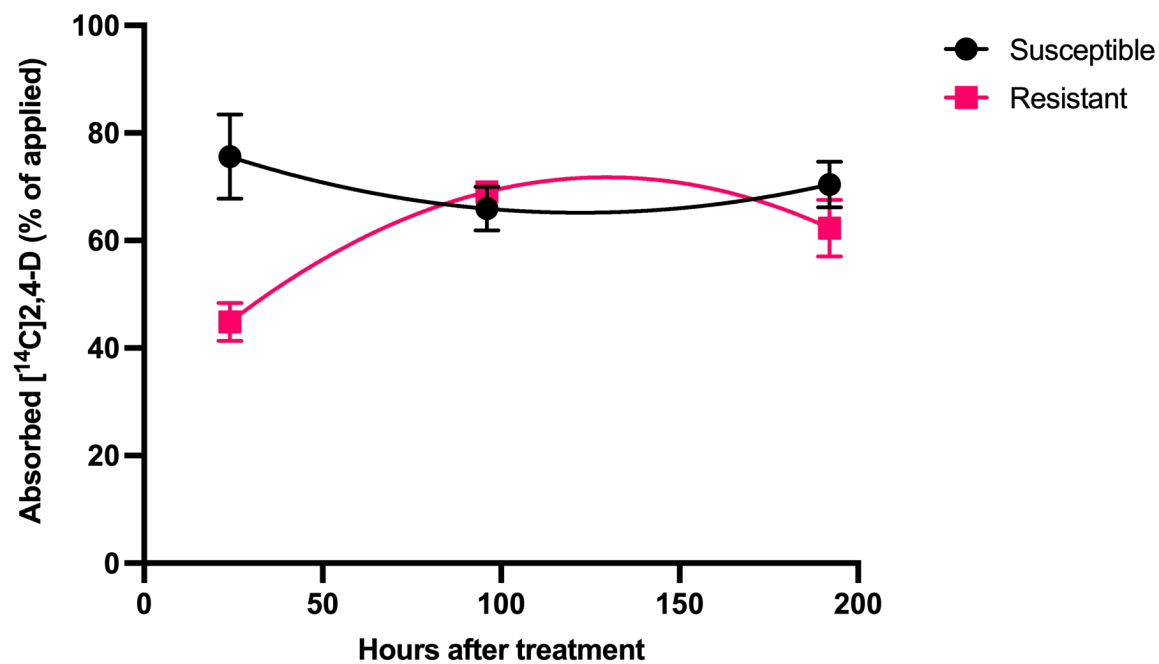


Figure 2.1. Percentage of applied [ $^{14}\text{C}$ ] from 2,4-D absorbed by ecotype for each harvest period in the second run of the experiment. Data points are means and standard errors across five blocks. Percentage of [ $^{14}\text{C}$ ] from 2,4-D absorbed was calculated by the total amount of radioactivity applied minus radioactivity recovered in the leaf wash.

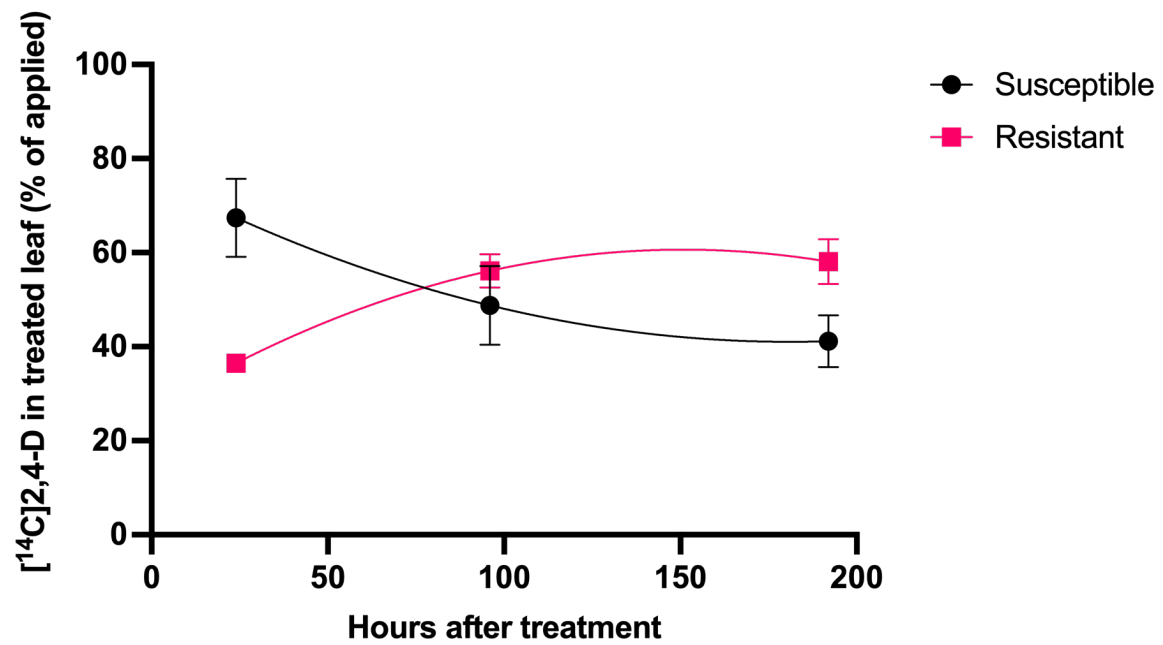


Figure 2.2. Percentage of applied  $[^{14}\text{C}]$  from 2,4-D recovered from the treated leaf by ecotype for each harvest period in the second run of the experiment. Data points are means and standard errors are shown across five blocks.

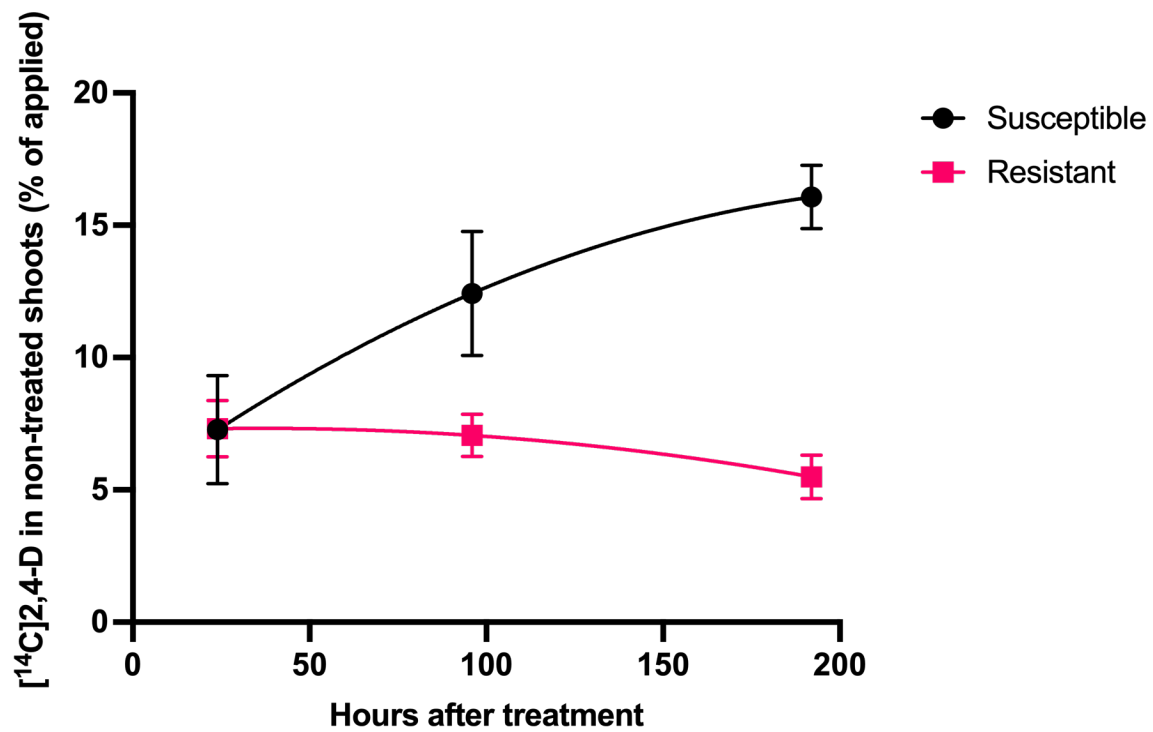


Figure 2.3. Percentage of applied [<sup>14</sup>C] from 2,4-D recovered from the non-treated shoots in the resistant and susceptible ecotypes over a 192 hr period. Data points are means and standard errors are shown across 2 experimental runs and 5 blocks (n = 10).



## 2.5 Discussion

The clearest difference between the resistant and susceptible ecotype was the interaction between ecotype and harvest period for [ $^{14}\text{C}$ ] from 2,4-D in the non-treated shoots. The resistant and susceptible ecotypes had translocated a similar amount (7.3%) of the applied [ $^{14}\text{C}$ ] from 2,4-D after 24 hr. After 96 hr, while still similar according to Tukey's HSD, a divergent trend begins between the susceptible (12.4%) and resistant (7.1%) ecotypes for the amount of [ $^{14}\text{C}$ ] from 2,4-D in the non-treated shoots, although the standard errors of the mean do not overlap. By 192 hr, the susceptible ecotype (15.7%) translocated more [ $^{14}\text{C}$ ] from 2,4-D to the non-treated shoots than the resistant ecotype (5.4%) at 192 hr. Further, a similar amount of [ $^{14}\text{C}$ ] from 2,4-D was recovered from the non-treated shoots from the resistant ecotype for all three harvest periods, indicating that translocation was not increasing with time.

Reduced translocation is further evidenced by the differences in [ $^{14}\text{C}$ ] from 2,4-D recovered from the treated leaf. A higher amount of [ $^{14}\text{C}$ ] from 2,4-D was recovered from the treated leaf of the resistant ecotype in the first run across all harvest periods. Additionally, the susceptible ecotype at 24 hr had a higher amount of [ $^{14}\text{C}$ ] from 2,4-D in the treated leaf than both the resistant ecotype at 24 hr and the susceptible ecotype at 192 hr in the second run. Differences in the treated leaf recovery between ecotypes in the first run as well as between harvest periods for the susceptible but not the resistant ecotype in the second run were likely, in part, a result of the reduced translocation in the resistant ecotype. The [ $^{14}\text{C}$ ] from 2,4-D absorbed by the resistant ecotype largely remained in the treated leaf, whereas the susceptible ecotype translocated the absorbed [ $^{14}\text{C}$ ] from 2,4-D to the non-treated shoots over time.

In the second run, the susceptible ecotype absorbed more [ $^{14}\text{C}$ ] from 2,4-D than the resistant ecotype, which was also a likely contributor to the differences in the treated leaf [ $^{14}\text{C}$ ] from 2,4-D recovery between the ecotype and harvest period interaction in that run. The resistant ecotype absorbed less [ $^{14}\text{C}$ ] from 2,4-D after 24 hr than the susceptible ecotype after 24 hr. There were no differences for the amount of [ $^{14}\text{C}$ ] from 2,4-D recovered from the caudex + roots between ecotype, harvest period, or the interaction between main effects.

While this research does not clearly elucidate the mechanism of 2,4-D resistance, it provides additional evidence that the resistance mechanism is non-target-site in nature for this particular ecotype and is likely due, in part, to reduced translocation. Similar to this experiment, Riar et al. (2011) noted reduced absorption and translocation of 2,4-D in resistant *Lactuca serriola*.

In that study, compared to the susceptible ecotype, the resistant ecotype absorbed less applied 2,4-D, retained more 2,4-D in the treated portion of the leaf, and translocated less applied 2,4-D to leaves above the treated leaf as well as the crown after 96 hr (Riar et al., 2011). However, they reported no differences in metabolism (Riar et al., 2011). Ground ivy (*Glechoma hederacea* L.) tolerant to 2,4-D was found to absorb less 2,4-D than a susceptible population as well as translocate less 2,4-D to the apical meristem of the primary stolon (Kohler et al., 2004). Reduced translocation of 2,4-D has also been noted in *Raphanus raphanistrum* (Goggin et al., 2016), *Sisymbrium oriental* (Dang et al., 2018), *Carduus nutans* (Harrington and Woolley, 2010), *Amaranthus hybridus*, *Hirschfeldia incana*, *Parthenium hysterophorus*, and *Papaver rheas* (Palma-Bautista et al., 2020).

McElroy et al. (2018) performed RNA-Seq analysis on the same resistant and susceptible ecotypes in this study and found more gene level transcripts of cytochrome P450s, glutathione S-transferases and ABC transporters were upregulated in the susceptible ecotype than the resistant ecotype 24 hr after 2,4-D application. Differences in [<sup>14</sup>C]2,4-D translocation between the resistant and susceptible ecotype in this experiment may be due to the differential upregulation of ABC transporters reported by McElroy et al. (2018). In another related research project, malathion, a cytochrome P450 inhibitor, synergized 2,4-D activity in this resistant ecotype (Chapter 3; Law, 2021). Thus, while reduced translocation was the most apparent difference in this experiment, absorption and/or metabolism may also contribute to 2,4-D resistance in this ecotype.

These findings warrant future research on 2,4-D resistance in buckhorn plantain. A radiolabeled degradation experiment may be necessary to elucidate a potential differential metabolism mechanism. A similar project, including additional resistant ecotypes reported by Law (Chapter 4; 2021), could be performed to screen for similar or different 2,4-D resistance mechanisms within ecotypes of the species.

## 2.6 References

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## **CHAPTER 3. CYTOCHROME P450 INHIBITION PARTIALLY REVERSES 2,4-D HERBICIDE RESISTANCE IN *PLANTAGO* *LANCEOLATA***

### **3.1 Abstract**

Investigating the mechanism(s) of 2,4-D resistance in buckhorn plantain may help to inform management practices and preserve herbicidal efficacy. A majority of the reported mechanisms of 2,4-D resistance thus far have been non-target-site in nature, and many of which involve some type of cytochrome P450 metabolism. As such, the objective of this research was to determine if metabolism via cytochrome P450 plays a role in 2,4-D resistance in buckhorn plantain. To do so, a 2,4-D dose-response experiment was conducted using malathion as a cytochrome P450 inhibitor. Epinasty results suggest that cytochrome P450 metabolism may play a role, albeit small, in 2,4-D resistance in this ecotype of buckhorn plantain. Malathion pre-treatment did not fully revert the resistant ecotype back to susceptible. If cytochrome P450 metabolism is part of the 2,4-D resistance mechanism of this buckhorn plantain ecotype, it is a contributor and not the sole mechanism of resistance. Additional research is needed to fully elucidate the mechanism of resistance in this 2,4-D resistant buckhorn plantain ecotype.

### 3.2 Introduction

Commercialized in 1945, 2,4-Dichlorophenoxyacetic acid (2,4-D) revolutionized chemical weed control (Peterson et al., 2016). The synthetic auxin herbicide provides selective control of broadleaf weeds and is relatively safe on grasses. The herbicide has been used worldwide on small grains, fruits, nuts, vegetables, pastures, rangelands, turfgrasses, rights-of-way, aquatic habitats, and forestry sites (Peterson et al., 2016). An estimated 65% of the 2,4-D used annually between 1992 and 2000 in the United States was for agriculture and another 25% was used on home lawns (Borges et al., 2004). Thus, the turfgrass industry is a large market for 2,4-D use, and an estimated 5.2 million kg of active ingredient are applied to lawns annually (Borges et al., 2004).

The first reported cases of 2,4-D resistance occurred in 1957 on climbing dayflower (*Commelina diffusa* Burm. f.) and wild carrot (*Daucus carota* L.) (Heap, 2021). Since then, a total of at least 45 cases of 2,4-D resistance across 25 weed species have been described (Heap, 2021). While the incidence of weed resistance is lower for synthetic auxins than other herbicide modes of action (Busi et al., 2017), over half of the reported cases of 2,4-D resistance identified in the last 20 years (Heap, 2021). Further, 2,4-D use is predicted to increase as 2,4-D tolerant crops are adopted (Peterson et al., 2016) and resistance issues with other herbicides develop/persist.

Patton et al. (2018) were the first to report synthetic auxin resistance in a turfgrass system after confirming 2,4-D resistance in buckhorn plantain (*Plantago lanceolata* L.). Since then, 2,4-D resistant ecotypes of buckhorn plantain have been identified in Indiana (Law, 2021), Ohio (Law, 2021), Pennsylvania (Russell et al., 2020), Georgia (P.E. McCullough, personal communication), and Virginia (S.D. Askew, personal communication). These resistant buckhorn plantain ecotypes were located in multiple turfgrass systems, including a cemetery, home lawn, university campus, sports field, and golf course.

Few options exist for herbicides that provide excellent postemergence buckhorn plantain control in turf, especially those labeled for use in cool-season residential turf (Patton and Elmore, 2021). Most herbicides that provide excellent postemergence control of buckhorn plantain in turf contain 2,4-D (Patton and Elmore, 2021), and the herbicides offering excellent control that do not contain 2,4-D are more expensive (Patton et al., 2018), not labeled for home lawns, and/or not labeled for cool-season turf (Patton and Elmore, 2021). As a likely result, 2,4-D was routinely applied in lieu of herbicide rotation, selecting for herbicide resistant individuals and eventually ecotypes (i.e., populations).

Investigating the mechanism(s) of 2,4-D resistance in buckhorn plantain may help to inform management practices and preserve herbicidal efficacy (Busi et al., 2017). Just as the mechanism of herbicidal action is complex, so are the apparent mechanisms of 2,4-D resistance (Goggin et al., 2018). Neither are fully understood. Herbicide resistance mechanisms fall into two broad categories: target-site and non-target-site (Murphy and Tranel, 2019). While there are reports of at least partial target-site 2,4-D resistance (LeClere et al., 2018), others hypothesize that potential fitness penalties, the recessive nature of certain auxin herbicide resistance genes, functional redundancy of auxin receptors, and/or the complex nature of synthetic auxin herbicide mode of action may contribute to the lack of a target site resistance mechanism (Peterson et al., 2016). Interestingly, LeClere et al. (2018) reported a fitness cost associated with their target-site resistance mechanism.

Thus far, a majority of the reported mechanisms of 2,4-D resistance have been non-target-site in nature. This includes reduced transport, enhanced metabolism, and a combination of transport and metabolism. Ring hydroxylation of 2,4-D has been proposed as the pathway for metabolism of 2,4-D in tolerant species (Peterson et al., 2016), which is mediated by cytochrome P450 enzymes (Hatzios et al., 2005). “Products from the ring hydroxylation metabolic pathway are more hydrophilic, non-phytotoxic, and polar compared with 2,4-D and cannot be hydrolyzed back to 2,4-D (Cobb and Reade, 2010a).” (Peterson et al., 2016). Malathion, an organophosphorus insecticide, inhibits cytochrome P450 monooxygenases involved in herbicide metabolism (Kreuz et al., 1992). As such, it can be used as a cytochrome P450 inhibitor in 2,4-D herbicide resistance research (Figueiredo et al., 2018; Shergill et al., 2018; Palma-Bautista et al., 2020).

Cytochrome P450 enzymes can metabolize 2,4-D via ring hydroxylation in tolerant and resistant plants. Cytochrome P450 likely mediates 2,4-D metabolism in resistant waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] (Figueiredo et al., 2018; Shergill et al., 2018), Canadian horseweed [*Conyza canadensis* (L.) Cronquist], and tall fleabane [*Conyza sumatrensis* (Retz.) E. H. Walker] (Palma-Bautista et al., 2020), as pre-treatment with a cytochrome P450 inhibitor (i.e., malathion) reversed the phenotype from resistant to susceptible in 2,4-D resistant ecotypes of these species. Further, cytochrome P450 likely plays a partial role in the 2,4-D resistance mechanism(s) for corn poppy (*Papaver rhoeas* L.), smooth pigweed (*Amaranthus hybridus* L.), and Santa Maria feverfew (*Parthenium hysterophorus* L.) (Palma-Bautista et al., 2020). While malathion pre-treatment did not fully reverse the resistant phenotype in these species, it reduced the herbicide

resistance factor by around 50% compared to plants treated only with 2,4-D (Palma-Bautista et al., 2020).

Given that Patton et al. (2018) reported a relatively low level of 2,4-D resistance for buckhorn plantain (resistance factor between 6 and 30, depending on metric) and that a majority of other 2,4-D resistance mechanisms are non-target-site in nature, investigating 2,4-D metabolism via cytochrome P450 in a resistant buckhorn plantain ecotype may provide clues as to the mechanism of resistance. Thus, the objective of this research was to determine if metabolism via cytochrome P450 plays a role in 2,4-D resistance in buckhorn plantain. To do so, a 2,4-D dose-response experiment was conducted using malathion as a cytochrome P450 inhibitor.

### 3.3 Materials and Methods

Research was performed in a greenhouse at the Purdue Horticulture Plant Growth Facility in West Lafayette, IN (40.421°N, 86.914°W). Individual resistant and susceptible buckhorn plantain rosettes were vegetatively transplanted into 7.6 cm square pots with a sandy loam soil and allowed to establish before the experiment was initiated. The resistant and susceptible ecotypes used are described by Patton et al. (2018). Plants were watered to prevent wilt (approximately daily) and fertigated every 2 wk using two water-soluble fertilizers (3:1 mixture of 15 N–2.2 P–12.5 K and 21 N–2.2 P–16.6 K, respectively; Scotts Company LLC, Marysville, OH 43040) to provide the following (in mg L<sup>-1</sup>): 200 N, 26 P, 163 K, 50 Ca, 20 Mg, 1.0 Fe, 0.5 Mn and Zn, 0.24 Cu and B, and 0.1 Mo. Seventy-six percent of the nitrogen provided was in the nitrate form.

The experiment was a randomized complete block design with a 2×2×6 factorial arrangement, four blocks, and two runs. Treatments were comprised of ecotype (resistant and susceptible), malathion rate (0 and 2.24 kg a.i. ha<sup>-1</sup>), and 2,4-D rate (0, 0.168, 1.68, 8.4, 16.8, 42.0 kg ae ha<sup>-1</sup>). Ortho® MAX® Malathion (Scotts Company LLC, Marysville, OH) and Nufarm Weedar® 64 2,4-Dichlorophenoxyacetic acid, dimethamine salt (Nufarm Inc., Alsip, IL) were the sources of malathion and 2,4-D used in the experiment, respectively. The first and second runs were initiated on 20 Nov. 2018 and 5 June 2019, respectively. Plant diameters were approximately 20 cm at application.

In the second run of the experiment, an additional malathion drench treatment was added to the 0, 8.4, and 16.8 kg ae ha<sup>-1</sup> 2,4-D rates. This drench ancillary experiment had a 2×3×3 factorial arrangement and was analyzed separately. The rationale for this ancillary experiment is explained



below. The drench treatment received the same 2.24 kg a.i. ha<sup>-1</sup> malathion pre-treatment followed by a soil drench of 5 mM malathion solution of 50 mL pot<sup>-1</sup> 2 d after herbicide treatment and every 7 d thereafter until the 6 WAA harvest.

Treatments were applied using compressed air in a track spray chamber (Generation III Research Sprayer, DeVries Manufacturing, Hollandale, MN 56045) calibrated to deliver a volume of 140 L ha<sup>-1</sup> using a TeeJet® 8002EVS nozzle (TeeJet Technologies, Spraying Systems, Wheaton, IL 60187) at 275 kPa. Malathion was used as a cytochrome P450 inhibitor (Torra et al., 2017), and plants receiving malathion were treated 18 hr prior to 2,4-D application.

Weed epinasty was visually assessed 0.5, 1, 2, 3, 4, 5, 6 WAA on a 0 to 100% scale, where 0% was no epinasty and 100% represented complete epinasty with the entire plant exhibiting symptoms, including twisting or bending of stems and curling of leaves. Weed control was visually assessed 0.5, 1, 2, 3, 4, 5, 6 WAA on a 0 to 100% scale, where 0% was no control and 100% represented a completely dead plant with no green tissue.

Digital images (1.92 megapixels at 180 dpi resolution) were taken of the individual buckhorn plantain plants immediately prior to application as well as 0.5, 1, 2, 3, 4, 5, 6, and 10 WAA using a camera (Canon PowerShot SX250 HS, Canon USA, Melville, NY 11747) and light box similar to that described by Ghali et al. (2012). Images were collected with the light box using camera settings of F-stop equal to f/3.5, 1/25-s exposure, and ISO speed equal to 100. Images were analyzed for percent green pixels with ImageJ (v. 1.53a, National Institutes of Health, Bethesda, MD 20892)(Schneider et al. 2012) using color threshold settings of hue = 42 to 95, saturation = 57 to 255, and brightness = 51 to 255 in a modified macro (Soldat et al. 2012). Images were taken of a green calibration disk, and data were converted from selected green pixels to plant area (cm<sup>2</sup>).

At 6 WAA, plants were harvested approximately 1 to 2 cm above the soil surface to obtain a leaf fresh weight and also a leaf dry weight after 3 d of drying in a forced-air dryer at 60 C. The caudex and root system were left intact and allowed to regrow for an additional 4 wk. At 10 WAA, plants were harvested a second time in the same manner, and fresh and dry leaf weights were measured as before. Harvest data were analyzed as a percent of the non-treated control within each block for each ecotype and malathion rate, save for the ancillary drench experiment.

All data were analyzed using PROC GLIMMIX in SAS software (SAS Institute, Cary, NC). Means were separated using Tukey's honest significant difference test ( $\alpha = 0.05$ ). Run by treatment interactions were not significant ( $P > 0.05$ ), so data were pooled across runs. The ancillary drench

experiment was analyzed separately, as it was only performed for one run and did not include all 2,4-D rates. When a significant ecotype by 2,4-D rate interaction existed ( $P \leq 0.05$ ), data were subject to nonlinear (i.e., sigmoidal) regression and analysis using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA 92037).

### 3.4 Results

There was not an ecotype by malathion rate interaction for fresh weight 6 WAA ( $P = 0.5599$ ), dry weight 6 WAA ( $P = 0.7397$ ), regrowth fresh weight 10 WAA ( $P = 0.3703$ ), or regrowth dry weight 10 WAA ( $P = 0.1379$ ). Nor was there a three-way interaction between ecotype, malathion rate, and 2,4-D rate for fresh weight 6 WAA ( $P = 0.1897$ ), dry weight 6 WAA ( $P = 0.5970$ ), fresh weight 10 WAA ( $P = 0.1913$ ), or dry weight 10 WAA ( $P = 0.0913$ ). For visual control 6 WAA, the ecotype by malathion rate  $P$ -value was 0.0468, but Tukey's honest significant difference test did not separate the interactive effects. Additionally, there was not a three-way interaction between ecotype, malathion rate, and 2,4-D rate for visual control 6 WAA ( $P = 0.0958$ ). Differences for one of these interactions may have indicated potential synergism between 2,4-D and malathion for the resistant ecotype. The non-significant ecotype by malathion rate interaction across 2,4-D doses is visualized in Figure 3.1. These results were similar to leaf area via digital image analysis (data not shown).

However, there was an interaction between ecotype and malathion rate for epinasty 0.5 WAA ( $P = 0.0021$ ). On this early rating date, malathion synergized 2,4-D activity in the resistant ecotype and caused more epinasty than 2,4-D alone (Figure 3.2). Given differences in early injury that did not translate to differences in control, we hypothesized that cytochrome P450 inhibition from the malathion may not have persisted for 6 wk. Thus, we added the drench ancillary experiment to run in conjunction with the second run of the full experiment.

In the drench ancillary experiment, which was analyzed separately, there was not an ecotype by malathion rate interaction for fresh weight 6 WAA ( $P = 0.6248$ ) or dry weight 6 WAA ( $P = 0.7401$ ). However, there was an ecotype by malathion rate interaction for regrowth fresh weight 10 WAA ( $P = 0.0001$ ) and regrowth dry weight 10 WAA ( $P = 0.0002$ ). In both cases, the resistant ecotype with a malathion drench was similar to all of the susceptible ecotype by malathion rate treatments and also different from the other resistant ecotype by malathion rate treatments (Figure 3.3).

As expected, the interaction between ecotype and 2,4-D rate was significant for control 6 WAA ( $P < 0.0001$ ), fresh weight 6 WAA ( $P = 0.0005$ ), regrowth fresh weight 10 WAA ( $P < 0.0001$ ), and regrowth dry weight 10 WAA ( $P < 0.0001$ ). However, the ecotype by 2,4-D rate interaction was not significant for dry weight 6 WAA ( $P = 0.2919$ ). In general, the resistant ecotype survived higher rates of 2,4-D than the susceptible ecotype.

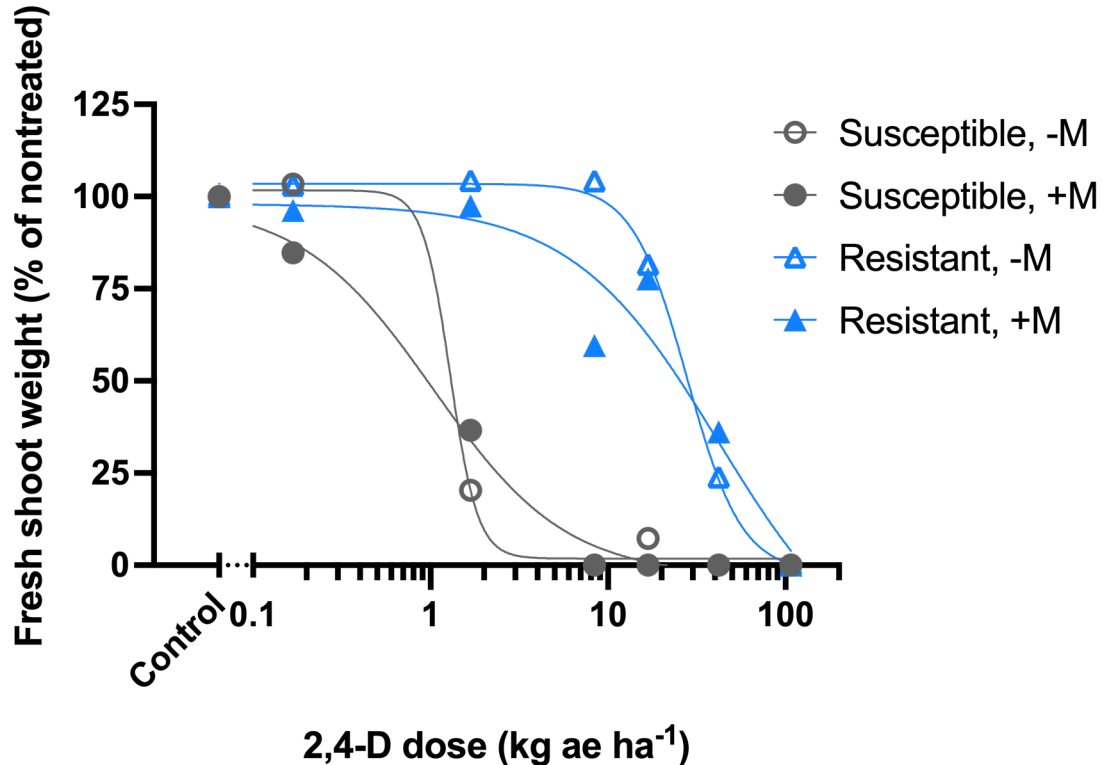


Figure 3.1. Fresh shoot regrowth 10 WAA, as percent of nontreated, for the susceptible and resistant ecotypes with (+M) and without (-M) 2.24 kg ai ha<sup>-1</sup> malathion pretreatment across four blocks and two experimental runs. The GR<sub>50</sub> values calculated from sigmoidal regression were 42 and 28 kg ae ha<sup>-1</sup> for the resistant ecotypes with and without malathion pretreatment, respectively, and the GR<sub>50</sub> values were 1.04 and 1.29 kg ae ha<sup>-1</sup> for the susceptible ecotypes with and without malathion pretreatment, respectively. The maximum labeled application rate of 2,4-D is 1.68 kg ae ha<sup>-1</sup>.

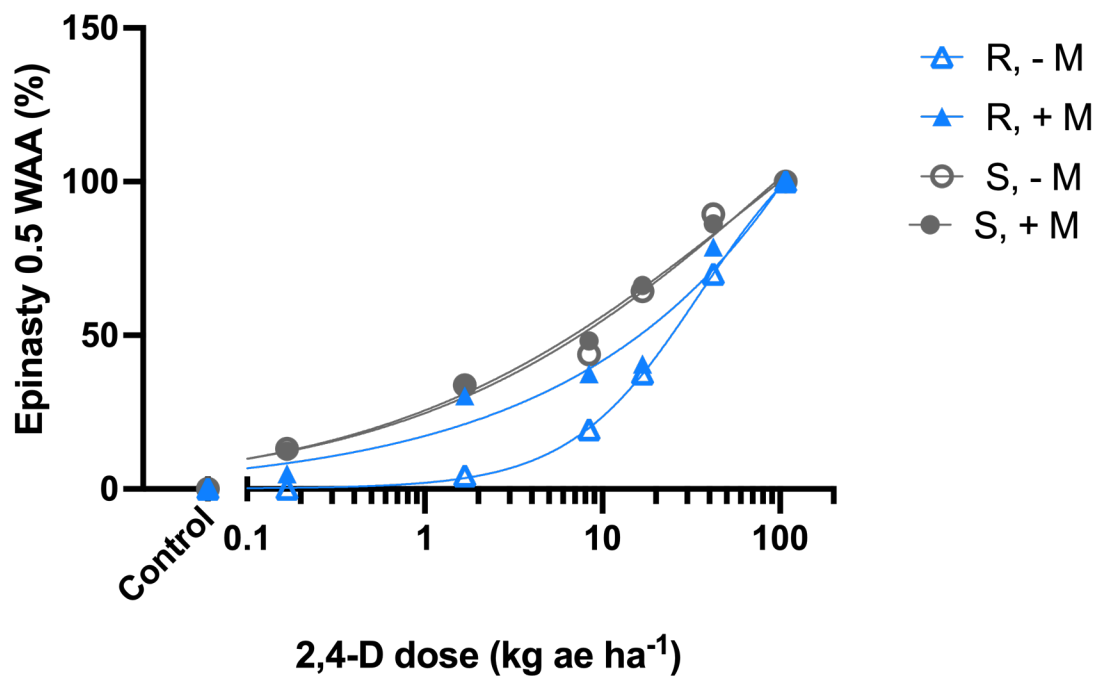


Figure 3.2. Epinasty (%) as visually rated 0.5 WAA for the susceptible and resistant ecotypes with (+M) and without (-M) 2.24 kg ai ha<sup>-1</sup> malathion pretreatment across four blocks and two experimental runs. Data were subject to nonlinear (sigmoidal) regression. Epinasty was rated on a 0 to 100% scale, where 0% was no epinasty and 100% represented complete epinasty with the entire plant exhibiting symptoms, including twisting or bending of stems and curling of leaves.

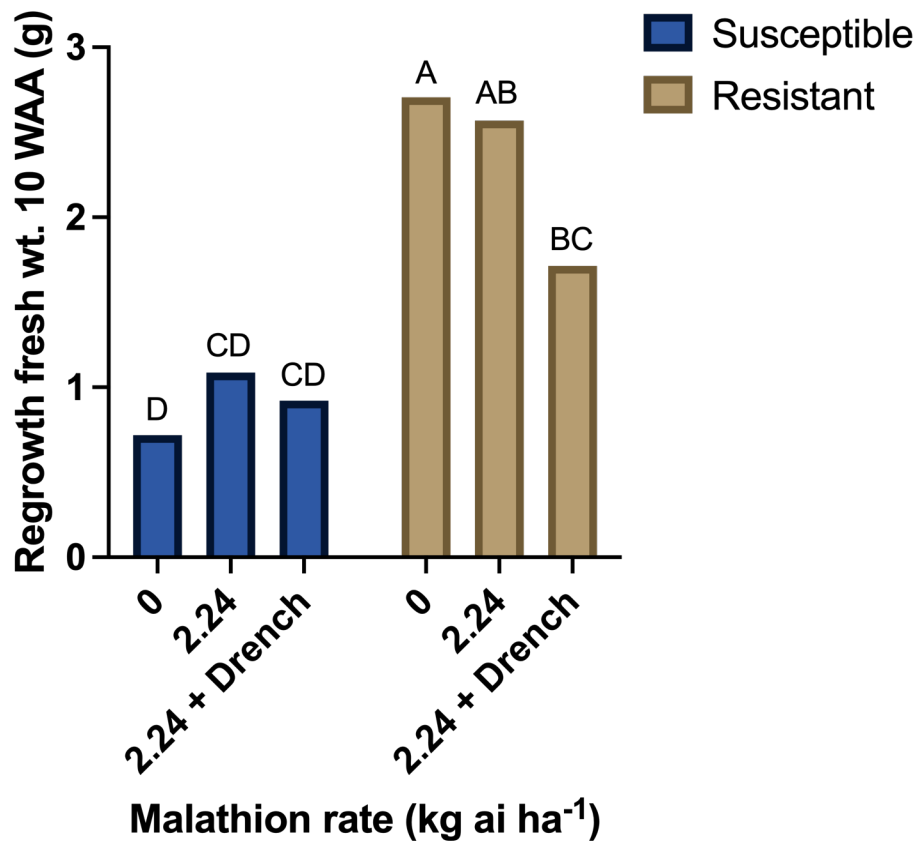


Figure 3.3. Regrowth fresh weight 10 WAA for the ancillary drench experiment across 0, 8.4, and 16.8 kg ae ha<sup>-1</sup> 2,4-D rates. The drench treatment received the same 2.24 kg ai ha<sup>-1</sup> malathion pre-treatment followed by a soil drench of 5 mM malathion solution with 50 ml pot<sup>-1</sup> 2 d after herbicide treatment and every 7 d until the 6 WAA harvest.

### 3.5 Discussion

The interaction between ecotype and 2,4-D rate confirms the previous findings of Patton et al. (2018) that this ecotype of buckhorn plantain is indeed resistant to 2,4-D. Early epinasty results suggest that cytochrome P450 metabolism may play a role, albeit small, in 2,4-D resistance in this ecotype of buckhorn plantain. However, malathion pre-treatment did not revert the resistant ecotype back to susceptible. For example, the GR<sub>50</sub> values were 28 and 42 kg ae ha<sup>-1</sup> for the resistant ecotypes with and without malathion pretreatment for fresh shoot regrowth weight 10 WAA, respectively (Figure 3.1).

If cytochrome P450 metabolism is part of the 2,4-D resistance mechanism of this buckhorn plantain, it is likely a contributor and not the sole mechanism of resistance. Preliminary screening of an additional 2,4-D resistant ecotype of buckhorn plantain described by Law (Chapter 4; 2021) yielded similar results to this experiment. A 2.24 kg ai ha<sup>-1</sup> pretreatment of malathion 18 h prior to an 8.4 kg ae ha<sup>-1</sup> application of 2,4-D produced differences in early symptomology compared to 2,4-D alone (Figure 3.2), but it did not reduce yield or improve control 6 WAA (data not shown).

Palma-Bautista et al. (2020) reported a range in the level of phenotypic reversal (i.e., from resistant to susceptible) for different species when pre-treated with malathion: *Hirschfeldia incana* (L.) did not respond to malathion pre-treatment, whereas Canadian horseweed and tall fleabane essentially lost their 2,4-D resistance with a malathion pre-treatment; 2,4-D was partially synergized by malathion in corn poppy, smooth pigweed, and Santa Maria feverfew, with an approximate 50% reduction in the resistance factor compared to plants treated only with 2,4-D (Palma-Bautista et al., 2020).

While this research does not provide definitive evidence that cytochrome P450 mediates 2,4-D resistance in this ecotype of buckhorn plantain, it seemingly hints that it may be a contributing factor. McElroy et al. (2018) performed RNA-Seq analysis on the same resistant and susceptible ecotypes and found more gene level transcripts of cytochrome P450s, glutathione S-transferases and ABC transporters were upregulated in the susceptible ecotype than the resistant ecotype 24 hr after 2,4-D application. Thus, if cytochrome P450 metabolism contributes to 2,4-D resistance in buckhorn plantain, it must be independent of upregulation.

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## **CHAPTER 4. LACK OF EVIDENCE OF A FITNESS PENALTY ACROSS MULTIPLE 2,4-D RESISTANT ECOTYPES OF *PLANTAGO LANCEOLATA***

### **4.1 Abstract**

After confirming 2,4-D resistance in one ecotype of buckhorn plantain (*Plantago lanceolata*), failure to control other distinct ecotypes with 2,4-D has prompted additional research. Identifying potential fitness penalties in resistant ecotypes may lend clues as to the mechanism of resistance as well as effective cultural control methods. A two-part experiment was conducted to 1) determine the 2,4-D sensitivity of 13 buckhorn plantain ecotypes in a bioassay greenhouse trial and 2) evaluate and characterize the morphological and reproductive variation among 12 populations of buckhorn plantain to determine similarity or dissimilarity among 2,4-D resistant populations. Two runs of a bioassay experiment were conducted in a greenhouse, and two runs of a garden study were conducted in the field. Five ecotypes were found to be resistant, which were 7.9 to 25.5 times less sensitive to 2,4-D than the susceptible biotype. When planted in the field, differences between ecotypes were present, but the 2,4-D resistant ecotypes did not cluster for individual measurements nor principal component analysis. These additional cases of 2,4-D resistance arose independently and hint that there may be more. Confirming resistance in additional ecotypes highlights the importance of herbicide resistance research as well as educating turfgrass practitioners about resistance prevention. While no fitness penalties were observed, that does not confirm they are not present, just that they were not measured.

## 4.2 Introduction

Buckhorn plantain (*Plantago lanceolata* L.), also known as English plantain, narrow-leaved plantain, ribwort plantain, and lamb's tongue, is a cool-season perennial, seldom annual or biennial, broadleaf that is a common weed in turfgrass systems (Christians et al., 2016) as well as in hayfields, pastures, farmyards, waste places, and roadsides (Cavers et al., 1980). Buckhorn plantain is a noxious, invasive, or restricted weed species in many U.S. states. The species originates from Eurasia (Cravers et al., 1980), but it has since established throughout the world save for subarctic and low-lying tropical areas (Tessene, 1968).

Despite being a dicotyledonous plant, the cotyledons of a germinating buckhorn plantain seedling appear grass-like. Mature leaves are lanceolate-shaped [3-40 cm long (including the petiole) by 0.5 to 3.5 cm wide (Cavers et al., 1980)], which is why the species name (*lanceolata*) was chosen. Leaves have prominent parallel veins, though closer inspection reveals reticulated veins that dicots are notorious for. Leaf margins vary from entire to serrated, and leaf blades, though generally glabrous, can be pubescent. Buckhorn plantain forms and grows from a central, basal rosette. Long, silky hairs are often present at the growing point. There is no visible aboveground stem, but a semi-woody caudex and/or undeveloped taproot is often present. Plants develop a few deep roots (1 m or more) as well as a mass of short (5-10 cm) fibrous roots near the soil surface (Sagar and Harper, 1964).

Buckhorn plantain has  $2n = 12$  chromosomes (Sagar and Harper, 1964) and reproduces by both seeds and basal shoots. Buckhorn plantain is a long day species (Snyder, 1948), flowering from mid-spring through early fall in the Midwest. Buckhorn plantain is a wind-pollinated, self-incompatible, obligate outcrosser (Sharma et al., 1992) with a relatively high level of gene flow (Van Dijk et al., 1988). It is a gynodioecious species (Ross, 1969) in which flowers may be pistillate, staminate, or hermaphroditic (Tessene, 1968). Also a protogynous species, the stigma protrudes 4-5 d prior to flower opening (Sharma et al., 1992). Inflorescences are long, leafless stalks (up to 45 cm in length) that bear dense, cylinder-shaped spikes (1-8 cm in length); single flowers are inconspicuous (Cavers et al., 1980; Warwick and Briggs, 1979).

Fruiting capsules yield one or two narrowly oval, brown to black, shiny seeds indented on one side (2 mm in length)(Cavers et al., 1980). Buckhorn plantain seed yield can be multiplicatively determined by the number of inflorescences per plant, capsule number per inflorescence, seed number per capsule, and seed mass (Primack and Antonovics, 1981). Seed

weight ranges from 0.7 to 2.0 mg, with an average of up to 470 seeds per flowering plant (Mook et al., 1992). Approximately 20% of seedlings survive to adulthood, and the average half-life for adults is 2.7 yr (Mook et al., 1992).

Compared to other *Plantago* species (i.e., *P. coronopus*, *P. major*, and *P. media*), buckhorn plantain has the widest ecological amplitude (Mook et al., 1992). Buckhorn plantain tolerates adverse growing conditions, including compaction, low fertility (Hoveland et al., 1976), a wide range of soil pH values (Buchanan et al., 1975), and an adaptive lead tolerance that is transmitted to seed progeny (Wu and Antonovics, 1976). Fertilization can have mixed effects on buckhorn plantain populations. In a hayfield, fertilization reduced buckhorn plantain biomass in undisturbed vegetation but increased biomass in a buckhorn plantain monoculture (Berendse, 1983).

Morphological differences between buckhorn plantain ecotypes can vary based on habitat and management. Left unmown, buckhorn plantain grows long, erect leaves and appears bush-like. As mowing height decreases and frequency increases, smaller, more prostrate leaves and plants result. Warwick and Briggs (1979) measured field samples of various ecotypes and found that leaf length, leaf width, scape length, and spike length were shorter/narrower for buckhorn plantain collected from a lawn compared to roadside and waste areas. However, when plants from these same ecotypes were cultivated, the between-habitat differences in ecotypes disappeared (Warwick and Briggs, 1979).

There may be genetic variation in buckhorn plantain plasticity (Schmitt et al., 1992). Environmental effects on life-history are large relative to genetic differences (Antonovics and Primack, 1982), and such environmental maternal effects may affect offspring fitness as well as contribute to differences among populations in seed and seedling demography (Schmitt et al., 1992). Gaspar et al. (2019) found that, “Epigenetic differences [of buckhorn plantain] were consistently related to genetic and environmental variation, and to a lesser degree to phenotypic variation and land use, with more grazed populations harboring greater epigenetic diversity.” In a related study, increased mowing intensity of the seed source was related to larger plant sizes, lower specific leaf areas, and lower levels of intrapopulation phenotypic variation in the offspring (Gaspar et al., 2020).

Recently, the first report of 2,4-D resistance in buckhorn plantain as well as the first report of 2,4-D resistance in turf was published (Patton et al., 2018). This resistant ecotype was

identified after an application of 2,4-D + mecoprop + dicamba at 1.3 + 0.4 + 0.1 kg ae ha<sup>-1</sup> failed to control buckhorn plantain. Pesticide spray records revealed 30 yr of 2,4-D-containing herbicide applications, and the site offered a few other signs of herbicide resistance. There was little to no synthetic auxin herbicide symptomology present on the buckhorn plantain, 10 to 40% buckhorn plantain ground coverage, and no other perennial broadleaf weeds present (Patton et al., 2018).

Upon communicating this confirmation of 2,4-D resistance in buckhorn plantain to turfgrass practitioners, other potentially resistant populations were brought to the attention of the Purdue Turfgrass Extension team. In a preliminary bioassay, a total of five ecotypes survived 2,4-D rates of 16.8 kg ae ha<sup>-1</sup>, which is 10× the standard application rate of 1.68 kg ae ha<sup>-1</sup>. Four of these ecotypes, including the ecotype confirmed to be 2,4-D resistant by Patton et al. (2018), were from Indiana, and the fifth ecotype was from Ohio (data not shown). Additionally, 2,4-D resistant buckhorn plantain ecotypes have been identified in Pennsylvania (Russell et al., 2020), Georgia (P.E. McCullough, personal communication), and Virginia (S.D. Askew, personal communication). These resistant buckhorn plantain ecotypes were located in multiple turfgrass systems, including a cemetery, home lawn, university campus, sports field, and golf course. Glyphosate resistance has also been confirmed in buckhorn plantain (Heap, 2021).

Few options exist for herbicides that provide excellent postemergence buckhorn plantain control in turf, especially those labeled for use in cool-season home lawns (Patton and Elmore, 2021). Most herbicides that provide excellent postemergence control of buckhorn plantain in turf contain 2,4-D (Patton and Elmore, 2021), and the herbicides offering excellent control that do not contain 2,4-D are more expensive (Patton et al., 2018), not labeled for home lawns, and/or not labeled for cool-season turf (Patton and Elmore, 2021). As a likely result, 2,4-D was routinely applied in lieu of herbicide rotation, selecting for herbicide resistant individuals and eventually ecotypes (i.e., populations). Although the incidence of weed resistance is reportedly lower for synthetic auxins than other herbicide modes of action (Busi et al., 2017), the recent identification of numerous 2,4-D resistant buckhorn plantain ecotypes is alarming. Furthermore, the release and adoption of 2,4-D tolerant (Enlist™) soybean (*Glycine max*) is predicted to increase 2,4-D use (Peterson et al., 2016). Thus, the investigating 2,4-D resistance in buckhorn plantain is justified.

Given the significance of auxin in plant growth and development, a mutation conferring resistance to a synthetic auxin herbicide may cause a fitness penalty associated with plant morphology and/or reproduction. Further, identifying a fitness penalty associated with 2,4-D resistance may provide insight into the mechanism of resistance as well as effective cultural control practices. As such, the objectives of this research were to 1) determine the 2,4-D sensitivity of 13 buckhorn plantain ecotypes in a bioassay greenhouse trial and 2) evaluate and characterize the morphological and reproductive variation among 12 populations of buckhorn plantain to determine similarity or dissimilarity among 2,4-D resistant and susceptible populations.

### **4.3 Materials and Methods**

This experiment consisted of two parts: a greenhouse bioassay to screen for 2,4-D resistance as well as a garden study to monitor for fitness penalties. Cases of failure to control buckhorn plantain reported to Dr. Aaron Patton's Turfgrass Extension program were preliminarily investigated with a site visit, whole plant collection, and initial screening. Plants were then colonially propagated and maintained in the greenhouse for additional research. In the cases of failure to control buckhorn plantain, there were 20+ yr of previous herbicide applications, the populations were stable, and there was no obvious segregation between plants (within population) for their level of 2,4-D resistance.

To improve geographic diversity and capture a range of susceptible buckhorn plantain characteristics for this experiment, populations from other regions were collected from low maintenance turf areas with no known previous herbicide applications. Collected plants were in close proximity to one another and largely isolated from others. Thus, given the local adaption of these populations, especially in the case of 2,4-D resistance, these populations meet the definition of an ecotype and will be referred to as such going forward. Buckhorn plantain from 13 distinct ecotypes (Table 4.1) were clonally propagated, transplanted into a sandy loam soil, and allowed to establish in the greenhouse in preparation for the bioassay trial and the garden study.

Table 4.1. Buckhorn plantain ecotypes used in this experiment.

<b>Ecotype</b>	<b>Origin</b>	<b>GPS</b>	<b>Site description</b>
GA	Griffin, GA	33.262°N, 84.292°W	Commercial turf
IL	Urbana, IL	40.102°N, 88.223°W	University grounds turf
IN-Mun1**	Muncie, IN	40.210°N, 85.412°W	University grounds turf
IN-Mun2**	Muncie, IN	40.208°N, 85.412°W	Landscape bed
IN-Dec**	Decatur, IN	40.918°N, 84.997°W	Residential turf
IN-GW**	Greenwood, IN	39.616°N, 86.118°W	Cemetery
IN-Laf	Lafayette, IN	40.420°N, 86.873°W	Low maintenance turf
IN-Spd**	Speedway, IN	39.801°N, 86.256°W	High school grounds turf
IN-WL	West Lafayette, IN	40.416°N, 86.942°W	Park turf (Murdock Park)
MI	East Lansing, MI	42.710°N, 84.478°W	Low maintenance turf
MO	Columbia, MO	38.909°N, 92.282°W	Low maintenance turf
OH***	Convoy, OH	40.931°N, 84.702°W	Cemetery
TN	Knoxville, TN	35.902°N, 83.957°W	Low maintenance turf

\* The OH ecotype was not included in the garden study, only the bioassay.

\*\* Collected following a report of potential resistance.

#### **4.3.1 Bioassay**

Research was conducted in the Purdue University Horticulture Plant Growth Facility in West Lafayette, IN (40.421°N, 86.914°W) to determine the 2,4-D dimethylamine sensitivity of 13 buckhorn plantain ecotypes. Live plants were harvested from 13 sites (Table 4.1) and maintained in the greenhouse until individual rosettes were vegetatively propagated for experimental use in 7.6 cm square pots containing a sandy loam soil. Plants were irrigated with municipal tap water as needed and fertigated biweekly with two water-soluble fertilizers (3:1 mixture of 15-2.2-12.5 and 21-2.2-16.6 [N-P-K], respectively; The Scotts Co., Marysville, OH 43040) to provide the following (in mg L<sup>-1</sup>): 200 N, 26 P, 163 K, 50 Ca, 20 Mg, 1.0 Fe, 0.5 each Mn and Zn, 0.24 each Cu and B, and 0.1 Mo. Seventy-six percent of the nitrogen provided was in the nitrate form.

The first experimental run was initiated on 19 Dec. 2018 and the second on 31 Oct. 2019. The experimental design was a randomized complete block consisting of three blocks and a 13×4 factorial arrangement. Experimental factors included 13 ecotypes (Table 4.1) and four 2,4-D rates: 0, 1.68, 8.4, and 16.8 kg ae ha<sup>-1</sup>. In the first experimental run, the 16.8 kg ae ha<sup>-1</sup> 2,4-D rate seemed to be a discriminating dose in which susceptible plants were fully controlled and resistant plants survived. Thus, in the second run of the experiment, 2,4-D rates of 33.6 and 50.4 kg ae ha<sup>-1</sup> were added for the IN-BSU1, IN-Dec, IN-GW, IN-SHS, IN-WL, and OH ecotypes to determine if there were any differences in levels of resistance between the resistant ecotypes.

Day/night temperatures were 26/21 C with a 14 h photoperiod in the first experimental run and 29/23 C with a 16 h photoperiod in the second. Treatments were applied using compressed air in a track spray chamber (Generation III Research Sprayer; Devries Manufacturing, Hollandale, MN 56045) calibrated to deliver 815 L ha<sup>-1</sup> using a TeeJet® 8004EVS nozzle (TeeJet Technologies; Spraying Systems Co., Wheaton, IL 56045) at 275 kPA. Plant diameters were approximately 20 cm at application, and plants were not irrigated for 24 h after spray application to allow ample time for herbicide absorption.

Plant epinasty and control were visually assessed 0.5, 1, 2, 3, 4, 5, and 6 wk after application (WAA) on a 0% to 100% scale. For epinasty, 0% was no epinasty and 100% represented complete epinasty, with all leaves exhibiting symptoms including twisting, bending, and curling of leaves. For control, 0% was no control and 100% represented complete plant death. Digital images were taken of the plants at 0, 0.5, 1, 2, 3, 4, 5, 6, and 10 WAA using a camera and

light box and analyzed for percentage green cover using ImageJ (version 1.53a; National Institutes of Health, Bethesda, MD 20892) (Schneider et al., 2012) as described by Patton et al. (2018).

At 6 WAA, aboveground plant tissue was harvested and fresh weights were measured. Plant tissues were then placed into a forced-air dryer at 60 C for 3 d before dry weights were measured. After harvest, plants were allowed to re-grow for another 4 wk, and aboveground plant tissue was harvested for a second time (10 WAA). Fresh and dry weights were measured as before.

Data were analyzed using PROC GLIMMIX in SAS (version 9.4; SAS Institute Inc., Cary, NC 27513). When a significant ecotype by 2,4-D rate interaction existed ( $P \leq 0.05$ ), data were subject to nonlinear (sigmoidal) regression and analysis using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA 92037).

#### **4.3.2 Garden study**

Individual rosettes from 12 ecotypes (Table 4.1) were vegetatively propagated in 10.2 cm square pots containing a sandy loam soil and given 6 wk to establish in the greenhouse. Twelve healthy, uniform plants were selected for each ecotype.

Field research was conducted at the William H. Daniel Turfgrass Research and Diagnostic Center in West Lafayette, IN (40.442°N, 86.9295°W). The two runs were completed in separate but nearby areas. Approximately 14 d prior to planting, the experimental areas were sprayed with a glyphosate + diquat dibromide premix (Roundup QuikPRO®) at a rate of 0.54 + 0.03 kg ae ha<sup>-1</sup>, respectively. The areas were then tilled to an approximate 10.2 cm depth then raked flat and clean of debris. The first run of the garden study was planted 29 Sept. 2017, and plants were installed on a 0.6×0.6 m spacing. The second run was planted on 3 Oct. 2018, and those plants were installed on a 0.6×0.9 m spacing. Twelve individual plants from 12 distinct ecotypes of buckhorn plantain (Table 4.1) were arranged as a Latin square.

Plants were allowed to establish prior to winter, and data collection began the following spring. Once buckhorn plantain growth began in the spring, winter survival was rated on a 0 and 1 scale, with 0 = dead and 1 = alive. Dead plants were marked with orange survey marking whiskers, and no further data was collected for them. Beginning on 1-May each year, prior to inflorescence initiation, anthered inflorescences were counted every 2 d to note the first day of flowering for each plant. Thus, 1-May was set “day one” in terms of days until flowering. If a plant had not flowered by 30-June, it was input as missing data.



In mid-July each year, maximum leaf length and maximum leaf width were measured with a ruler; total green leaf number was counted. In late July, total anthered inflorescences were counted, the longest scape for each plant was measured, and the spike length from the aforementioned scape was also measured. Fecundity potential was then calculated (spike length  $\times$  number).

Data were analyzed using PROC GLIMMIX in SAS (version 9.4; SAS Institute Inc., Cary, NC 27513) to determine if ecotypes differed for the individual morphological and reproductive features measured. Data were pooled across both runs. Principal component analysis was then performed using GraphPad Prism (version 9.0.1; GraphPad Software, Inc., La Jolla, CA 92037). Fecundity was not included in the analysis, due to its relatedness to spike length and total inflorescences. Data were standardized (i.e., scaled to have a mean of 0 and a standard deviation of 1). Principal components were selected to explain at least 75% of the total variance.

## 4.4 Results

### 4.4.1 Bioassay

There was an ecotype by 2,4-D rate interaction for control 6 WAA ( $P < 0.0001$ ), fresh weight 6 WAA ( $P < 0.0001$ ), dry weight 6 WAA ( $P < 0.0001$ ), and dry weight 10 WAA ( $P < 0.0001$ ). Across all of these instances, a higher dose of 2,4-D was required to control or reduce growth for the IN-Dec, IN-Mun1, IN-GW, IN-Spd, and OH ecotypes than all other ecotypes. These differences between the ecotypes were best illustrated by regrowth dry weight 10 WAA (Figure 4.1). The  $GR_{50}$  values (i.e., the 2,4-D dose that reduced dry weight by 50 percent), calculated from the regrowth dry weight 10 WAA, is presented on Table 4.2 for all 13 ecotypes used in the bioassay. Eight populations were found to be susceptible, with a  $GR_{50}$  of  $\leq 1.5$  kg ae ha<sup>-1</sup>, which is less than the maximum allowable 2,4-D application rate in turf of 1.68 kg ae ha<sup>-1</sup>.

In the ancillary study that included higher 2,4-D rates, the ecotype by 2,4-D rate interaction was significant for control 6 WAA ( $P < 0.0001$ ), fresh weight 6 WAA ( $P = 0.0035$ ), and dry weight 10 WAA ( $P < 0.0001$ ). Similarly, the IN-Dec, IN-Mun1, IN-GW, IN-Spd, and OH ecotypes required a higher dose of 2,4-D to control or reduce growth compared to the IN-WL ecotype (Figure 4.2). Five populations had  $GR_{50}$  values  $\geq 11.8$  kg ae ha<sup>-1</sup> and R/S ratio of 7.9-25.5 (Table 4.2).

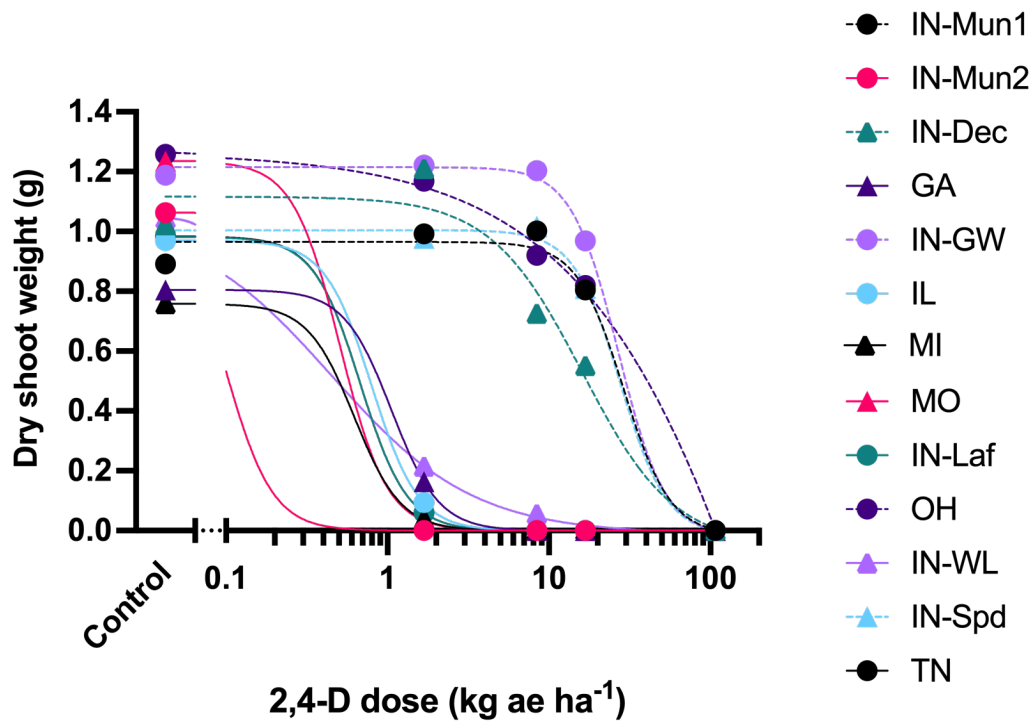


Figure 4.1. Dry shoot regrowth weight 10 WAA for the 13 ecotypes across two runs and three blocks. Data were subject to nonlinear (sigmoidal) regression. Resistant ecotypes are represented by a dashed line.

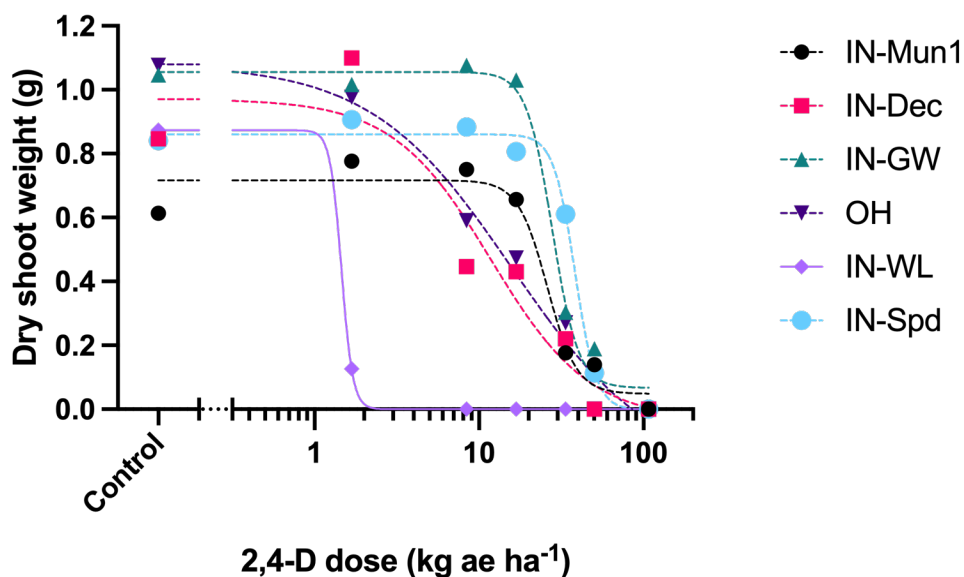


Figure 4.2. Dry shoot regrowth weight 10 WAA for the 6 ecotypes in the ancillary study with higher 2,4-D rates across three blocks. The IN-WL population served as a susceptible check for comparison. The maximum labeled rate for 2,4-D in turf is 1.68 kg ae ha<sup>-1</sup>. Data were subject to nonlinear (sigmoidal) regression. Resistant ecotypes are represented by a dashed line.

Table 4.2. The GR<sub>50</sub> and R/S ratios for the 13 ecotypes studied in this experiment. Values were calculated from dry shoot regrowth rate across both experimental runs, save for the ecotypes included in the ancillary study with higher 2,4-D rates.

<b>Ecotype</b>	<b>GR<sub>50</sub></b>	<b>R/S ratio</b>
GA	1.1	0.7
IL	0.8	0.5
IN-BSU1*	26.0	17.3
IN-BSU2	1.1	0.7
IN-Dec*	11.8	7.9
IN-GW*	27.9	18.6
IN-Laf*	0.7	0.5
IN-SHS*	38.3	25.5
IN-WL	1.5	1.0
MI	0.6	0.4
MO	0.5	0.3
OH*	15.8	10.5
TN	0.7	0.5

\* GR<sub>50</sub> values obtained from ancillary study with higher 2,4-D rates.

#### **4.4.2 Garden study**

Across both years, ecotype was significant for winterkill ( $P = 0.0027$ ), days until anthered inflorescence ( $P < 0.0001$ ), total inflorescences ( $P = 0.0013$ ), longest leaf ( $P < 0.0001$ ), widest leaf ( $P < 0.0001$ ), number of green leaves ( $P < 0.0001$ ), scape length ( $P < 0.0001$ ), spike length ( $P < 0.0001$ ), and fecundity ( $P < 0.0001$ ). Despite these differences, the resistant ecotypes did not all separate from all the susceptible ecotype for any of the phenotypic measures (Table 4.3).

Two principal components were selected that explained 84.8% of the total variance in phenotype. The loadings plot (Figure 4.3) shows a cluster of leaf length, leaf width, total flowers, leaf number, scape length, and spike length, indicating redundancy in these measurements. Days to flower was opposite these measurements, but still largely along the first principal component. Winterkill was the only metric to largely deviate from the first principal component. The resistant ecotypes do not cluster on the principal component scores plot (Figure 4.4). Thus, the resistant ecotypes do not have an apparent, consistent fitness penalty explained by a majority (i.e., 84.8%) of the variance.

Table 4.3. Means and standard deviations of garden study measurements by buckhorn plantain ecotype in West Lafayette, IN across two yr.

Ecotype	Winterkill (%)	Days to flower	Total flowers	Leaf length (cm)	Leaf width (cm)	Total leaves	Scape length (cm)	Spike length (cm)	Fecundity (cm)
GA	25	32 ± 4	131 ± 98	15.3 ± 8.2	1.8 ± 0.6	99 ± 81	28.4 ± 9.3	3.2 ± 2.0	566 ± 707
IL	4	23 ± 7	160 ± 76	19.3 ± 7.3	2.1 ± 0.6	146 ± 67	35.1 ± 9.2	5.3 ± 2.7	974 ± 748
IN-Mun1*	25	27 ± 6	128 ± 57	19.7 ± 6.0	2.2 ± 0.5	140 ± 52	36.3 ± 6.6	4.6 ± 2.4	569 ± 361
IN-Mun2	29	24 ± 8	106 ± 59	14.5 ± 8.0	1.8 ± 0.5	98 ± 59	25.6 ± 8.4	2.4 ± 1.5	328 ± 305
IN-Dec*	13	31 ± 5	162 ± 70	21.5 ± 5.8	2.3 ± 0.5	157 ± 63	36.6 ± 4.3	4.6 ± 2.0	800 ± 640
IN-GW*	4	29 ± 5	122 ± 87	15.5 ± 5.8	1.8 ± 0.5	103 ± 56	28.0 ± 8.6	3.0 ± 2.1	484 ± 815
IN-Laf	4	25 ± 7	170 ± 77	19.6 ± 7.2	2.1 ± 0.6	133 ± 64	34.1 ± 8.0	3.8 ± 2.1	724 ± 569
IN-Spd*	0	28 ± 7	125 ± 49	18.4 ± 5.2	2.1 ± 0.6	113 ± 55	31.1 ± 8.0	3.3 ± 1.4	439 ± 288
IN-WL	21	34 ± 3	98 ± 44	12.3 ± 5.4	1.6 ± 0.4	85 ± 52	27.6 ± 5.1	2.5 ± 1.7	293 ± 256
MI	21	30 ± 7	107 ± 44	13.6 ± 6.1	1.7 ± 0.6	92 ± 55	29.2 ± 7.2	2.6 ± 1.5	296 ± 219
MO	4	28 ± 5	138 ± 55	17.1 ± 7.0	2.0 ± 0.6	125 ± 66	34.1 ± 6.4	3.8 ± 1.5	568 ± 324
TN	29	25 ± 8	176 ± 68	19.9 ± 6.0	2.2 ± 0.6	155 ± 61	40.4 ± 6.7	6.0 ± 2.3	1176 ± 684

\* 2,4-D resistant ecotype.

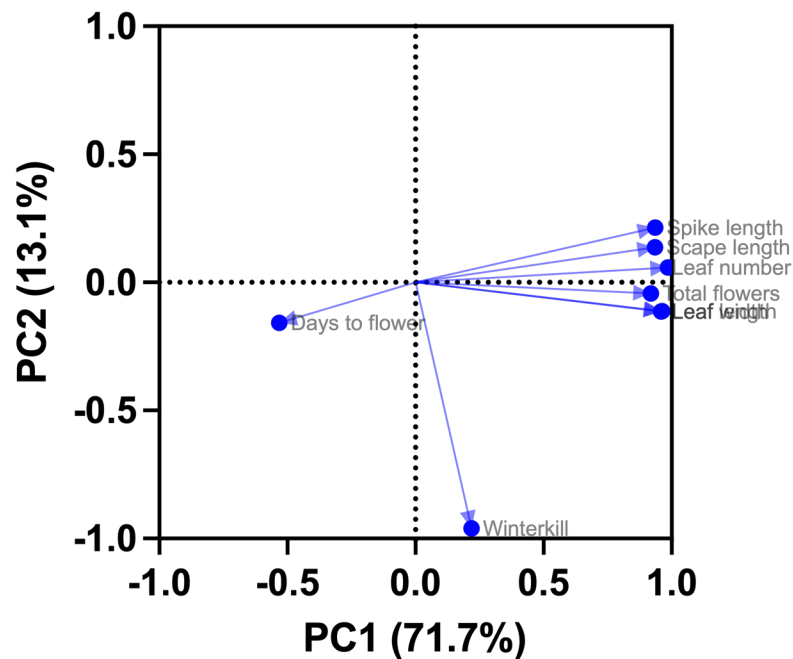


Figure 4.3. Loadings plot from the principal component analysis. The length and direction of the vectors show how much weight they have on each of the principal components. Spike length, scape length, leaf number, total flowers, leaf width, and leaf length are positively correlated with one another, which are all negatively correlated to days to flower. Winterkill is likely not correlated to the other vectors.

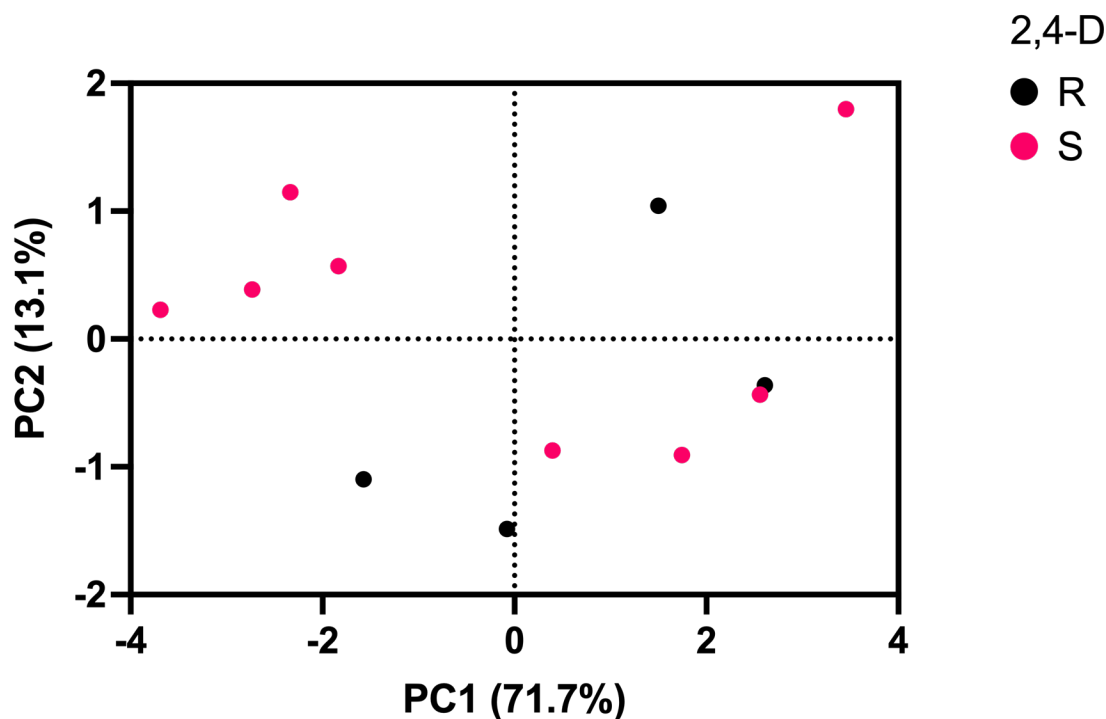


Figure 4.4. Principal component plot from the principal component analysis of plant phenotype. Resistant (R) ecotypes are displayed in black and susceptible (S) ecotypes in red.

#### 4.5 Discussion

This research confirms 2,4-D resistance in four buckhorn plantain ecotypes, in addition to the IN-GW resistant ecotype reported by Patton et al. (2018). Resistant ecotypes originated from university grounds turf, high school grounds turf, residential turf, and two cemeteries. Additionally, 2,4-D resistant buckhorn plantain ecotypes have been identified in Pennsylvania (Russell et al., 2020), Georgia (P.E. McCullough, personal communication), and Virginia (S.D. Askew, personal communication). Russell et al. (2020) reported their resistant ecotype was  $\geq 11.3$  times less sensitive to 2,4-D than the susceptible biotype. The resistant ecotypes in this experiment ranged from 7.9 to 25.5 times less sensitive to 2,4-D than the susceptible biotype. When considering the site age for the resistant ecotype origins in this experiment, IN-Spd is the newest. The facility (i.e., Speedway High School) was built in 1964. Previous management and 2,4-D applications are unknown, but even the youngest site from this research potentially received 50+ yr of 2,4-D applications before resistance was suspected by the site manager.

While the incidence of weed resistance is reportedly lower for synthetic auxins than other herbicide modes of action (Busi et al., 2017), over half of the reported cases of 2,4-D resistance have been identified in the last 20 years (Heap, 2021). In the short time since Patton et al. (2018) confirmed the first 2,4-D resistance case in buckhorn plantain, numerous other resistant ecotypes have been identified. These additional cases of 2,4-D resistance likely arose independently and hint that there may be more. Confirming resistance in additional ecotypes highlights the importance of herbicide resistance research as well as communicating new herbicide resistance issues with turfgrass practitioners.

As for the garden study, no metric measured in the experiment indicated that there was a fitness penalty. Leaf length, leaf width, scape length, and spike length fall within the values outlined by Cavers et al. (1980) and Kuiper and Bos (1992), both in terms of means as well as extremes. Not only were there differences between ecotypes for the garden measurements, but there were also differences within ecotypes. This is evidenced by the high standard deviations relative to the means.

It may be that there is no fitness penalty, the four resistant ecotypes have unique resistance mechanisms and do not share a fitness penalty, or the fitness penalty falls outside of what was measured in this experiment. These results agree with Watanabe et al. (1997), who could not distinguish between 2,4-D resistant and susceptible morphological characteristics in *Fimbristylis miliacea* (L.) Vahl. Goggin et al. (2019) also did not find an auxinic herbicide-resistance cost in wild radish. However, Cousens (2020) argues that experiments cannot prove the lack of an herbicide-resistance fitness penalty.

Radiolabeled research on the IN-GW ecotype indicated that reduced translocation plays a role in the mechanism of resistance (Chapter 2; Law, 2021). The reduced translocation mechanism of glyphosate resistance in *Lolium perenne* L. resulted in reduced plant height and leaf blade area (Yanniccari et al., 2017), both of which were roughly measured in this experiment and did not provide differences between resistant and susceptible biotypes.

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## **CHAPTER 5.     REDUCTION IN *PLANTAGO LANCEOLATA* COVERAGE VIA NITROGEN FERTILIZATION AND CLOSE MOWING**

### **5.1   Abstract**

Mowing height and nitrogen fertilization have both been shown to influence weed populations, and, in general, mowing high and supplementing with N provides both the best turf quality as well as reduces weeds. With confirmation of 2,4-D herbicide resistance in buckhorn plantain (*Plantago lanceolata* L.) and no documented cultural control options for the species, the objective of this 3 yr field trial was to determine the influence of mowing height and nitrogen rate on buckhorn plantain. The experimental design was a randomized complete block design in a 3×4 factorial arrangement (three mowing heights by four nitrogen fertility treatments) with four blocks. Treatments included mowing heights of 5.1, 7.6, and 10.2 cm, and nitrogen application rates of 0, 81, 163, and 244 kg N ha<sup>-1</sup> yr<sup>-1</sup>. Mowing height, nitrogen fertility, and the mowing height by nitrogen fertility interaction were all significant for buckhorn plantain coverage ( $P < 0.0001$ ). Plots mown at 10.2 cm and receiving no supplemental N had the highest buckhorn plantain coverage (20%), followed by the 7.6 cm mowing height with no supplemental N (16%). Conversely, the plots mown at 5.1 cm and 7.6 cm and receiving 244 kg N ha<sup>-1</sup> yr<sup>-1</sup> were amongst the lowest buckhorn plantain coverages in the experiment (7 and 8%, respectively). Based on these findings and to prevent encroachment of other problematic weeds at low mowing heights (i.e., annual bluegrass and crabgrass), increasing nitrogen fertility and mowing at or above 7.6 cm is the recommended cultural approach for reducing buckhorn plantain in managed turf. This research contributes additional evidence to the idea that healthy turf is the best strategy for combating weeds.

## 5.2 Introduction

Recently, the first report of 2,4-D resistance in buckhorn plantain as well as the first report of 2,4-D resistance in turf was published (Patton et al., 2018). Additional 2,4-D resistant buckhorn plantain ecotypes have been reported in Indiana (Law, 2021), Ohio (Law, 2021), Pennsylvania (Russell et al., 2020), Georgia (P.E. McCullough, personal communication), and Virginia (S.D. Askew, personal communication) in the short time since. These resistant buckhorn plantain ecotypes have originated from multiple turfgrass systems, including cemeteries, home lawns, a university campus, sports fields, and golf courses. Glyphosate resistance has also been confirmed in buckhorn plantain from a vineyard in South Africa (Heap, 2021).

Herbicide pre-mixtures containing the synthetic auxin herbicides 2,4-D, mecoprop, and dicamba are commonly used in residential turf to control common weeds, including dandelion (*Taraxacum officinale* F.H. Wigg.), white clover (*Trifolium repens* L.), and *Plantago major* L. (Neal, 1990). Most herbicide premixtures that provide excellent postemergence control of buckhorn plantain in turf contain 2,4-D (Patton and Elmore, 2021), and the herbicides offering excellent control that do not contain 2,4-D are more expensive (Patton et al., 2018), not labeled for residential turf, not labeled for cool-season turf (Patton and Elmore, 2021) and/or not available until recently (i.e., halauxifen-methyl). As a likely result, 2,4-D was routinely applied in lieu of herbicide rotation, selecting for herbicide resistant individuals and eventually ecotypes (i.e., populations). Although the incidence of weed resistance is reportedly lower for synthetic auxins than other herbicide modes of action (Busi et al., 2017), the recent identification of numerous 2,4-D resistant buckhorn plantain ecotypes is concerning. Furthermore, the release and adoption of 2,4-D tolerant (Enlist™) soybean [*Glycine max* (L.) Merr.] is predicted to increase 2,4-D use (Peterson et al., 2016).

With so few postemergence chemical control options for buckhorn plantain, particularly in the case of a 2,4-D resistant ecotype in cool-season residential turf, it is important to investigate alternative, nonchemical control methods. While no specific cultural or biological control methods of buckhorn plantain have been identified to date, maintaining a competitive stand of turf is one of the best defenses against weed encroachment (Christians et al., 2016). Mowing height and nitrogen fertility are two turfgrass management practices that have been shown to influence the presence and number of weeds (Busey, 2003).

Mowing height, frequency, and grass clippings management can all affect weed populations; most markedly, though, is mowing height (Busey, 2003). Low mowing heights are associated with more weeds in turf (Busey, 2003). However, many of the mowing height experiments have focused on crabgrass (*Digitaria* spp.). Broadleaf weeds [i.e. mockstrawberry [*Duchesnea indica* (Andrews) Teschem.] and common blue violet (*Viola sororia* Willd.)] can also be reduced with a higher mowing height (Gray and Call, 1993), though mowing height research with broadleaf weeds is limited. It is important to note that frequent, low mowing favors genetically shorter ecotypes (Warwick and Briggs, 1978, 1979).

In general, nitrogen fertilization reduces weeds in turf (Busey, 2003). Nitrogen is often the growth-limiting nutrient for turf (Christians et al., 2016), and stimulating turfgrass growth via nitrogen fertilization increases density, helps the turf to fill in voids in the canopy, and improves its competitiveness with encroaching weeds. Similar to mowing height, most of the nitrogen-related research has focused on crabgrass (Busey, 2003). However, nitrogen fertilization reduces the coverage of dandelion (Callahan and Overton, 1978), white clover (Templeton and Taylor, 1966), and ground ivy (*Glechoma hederacea* L.)(Kohler et al., 2004).

The Park Grass Experiment at the Rothamsted Experiment Station outside of London, England highlights the importance and demonstrates the effects of long-term fertilization practices: annual spring applications of 49 kg N ha<sup>-1</sup> as ammonium sulfate each year since 1856 has resulted in almost full turfgrass coverage (Woods and Rossi, 2011). However, the regular application of crushed limestone, phosphorus, or potassium increased broadleaf species abundance (Woods and Rossi, 2011). For example, potassium fertilization increased dandelion abundance, and potassium plus crushed limestone further increased dandelion abundance (Woods and Rossi, 2011).

A plant's biology and preferred habitat can lend clues as to which cultural practices may provide control (Bell, 2011), and exploitation of weed biology through cultural practices makes it possible to shift plant ecology in the desired direction. Buckhorn plantain is a cool-season perennial, seldom annual or biennial, broadleaf that is a common weed in turfgrass systems (Christians et al., 2016) as well as in hayfields, pastures, farmyards, waste places, and roadsides (Cavers et al., 1980). Buckhorn plantain is a noxious, invasive, or restricted weed species in many U.S. states. The species originates from Eurasia (Cravers et al., 1980), but it has since established throughout the world save for subarctic and low-lying tropical areas (Tessene, 1968). Buckhorn

plantain forms and grows from a central, basal rosette. Long, silky hairs are often present at the growing point. There is no visible aboveground stem, but a semi-woody caudex and/or undeveloped taproot is often present. Plants develop a few deep roots (1 m or more) as well as a mass of short (5-10 cm) fibrous roots near the soil surface (Sagar and Harper, 1964).

There are several ecological traits of buckhorn plantain that contribute to its successful colonization of low maintenance turf. First, the species is able to germinate across an array of environmental conditions. Not only can buckhorn plantain germinate without light, light does not favor germination (Kuiper and Bos, 1992). Thus, buckhorn plantain is able to germinate when buried and can establish in tall, dense layers of vegetation (Blom, 1978). However, seedling emergence is inhibited by burial depths of 6 cm or more (Benvenuti et al., 2001). Fresh buckhorn plantain seeds have a lower germination capacity than 1- to 5-year-old seeds, though viability can remain strong for six or more years (Kuiper and Bos, 1992).

Compared to other *Plantago* species (i.e., *P. coronopus*, *P. major*, and *P. media*), buckhorn plantain has the widest ecological amplitude (Mook et al., 1992). Buckhorn plantain tolerates adverse growing conditions, including compaction, low fertility (Hoveland et al., 1976), a wide range of soil pH values (Buchanan et al., 1975), and an adaptive lead tolerance that is transmitted to seed progeny (Wu and Antonovics, 1976). Fertilization can have mixed effects on buckhorn plantain populations. Fertilization reduced buckhorn plantain biomass in undisturbed vegetation but increased biomass in a buckhorn plantain monoculture (Berendse, 1983).

Morphological differences between buckhorn plantain ecotypes can vary based on habitat and management. Left unmown, buckhorn plantain grows long, erect leaves and appears shrub-like. As mowing height decreases and frequency increases, smaller, more prostrate leaves and plants result. Warwick and Briggs (1979) measured field samples of various ecotypes and found that leaf length, leaf width, scape length, and spike length were shorter/narrower for buckhorn plantain collected from a lawn compared to roadside and waste areas. However, when plants from these same ecotypes were cultivated, the between-habitat differences disappeared (Warwick and Briggs, 1979).

To help combat herbicide resistance, cultural control methods must be utilized (Harker and O'Donovan, 2013). Thus, the objective of this experiment is to determine the influence of mowing height and nitrogen fertility on the persistence of buckhorn plantain in Kentucky bluegrass (*Poa pratensis* L.) turf.

### 5.3 Materials and Methods

Research was conducted at the William H. Daniel Turfgrass Research and Diagnostic Center in West Lafayette, IN (40.442°N, 86.929°W). Buckhorn plantain seed was collected locally from a nearby field in the summer of 2014, propagated in the greenhouse, and then transplanted in the field at a density of 4.4 plants m<sup>-2</sup> the following spring in a 0.5 ha sward of Kentucky bluegrass. Following transplanting, plants were irrigated to ensure establishment. The experimental area was maintained similar to a residential lawn with weekly mowing at 5.1 cm with a ride-on rotary mower, except for an 8 wk period in August and September of 2015 when mowing was withheld to encourage seed production and additional buckhorn plantain recruitment. The area was not fertilized with N for at least five years previously. After an additional year of weekly mowing at 5.1 cm, the mowing and fertilization experiment described here was initiated in October 2016.

The soil type was a Starks silt loam (fine-silty mixed, superactive mesic Aeric Endoaqualf) with a pH of 6.7, 276 kg P ha<sup>-1</sup>, 728 kg K ha<sup>-1</sup>, and 39 g kg<sup>-1</sup> organic matter. In an effort to control white clover and remove its N fixation potential, an application of on quinclorac (Quinclorac 75 DF; Makhteshim Agan of North America, Inc., Raleigh, NC) was made at a rate of 0.84 kg ae ha<sup>-1</sup> on 29 Aug. 2016, with minor injury to buckhorn plantain. No other pesticides were applied during the course of the experiment.

Plots were 1×5 m in size, and blocks were laid to uniformly encompass buckhorn plantain. The experimental design was a randomized complete block design (RCBD) in a 3×4 factorial arrangement (three mowing heights by four nitrogen fertility treatments) with four blocks. Treatments included mowing heights of 5.1, 7.6, and 10.2 cm, and nitrogen application rates of 0, 81, 163, and 244 kg N ha<sup>-1</sup> yr<sup>-1</sup>. Plots were mown using a rotary mower (Troy-Bilt TriAction 53-cm wide deck walk-behind push mower, Modern Tool and Die Company, Cleveland, OH) with clippings returned. Mowing height treatments began in October 2016, and plots were mown approximately once or twice per week during the growing season. While the plots receiving low mowing and/or high nitrogen generally dictated when mowing occurred, all plots were mown for every mowing event. Nitrogen fertility treatments began in May 2017 and were comprised of three applications per year (May, September, and November) at one-third of the total annual rate. Polymer coated sulfur coated urea (27.95% slow release nitrogen) was applied in May and September, and urea (0% slow release nitrogen) was applied in November. Plots were mown and fertilized for three full growing seasons (i.e., through 2019).

Buckhorn plantain coverage, buckhorn plantain transects, turf quality, and turf coverage were collected monthly during each growing season (April-November). Buckhorn plantain coverage and turf coverage were visually determined. Transects were counted using a 0.75×1.5 m grid laid across each plot three times, which encompassed a 0.75×4.5 m area in the center of each plot with 135 total transects. Transect data is reported as the percent of transects covering a buckhorn plantain leaf or rosette. Turf quality was visually rated on a 1-9 scale, with 9 = ideal turf quality, 6 = minimum acceptable quality, and 1 = completely brown or dead turf (Morris and Sherman, 1998). The number of dandelion plants within each plot were also counted on 3 June 2019 after potential treatment differences were observed. Crabgrass coverage was visually rated on 22 July 2019.

Prior to winter leaf desiccation, three plants were randomly selected to measure plant diameter and leaf biomass on 26 Nov. 2019. Plant diameter (cm) was measured at the longest point, from leaf tip to leaf tip, for each plant. All green leaves from the same three plants in each plot were harvested and pooled. Leaves were clipped at the base of the petiole, just above the caudex. Harvested tissues were weighed, dried at 60 C for 48 h, and weighed again. Fresh and dry weights were recorded.

All data were analyzed using PROC GLIMMIX in SAS software (SAS Institute, Cary, NC). Means were separated using Tukey's honest significant difference test ( $\alpha = 0.05$ ). Buckhorn plantain coverage, buckhorn plantain transects, turf quality, turf coverage, and weed species richness were analyzed as repeated measures. Block was analyzed as a random variable for all analyses. GraphPad Prism (GraphPad Software, Inc., La Jolla, CA 92037) was used to display the interaction of treatments.

## 5.4 Results

Mowing height, nitrogen fertility, and the mowing height by nitrogen fertility interaction were all significant for visual buckhorn plantain coverage over time ( $P < 0.0001$ ). There was a total of 26 rating dates, which were also significant for visual buckhorn plantain coverage ( $P < 0.0001$ ). However, rating date did not interact with either nitrogen rate ( $P = 0.9965$ ) or nitrogen fertility ( $P = 0.4179$ ). The full interaction, mowing height by nitrogen fertility by rating date, was not significant ( $P = 0.9999$ ). For the main effects mowing height and nitrogen fertility, all

treatments were different from one another. Mowing at 10.2 cm resulted in the highest buckhorn plantain coverage (14%), followed by the 7.6 cm mowing height (11%), and the 5.1 cm mowing height had the lowest coverage (9%). For fertility, the non-treated plots had the highest buckhorn plantain coverage (16%), followed by 81 kg N ha<sup>-1</sup> yr<sup>-1</sup> (11%), followed by 163 kg N ha<sup>-1</sup> yr<sup>-1</sup> (10%), and 244 kg N ha<sup>-1</sup> yr<sup>-1</sup> had the lowest coverage (9%).

The results for the mowing height by nitrogen fertility interaction are shown in Figure 5.1. Plots mown at 10.2 cm and receiving no supplemental N had the highest buckhorn plantain coverage (20%), followed by the 7.6 cm mowing height with no supplemental N (16%). Conversely, the plots mown at 5.1 cm and 7.6 cm and receiving 244 kg N ha<sup>-1</sup> yr<sup>-1</sup> were amongst the lowest buckhorn plantain coverages in the experiment (7 and 8%, respectively). The graph of the interaction between mowing height and nitrogen rate largely echoes the main effects: low mowing and high nitrogen fertility reduces buckhorn plantain coverage compared to high mowing and low nitrogen fertility. Another way to visualize this data is presented in Figure 5.2. This heat map illustrates decreasing buckhorn plantain coverage with increasing N fertilization and lower mowing. Further, the 163 and 244 kg N ha<sup>-1</sup> yr<sup>-1</sup> fertility treatments were “green” across all mowing heights, indicating that N fertility is likely the best cultural control option, especially when the negative effects are considered.

Buckhorn plantain transect results were similar to visual coverage estimates. Mowing height ( $P < 0.0001$ ), nitrogen rate ( $P < 0.0001$ ), and the mowing height by nitrogen rate interaction ( $P < 0.0001$ ) were all significant. All trends were the same as coverage: low mowing reduced buckhorn plantain coverage, as did nitrogen fertilization. Plots mown at 10.2 cm and receiving no nitrogen had the highest coverage (27%), and plots mown at 5.1 cm and receiving 244 kg N ha<sup>-1</sup> had the lowest coverage (10%).



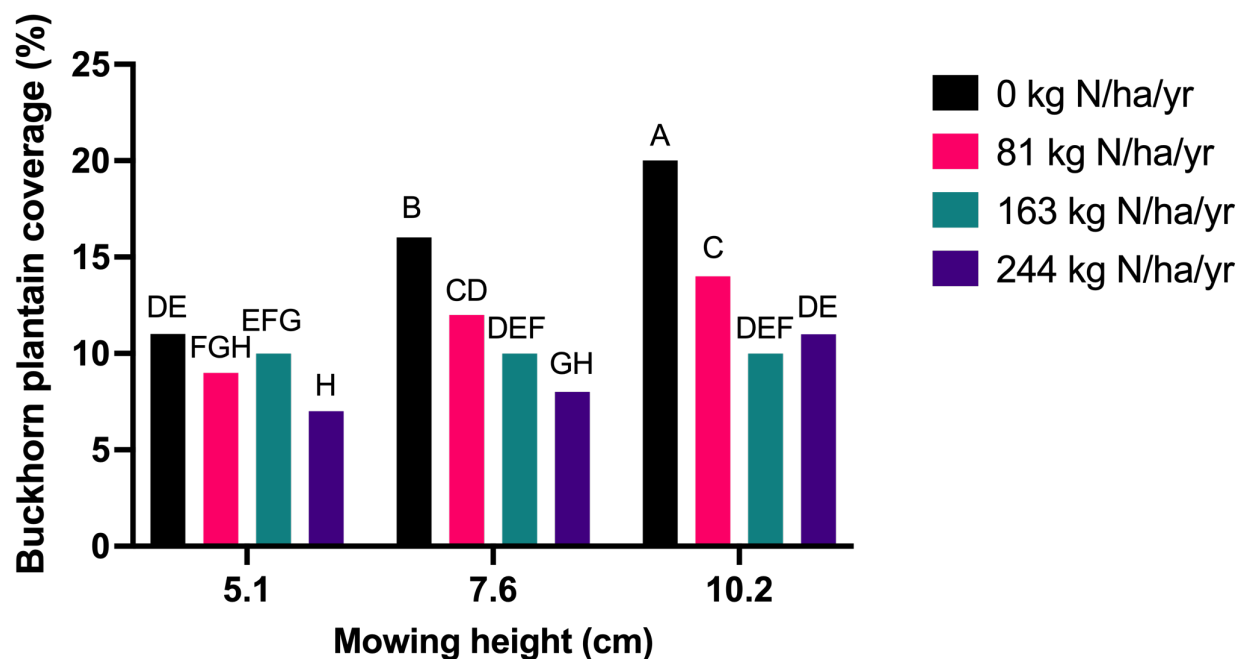


Figure 5.1. The interaction between mowing height and nitrogen rate for visual buckhorn plantain coverage in Kentucky bluegrass (*Poa pratensis* L.) turf. Each bar represents the mean of 4 blocks across 26 rating dates over 3 yr (n = 104). Means followed by the same letter are not significantly different according to Tukey's honest significant difference test ( $\alpha = 0.05$ ).

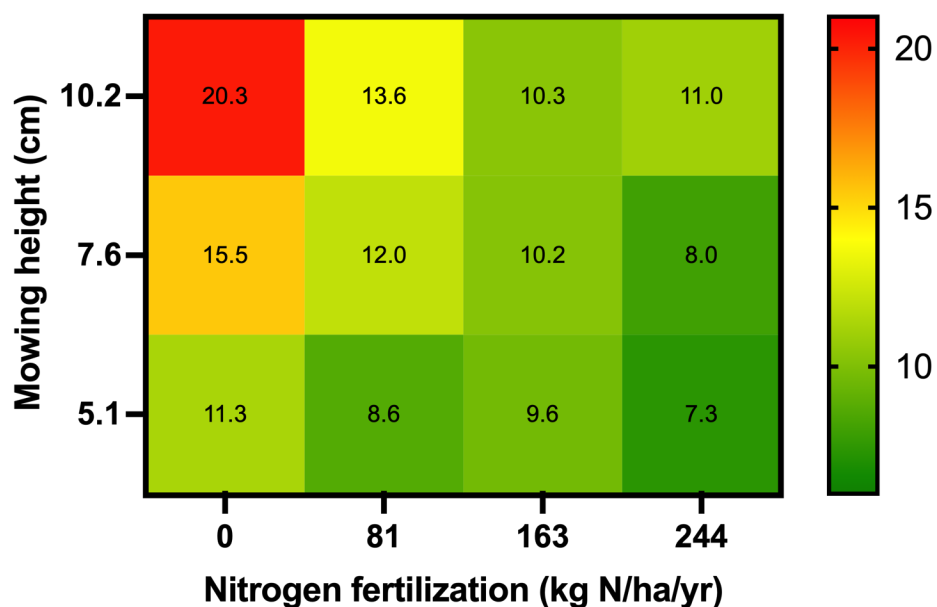


Figure 5.2. The interaction between mowing height and nitrogen rate for visual buckhorn plantain coverage in Kentucky bluegrass (*Poa pratensis* L.) turf. Each bar represents the mean of 4 blocks across 26 rating dates over 3 yr (n = 104).

After 3 yr of mowing and fertilization treatments (i.e., 26 Nov. 2019), mowing height was significant for plant diameter ( $P < 0.0001$ ), leaf biomass fresh weight ( $P < 0.0001$ ), and leaf biomass dry weight ( $P < 0.0001$ ). For all three metrics, the 10.2 cm mowing height had the largest plants (8.4 cm, 34.6 g, and 6.4 g, respectively), the 7.6 cm mowing height had intermediate plant sizes (7.1 cm, 22.5 g, and 4.1 g, respectively), and the 5.1 cm mowing height had the smallest plants (5.5 cm, 14.0 g, and 2.4 g, respectively). However, none of these metrics were significant for N fertility ( $P > 0.05$ ). There was an interaction between mowing height and N fertility for leaf biomass fresh weight ( $P = 0.0256$ ). However, the  $F$ -value of mowing height ( $F = 37.54$ ) was much higher than the interaction ( $F = 2.81$ ), and the overall trend was the same (data not shown).

Turf quality was significant for mowing height ( $P < 0.0001$ ), nitrogen rate ( $P < 0.0001$ ), and the mowing height by nitrogen rate interaction ( $P < 0.0001$ ). As expected, higher mowing and nitrogen fertilization both improved turf quality (Figure 5.3). High mowing along with nitrogen fertilization maintained acceptable turf quality, whereas low mowing without nitrogen was unacceptable.

The coverage of other common weeds in the plots was also assessed. Dandelion counts on 3 June 2019 were significant for both mowing height ( $P < 0.0001$ ) and N fertility ( $P < 0.0001$ ), but not for the interaction ( $P = 0.7511$ ). Plots mown at 7.6 and 10.2 cm had fewer dandelion plants than those mown at 5.1 cm (38 and 27 vs. 58 plants  $\text{m}^{-2}$ , respectively). Plots receiving more than 163 kg N  $\text{ha}^{-1}$   $\text{yr}^{-1}$  for 3 yr had fewer dandelion plants than the non-treated plots ( $\leq 6$  vs. 13 plants  $\text{m}^{-2}$ ). Crabgrass coverage was significant for mowing height ( $P < 0.0001$ ), nitrogen fertility ( $P < 0.0001$ ), and the mowing height by nitrogen rate interaction ( $P = 0.0031$ ). Mowing height had a much higher  $F$ -value (100.49) than nitrogen rate (27.22), indicating that it played a larger role in crabgrass coverage. Higher mowing and nitrogen fertilization reduced crabgrass coverage (Figure 5.4).

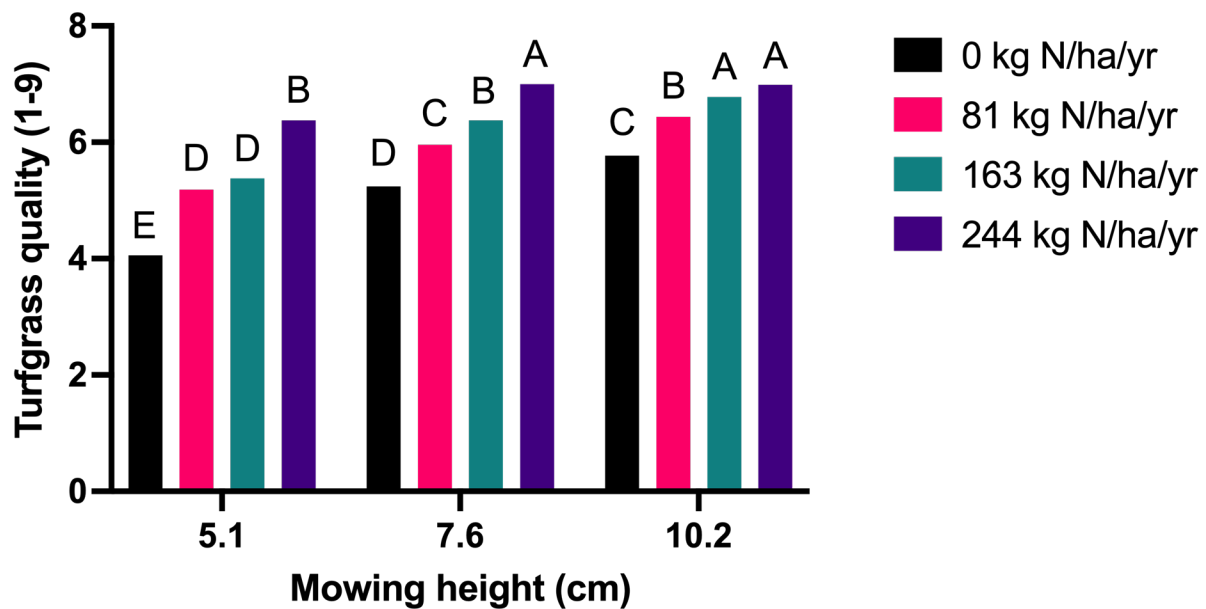


Figure 5.3. The interaction between mowing height and nitrogen rate for Kentucky bluegrass (*Poa pratensis* L.) turf quality. Each bar represents the mean of 4 blocks across 26 rating dates over 3 yr (n = 104). Means followed by the same letter are not significantly different according to Tukey's honest significant difference test ( $\alpha = 0.05$ ).

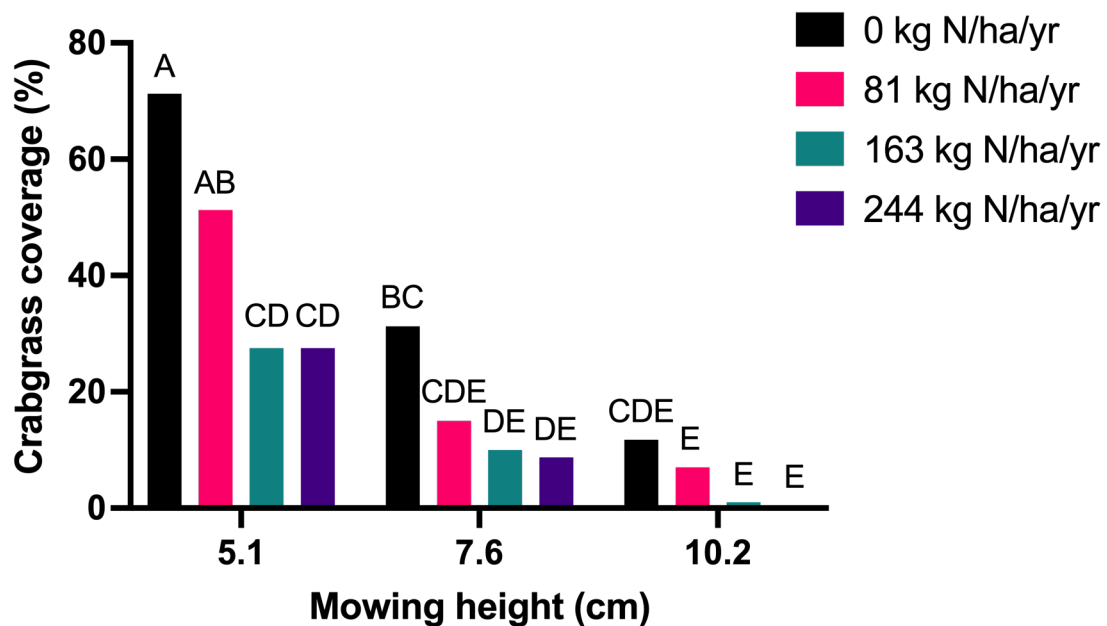


Figure 5.4. The interaction between mowing height and nitrogen rate for crabgrass (*Digitaria* spp.) coverage (%) on 22 July 2019, after 3 yr of mowing and fertilization treatments. Means followed by the same letter are not significantly different according to Tukey's honest significant difference test ( $\alpha = 0.05$ ).

## 5.5 Discussion

Similar to most other turf weeds, nitrogen fertilization reduced buckhorn plantain in Kentucky bluegrass turf when compared with a non-treated control. While high N rates reduced buckhorn plantain coverage in less time (data not shown), all N fertilization treatments (81, 163, and 244 kg ha<sup>-1</sup> yr<sup>-1</sup>) reduced buckhorn plantain coverage after three growing seasons. This is akin to Berendse (1983), who reported a reduction in buckhorn plantain in undisturbed vegetation after nitrogen was applied as part of a compound fertilizer over two years. Research has also demonstrated that higher rates of nitrogen fertilization reduce dandelion (Callahan and Overton, 1978) white clover (Templeton and Taylor, 1966), ground ivy (Kohler et al., 2004), and broadleaf weed populations (Haley et al., 1985; Voigt et al., 2001) in turf. Further, Dernoeden et al. (1993) and Dunn et al. (1981) saw a similar decrease in crabgrass coverage with increased mowing height and nitrogen fertility.

This is not to say that nitrogen fertilization controls buckhorn plantain, as previous research indicates otherwise. In a buckhorn plantain monoculture, fertilization actually increased yield (Berendse, 1983). Buckhorn plantain is oftentimes an indicator of low fertility soils (Patton et al., 2021) and can tolerate low fertility (Hoveland et al., 1976) as well as a wide range of soil pH values (Buchanan et al., 1975). More likely, nitrogen fertilization shifted the competitive advantage towards the Kentucky bluegrass. The Park Grass Experiment at the Rothamsted Experiment Station outside of London, England highlights the importance and demonstrates the effects of long-term nitrogen fertilization in turf: annual spring applications of 49 kg N ha<sup>-1</sup> as ammonium sulfate each year since 1856 has resulted in almost full turfgrass coverage (Woods and Rossi, 2011).

Unlike most other turf weeds, low mowing (i.e., 5.1 cm) was found to reduce buckhorn plantain coverage in Kentucky bluegrass turf over the course of this experiment (3 yr). Buckhorn plantain grows from a rosette, which enables it to tolerate defoliation. However, this research demonstrates that there may be a limit to how low it can be mown. While this finding may be unique, Patton et al. (2021) does not list buckhorn plantain as an indicator weed of low mowing, nor does Brosnan (2015) address the species as a management challenge on golf course putting greens, tees, or fairways (i.e., closely-mown turf). The reduced buckhorn plantain coverage at low mowing heights may also be due in part to the smaller plant size in these plots as measured in this experiment.

We caution the use of low mowing as a method of buckhorn plantain control. The reduction in buckhorn plantain via low mowing also resulted in lower turf quality, increased crabgrass coverage, and increased dandelion. While not measured in our study, lower mowing heights would also reduce the rooting and photosynthetic capacity of the Kentucky bluegrass (Christians et al., 2016). However, if no other buckhorn plantain control methods are available, low mowing can reduce but not eliminate this weed. Phenotypic plasticity as well as potential genotypic shifts of buckhorn plantain may change the influence of low mowing on the species over an extended period of time (i.e.,  $\geq 10$  yr). Given the shorter/narrower leaf length, leaf width, scape length, and spike length of buckhorn plantain collected from a lawn compared to roadside and waste areas (Warwick and Briggs, 1979) as well as the adaptive stress tolerance transmitted to seed progeny by the species [e.g., lead (Wu and Antonovics, 1976)], long-term low mowing may select for ecotypes that tolerate low mowing stress.

Previous management of the experimental area may have also played a role in the response of buckhorn plantain to the different mowing heights and N fertility treatments. The area was not previously fertilized with N for more than 5 yr previously and mown at 5.1 cm. Thus, the turf was likely deficient in N and responded vigorously to the N applied in the experiment. A more N-rich soil may not respond as well, but it also would likely have dense turf and fewer buckhorn plantain plants. Competition from other weed species should also be considered. Buckhorn plantain would have received more competition from crabgrass and dandelion at the 5.1 cm mowing height compared to 10.2 cm, which may explain some of the reduction in buckhorn plantain at the lower mowing height. However, competition from crabgrass and dandelion would not explain the influence of N fertility, as buckhorn plantain responded similarly to the other weeds.

This research contributes additional evidence to the idea that promoting healthy turf is the best strategy for combating weeds. In the absence of pesticides, nitrogen fertilization increased turf quality while reducing buckhorn plantain, dandelion, and crabgrass coverage. While low mowing reduced buckhorn plantain, the negative consequences of likely offset the benefits.

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## CHAPTER 6. FREQUENT MOWING REDUCES *PLANTAGO LANCEOLATA* SEED PRODUCTION

### 6.1 Abstract

Mowing frequently enough such that weed inflorescences are cut prior to seed maturation can help to prevent seed rain and seed bank deposition. With confirmation of 2,4-D herbicide resistance in buckhorn plantain (*Plantago lanceolata* L.) and no documented cultural control options for the species, the objective of this 2 yr field trial was to determine the influence of mowing frequency on buckhorn plantain in Kentucky bluegrass (*Poa pratensis* L.) turf. The experimental design was a randomized complete block design with four blocks; treatments included mowing frequencies of 0.5, 1, 2, 4, and 8 wk. Three buckhorn plantain plants were randomly selected and marked within every plot each year, and the inflorescences were categorized, counted, and collected for later germination testing. A mowing interval  $\leq 2$  wk reduced the number of anthered inflorescences, inflorescence yield in both years, and viable seeds in both years compared to a mowing interval of 8 wk. However, mowing frequency did not influence the total number of inflorescences produced. After 2 yr, the shortest mowing interval (i.e., 0.5 wk) had amongst the highest amount of buckhorn plantain coverage, whereas the 4 wk had amongst the lowest. Thus, the reduction in viable seed production due to frequent mowing did not translate to reduced buckhorn plantain coverage but instead encouraged vegetative spread. This research further demonstrates the weediness of buckhorn plantain.

## 6.2 Introduction

Recently, the first report of 2,4-D resistance in buckhorn plantain as well as the first report of 2,4-D resistance in turf was published (Patton et al., 2018). Additional 2,4-D resistant buckhorn plantain ecotypes have been reported in Indiana (Chapter 4; Law, 2021), Ohio (Chapter 4; Law, 2021), Pennsylvania (Russell et al., 2020), Georgia (P.E. McCullough, personal communication), and Virginia (S.D. Askew, personal communication) in the short time since. These resistant buckhorn plantain ecotypes have originated from multiple turfgrass systems, including cemeteries, home lawns, a university campus, sports fields, and golf courses. Glyphosate resistance has also been confirmed in buckhorn plantain from a vineyard in South Africa (Heap, 2021).

Herbicide pre-mixtures containing the synthetic auxin herbicides 2,4-D, mecoprop, and dicamba are commonly used in residential turf to control common weeds, including dandelion (*Taraxacum officinale* F.H. Wigg.), white clover (*Trifolium repens* L.), and *Plantago major* L. (Neal, 1990). Most herbicide premixtures that provide excellent postemergence control of buckhorn plantain in turf contain 2,4-D (Patton and Elmore, 2021), and the herbicides offering excellent control that do not contain 2,4-D are more expensive (Patton et al., 2018), not labeled for residential turf, not labeled for cool-season turf (Patton et al., 2021) and/or not available until recently (i.e., halauxifen-methyl). As a likely result, 2,4-D was routinely applied in lieu of herbicide rotation, selecting for herbicide resistant individuals and eventually ecotypes (i.e., populations). Although the incidence of weed resistance is reportedly lower for synthetic auxins than other herbicide modes of action (Busi et al., 2017), the recent identification of numerous 2,4-D resistant buckhorn plantain ecotypes is concerning. Furthermore, the release and adoption of 2,4-D tolerant (Enlist™) soybean [*Glycine max* (L.) Merr.] is predicted to increase 2,4-D use (Peterson et al., 2016).

With so few postemergence chemical control options for buckhorn plantain, particularly in the case of a 2,4-D resistant ecotype in cool-season residential turf, it is important to investigate alternative, nonchemical control methods. While no specific cultural or biological control methods of buckhorn plantain have been identified to date, maintaining a competitive stand of turf is one of the best defenses against weed encroachment (Christians et al., 2016). A plant's biology and preferred habitat can lend clues as to which cultural practices may provide control (Bell, 2011). Buckhorn plantain is a cool-season perennial, seldom annual or biennial,

broadleaf that is a common weed in turfgrass systems (Christians et al., 2016) as well as in hayfields, pastures, farmyards, waste places, and roadsides (Cavers et al., 1980). Buckhorn plantain is a noxious, invasive, or restricted weed species in many U.S. states. The species originates from Eurasia (Cravers et al., 1980), but it has since established throughout the world save for subarctic and low-lying tropical areas (Tessene, 1968). Buckhorn plantain forms and grows from a central, basal rosette. Long, silky hairs are often present at the growing point. There is no visible aboveground stem, but a semi-woody caudex and/or undeveloped taproot is often present. Plants develop a few deep roots (1 m or more) as well as a mass of short (5-10 cm) fibrous roots near the soil surface (Sagar and Harper, 1964).

There are several ecological traits of buckhorn plantain that contribute to its successful colonization of low maintenance turf. First, the species is able to germinate across an array of environmental conditions. Not only can buckhorn plantain germinate without light, light does not favor germination (Kuiper and Bos, 1992). Thus, buckhorn plantain is able to germinate when buried and can establish in tall, dense layers of vegetation (Blom, 1978). However, seedling emergence is inhibited by burial depths of 6 cm or more (Benvenuti et al., 2001). Fresh buckhorn plantain seeds have a lower germination capacity than 1- to 5-year-old seeds, though viability can remain strong for six or more years (Kuiper and Bos, 1992). Compared to other *Plantago* species (i.e., *P. coronopus*, *P. major*, and *P. media*), buckhorn plantain has the widest ecological amplitude (Mook et al., 1992).

Morphological differences between buckhorn plantain ecotypes can vary based on habitat and management. Left unmown, buckhorn plantain grows long, erect leaves and appears bush-like. As mowing height decreases and frequency increases, smaller, more prostrate leaves and plants result. Warwick and Briggs (1979) measured field samples of various ecotypes and found that leaf length, leaf width, scape length, and spike length were shorter/narrower for buckhorn plantain collected from a lawn compared to roadside and waste areas. However, when plants from these same ecotypes were cultivated, the between-habitat differences disappeared (Warwick and Briggs, 1979).

Buckhorn plantain has  $2n = 12$  chromosomes (Sagar and Harper, 1964) and reproduces by both seeds and basal shoots. Buckhorn plantain is a long day species (Snyder, 1948), flowering from mid-spring through early fall in the Midwest. Buckhorn plantain is a wind-pollinated, self-incompatible, obligate outcrosser (Sharma et al., 1992) with a relatively high

level of gene flow (Van Dijk et al., 1988). It is a gynodioecious species (Ross, 1969) in which flowers may be pistillate, staminate, or hermaphroditic (Tessene, 1968). Also a protogynous species, the stigma protrudes 4-6 d prior to flower opening (Sharma et al., 1992). Flowers are long, leafless stalks (up to 45 cm in length) that bear dense, cylinder-shaped spikes (1-8 cm in length); single flowers are inconspicuous (Cavers et al., 1980; Warwick and Briggs, 1979).

Fruiting capsules yield one or two narrowly oval, brown to black, shiny seeds indented on one side (2 mm in length)(Cavers et al., 1980). Buckhorn plantain seed yield can be multiplicatively determined by the number of inflorescences per plant, capsule number per inflorescence, seed number per capsule, and seed mass (Primack and Antonovics, 1981). Seed weight ranges from 0.7 to 2.0 mg, with an average of up to 470 seeds per flowering plant (Mook et al., 1992). Approximately 20% of seedlings survive to adulthood, and the average half-life for adults is 2.7 yr (Mook et al., 1992).

Turfgrasses have specialized growing points that allow them to survive frequent mowing (Christians et al., 2016). Thus, timely mowing is able to eventually kill most dicotyledon species and provides a competitive advantage for the desired turf. Mowing frequently enough such that weed inflorescences are cut prior to seed maturation can help to prevent seed rain and eventual seed bank deposition. For example, delayed mowing of a cool-season pasture increased the weed seed bank (Williams, 1984). However, the influence of mowing frequency is not steadfast and can be weed species-specific. Fortnightly mowing removed field bindweed (*Convolvulus arvensis* L.) from warm-season turf but did not prevent dandelion colonization (Timmons, 1950).

Buckhorn plantain germination is seemingly less affected by temperature than other *Plantago* species. Buckhorn plantain germination is temperature-independent between 15 and 25°C, unlike the increase in germination for *P. major* and *P. media* between those temperatures (Sagar and Harper, 1960). Further, a 5°C prechilling treatment results in smaller differences in germinability for buckhorn plantain compared to *P. major* (Sagar and Harper, 1960). Fresh buckhorn plantain seeds have a lower germination capacity than 1- to 5-year-old seeds, which is also true for *P. coronopus*, *P. major*, and *P. media* (Kuiper and Bos, 1992). While *P. coronopus*, *P. major*, and *P. media* germination decreased after four or five years, buckhorn plantain germination did not decrease after six years (Kuiper and Bos, 1992). Buckhorn plantain can also germinate across a range of soil moistures, with approximately 70% germination at soil moistures as low as 8% (water by volume) in a dune sand (Blom, 1979; Kuiper and Bos, 1992).

In that same study, maximum germination, approximately 90%, was achieved at a soil moisture of 21% (water by volume)(Blom, 1979; Kuiper and Bos, 1992).

Given that buckhorn plantain generally cannot set seed below common lawn mowing heights and that anther production in the species lags 4-6 d behind the stigma, frequent mowing may prevent viable seed production and eventually reduce buckhorn plantain coverage. Thus, the objective of this experiment was to determine the influence of mowing frequency on the persistence as well as seed production and viability of buckhorn plantain in Kentucky bluegrass (*Poa pratensis* L.) turf.

### **6.3 Materials and Methods**

Research was conducted at the William H. Daniel Turfgrass Research and Diagnostic Center in West Lafayette, IN (40.442°N, 86.9295°W). Buckhorn plantain seed was collected locally from a nearby field in the summer of 2014, propagated in the greenhouse, and then transplanted in the field at a density of 4.4 plants m<sup>-2</sup> the following spring in a 0.5 ha sward of Kentucky bluegrass. Following transplanting, plants were irrigated to ensure establishment. The experimental area was maintained similar to a residential lawn with weekly mowing at 5.1 cm with a rotary mower, except for an 8 wk period in August and September of 2015 and another 8 wk period in August and September of 2017, when mowing was withheld to encourage seed production and additional buckhorn plantain recruitment. The area was not fertilized with N for at least five years previously. The area was mown at 5.1 cm in 2016 and 10.2 cm in 2017, and it was mown at 10.2 cm for the experiment explained below, which began in April 2018.

The soil type was a Starks silt loam (fine-silty mixed, superactive mesic Aeric Endoaqualf) with a pH of 6.7, 276 kg P ha<sup>-1</sup>, 728 kg K ha<sup>-1</sup>, and 39 g kg<sup>-1</sup> organic matter. In an effort to control white clover and remove its N fixation potential, an application of quinclorac (Quinclorac 75 DF; Makhteshim Agan of North America, Inc., Raleigh, NC) was made at a rate of 0.84 kg ae ha<sup>-1</sup> on 29 Aug. 2016, with minor injury to buckhorn plantain. No other pesticides were applied during the course of the experiment.

Plots were 1×4 m in size, and blocks were laid to uniformly encompass buckhorn plantain. The experimental design was a randomized complete block design (RCBD) with four blocks. The five experimental treatments were made up of mowing frequencies, mowing every 0.5, 1, 2, 4, and 8 wk. Plots were mown using a rotary mower with a mulching deck and blade (Troy-Bilt TriAction

53-cm wide deck walk-behind push mower, Modern Tool and Die Company, Cleveland, OH) with clippings returned to their respective plot. Prior to flowering each year, three individual buckhorn plantain plants were randomly selected and marked with orange survey marking whiskers (Swanson Tool Company, Inc., Frankfort, IL) trimmed below the mowing height and pinned to the ground with a 15.2 cm 2-gauge aluminum nail. Due to plant loss as well as variability between plants within plots, three new plants were randomly selected in the second year of the experiment.

To keep all treatments on the same harvest and mowing schedule and set a “day zero”, the experimental area was mown at the earliest sign of buckhorn plantain flowering yet before any inflorescences had reached 10.2 cm (i.e., the mowing height). Thereafter, prior to each mowing event for individual plots, as based on mowing frequency, buckhorn plantain inflorescences above the mowing height for each of the three marked plants in each plot were categorized, counted, collected, and later dried on a greenhouse bench. Buckhorn plantain inflorescences were categorized by the lack or presence of anthers in an attempt to identify any maturity differences, and the number in each category were counted and recorded separately. Once dried, stems were removed, and the remaining portion of the inflorescences were pooled for each plot and stored at 5 C until germination testing could be performed. Total yield was measured at the end of each year.

Buckhorn plantain coverage, buckhorn plantain transects, and turf quality were collected monthly during each growing season (April-November). Buckhorn plantain coverage and turf coverage were visually determined. Transects were counted using a 0.75×1.5 m grid laid across each plot two times, which encompassed a 0.75×3 m area in the center of each plot with 90 total transects. Transect data is reported as the percent of transects covering a buckhorn plantain leaf or rosette. Turf quality was visually rated on a 1-9 scale, with 9 = ideal turf quality, 6 = minimum acceptable quality, and 1 = completely brown or dead turf (NTEP). On 24 June 2019, 8 wk after the initial mowing for 2019, leaf tissue above the clipping height for each of the marked plants was harvested, pooled by plot, dried, and weighed.

To perform germination testing, two random, uniform aliquots were collected and weighed from each plot for both years. Two 4-wk runs of germination testing were performed, beginning on 22 April 2020 and 20 May 2020. Germination testing was performed in an incubator (I-30BLL, Percival Scientific, Inc., Perry, IA) with a light intensity of 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , 12 h photoperiod, and day/night temp. of 20/15 C, respectively. Individual aliquots were placed on 8.3-cm unbleached blotter paper circles (Hoffman Manufacturing, Inc., Corvallis, OR) in 100 by 15 mm

polystyrene Petri dishes (VWR International, LLC., Radnor, PA). A 2.0 g KNO<sub>3</sub> L<sup>-1</sup> solution was used to moisten and maintain a consistent moisture status for the filter paper and inflorescence material. Germination counts were performed every week. A seed was determined to have germinated when both cotyledons plus a radicle were visible. Once a seed had germinated, it was counted, recorded, and removed from the petri dish.

All data were analyzed using PROC GLIMMIX is SAS software (SAS Institute, Cary, NC). Means were separated using Tukey's honest significant difference test ( $\alpha = 0.05$ ). Buckhorn plantain coverage, buckhorn plantain transects, and turf quality were originally analyzed as repeated measures, but significant treatment by rating date interactions ( $P > 0.05$ ) prevented presentation that way. Given that total inflorescences and anthered inflorescences are count datasets, they were square root transformed ( $Y^{0.5}$ ). Similarly, viable seed is count data containing zeros, so it was square root transformed  $[(Y+0.5)^{0.5}]$ . Transformed data were statistically analyzed, but the non-transformed data is presented for reader clarity. The year by treatment interaction was not significant for total inflorescences nor anthered inflorescences, so treatment means across years will be discussed. However, the year by treatment interactions were significant for inflorescence yield ( $P = 0.0019$ ) as well as viable seeds ( $P = 0.0004$ ), so treatment means are presented for each year.

## 6.4 Results

Mowing frequency was significant for anthered inflorescences across both years ( $P < 0.0001$ ), inflorescence yield in 2018 ( $P = 0.0004$ ), inflorescence yield in 2019 ( $P < 0.0001$ ), viable seeds in 2018 ( $P < 0.0001$ ), and viable seeds in 2019 ( $P < 0.0001$ ). Interestingly, mowing frequency did not influence total inflorescence production ( $P = 0.1808$ ). Frequent mowing (i.e., a mowing interval  $\leq 2$  wk) reduced the number of anthered inflorescences, inflorescence yield in both years, and viable seeds in both years compared to a mowing interval of 8 wk (Table 6.1).

There were interactions between buckhorn plantain coverage and rating date ( $P < 0.0001$ ) and buckhorn plantain transects and rating date ( $P < 0.0001$ ), which was likely a function of when the infrequently plots had been last mown. For example, buckhorn plantain coverage was amongst the highest in the plots mown every 8 wk in June 2019, but those same plots were amongst the lowest coverage in July 2019 following the scheduled mowing event (data not shown). All plots, including the 8 wk plots, were mown 24 June 2019, in between the June and July 2019 rating dates.

Table 6.1. Total buckhorn plantain inflorescences, anthered inflorescences, inflorescence yield, and viable seeds from this experiment in 2018 and 2019 in West Lafayette, IN.

	2018 and 2019 combined		2018	2019	2018	2019
	Total inflorescences	Anthered inflorescences	Inflorescence yield	Inflorescence yield	Viable seeds	Viable seeds
Mowing interval	----- # plant <sup>-1</sup> year <sup>-1</sup> -----		g plant <sup>-1</sup>	g plant <sup>-1</sup>	Seeds plant <sup>-1</sup>	Seeds plant <sup>-1</sup>
0.5 wk	301	29 c	4.1 b	1.5 c	0 c	0 b
1 wk	280	85 bc	4.5 b	1.9 c	0 c	0 b
2 wk	269	134 b	7.2 b	3.1 c	15 c	0 b
4 wk	348	245 a	15.8 b	7.7 b	858 b	458 a
8 wk	382	338 a	37.1 a	11.3 a	2842 a	524 a
<i>P</i> -value	<i>P</i> = 0.1808*	<i>P</i> < 0.0001*	<i>P</i> = 0.0004	<i>P</i> < 0.0001	<i>P</i> < 0.0001**	<i>P</i> < 0.0001**

\* Square root transformed with non-transformed data presented.

\*\* Square root transformed [(x+0.5)<sup>0.5</sup>] with non-transformed data presented.



A similar change occurred between the July and August 2018 rating dates, when the 8 wk mowing treatment was applied between those two rating dates (data not shown). This is further evidenced by the leaf tissue above the mowing height for the 24 June 2019 harvest, which is discussed below.

The larger plants in the infrequently mown plots, towards the end of their mowing interval, occupied more space (i.e., coverage) than the frequently mown plants. Thus, given the difficulty to make a fair comparison, only coverage and transects results from the 11 Nov. 2019 rating date will be presented. There was also an interaction between mowing frequency and rating date for turf quality ( $P < 0.0001$ ). This interaction was due in large part to the significant reduction in turf quality for the 4 wk and 8 wk treatments, once they were mown. The 4 and 8 wk intervals between mowing events allowed for turf growth well beyond 15.5 cm tall, which is when turf clipped at 10.2 cm should be mown when following the one-third mowing rule-of-thumb. As a result, the turf in these plots were scalped when mown, and turf quality suffered due to the reduction in verdure.

On 11 Nov. 2019, buckhorn plantain coverage ( $P = 0.0022$ ), buckhorn plantain transects ( $P = 0.0496$ ), and turfgrass quality ( $P < 0.0001$ ) varied by mowing frequency. For both buckhorn plantain visual coverage and transects, the shortest mowing interval (i.e., 0.5 wk) had amongst the highest amount of buckhorn plantain, whereas the 4 wk had amongst the lowest amount of buckhorn plantain (Figure 6.1). While no plots were of ideal quality, turf quality differed by mowing frequency on 11 Nov. 2019 ( $P < 0.0001$ ). After two growing seasons of mowing treatments, the 0.5, 1, and 2 wk mowing frequencies were still above acceptable quality (i.e.,  $\geq 6$ ). Plots mown every 8 wk had the lowest quality (4.5).

As expected, mowing frequency influenced the amount of buckhorn plantain leaf tissue above the mowing height when harvested on 24 June 2019 ( $P < 0.0001$ ). Plots mown on an 8 wk interval had more buckhorn plantain leaf tissue above the mowing height than any other mowing interval (37.1 vs. 2.6-13.7 g, respectively). Recall, plots mown at a frequency of 0.5 wk only had 0.5 wk of growth, compared to 8 wk of growth for that respective mowing frequency.

## **6.5 Discussion**

Frequent mowing reduced buckhorn plantain anthered inflorescences, inflorescence yield, and seed production, but those results did not translate to reductions in buckhorn plantain coverage. Buckhorn plantain can reproduce via both seeds and basal shoots; ramets have also been described

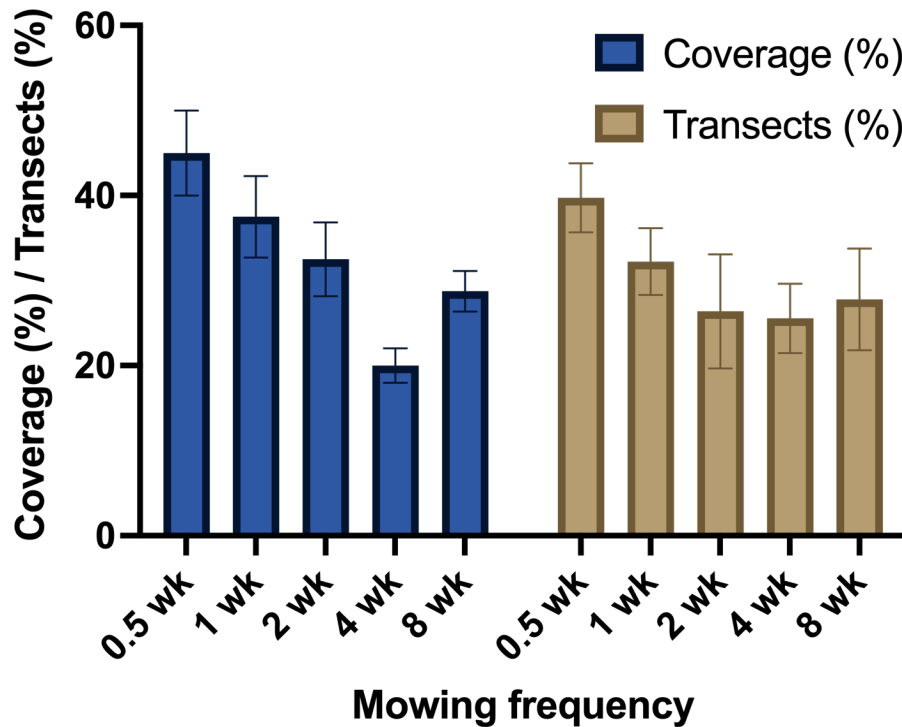


Figure 6.1. Buckhorn plantain coverage (%) and transects (%) on 11 Nov. 2019, after 2 yr of mowing frequency treatments. Data points are means, and standard errors of the means are presented across four blocks.

in favorable conditions (Cavers et al., 1980). Additionally, personal observations in the greenhouse and field have indicated the presence of rhizome-like structures. Thus, focusing buckhorn plantain control efforts on seed production alone will not remove existing plants or prevent vegetative reproduction and spread. Buckhorn plantain seed longevity can extend beyond 6 yr, while this study was conducted over only 2 yr. Frequent mowing extending beyond the seed longevity of buckhorn plantain may have eventually reduced buckhorn plantain coverage, once the soil seedbank had been depleted.

It is also possible that frequently mown buckhorn plantain may survive longer than infrequently mown plants. When buckhorn plantain was allowed to grow for 8 wk between mowing events, a large portion of the leaf tissue was removed when mown. Subsequent leaf tissue dieback with limited regrowth then resulted (personal observation; Figure 6.2). Furthermore, the cemetery where 2,4-D resistant buckhorn plantain was first identified was infested with the species despite frequent mowing (Patton et al., 2018). The author theorizes that spread of the 2,4-D ecotype



Figure 6.2. Image of a buckhorn plantain plant from the 8 wk mowing interval 4 d after mowing. Image taken 28 June 2019. Note the subsequent tissue dieback with limited regrowth.

at that location was through both vegetative spread and sexual reproduction resulting in viable seed. In drought conditions, turfgrass growth slows and mowing is often delayed. The more drought-tolerant buckhorn plantain would then be free to sexually reproduce in the brief absence of mowing.

Buckhorn plantain cannot produce viable seed below conventional mowing heights, unlike other turfgrass weeds (e.g., *Poa annua* and dandelion). Further, plant biology contributes to the effectiveness of frequent mowing on preventing seed production. To facilitate outcrossing, buckhorn plantain is a protogynous species, where the stigma protrudes 4-6 d prior to flower opening (Sharma et al., 1992). Clipping (i.e., mowing) before the flowers open prevents pollination and subsequent seed production. Although frequent mowing prevented inflorescence maturation and reduced subsequent viable seed production, it did not affect the total number of inflorescences produced. Thus, frequent mowing must be maintained throughout the flowering period for buckhorn plantain to effectively reduce seed production and subsequent seed rain. In a preliminary germination test outside of this experiment, a seed from a 1 wk mowing frequency plot germinated

(data not shown), demonstrating that even a 1 wk mowing frequency didn't entirely halt viable seed production.

While the heritability of herbicide resistance traits from sexual reproduction in buckhorn plantain is unknown, vegetative reproduction produces genetic clones and thus passes along genetic herbicide resistance traits to daughter plants. Buckhorn plantain is wind pollinated, so frequent mowing may help to reduce long-distance spread of herbicide resistance traits. However, it will not halt the spread herbicide resistance entirely. Heagy and Cavers (unpublished) noted >10,000 seeds on >30 spikes in a rich arable field without competition (Cavers et al., 1980), which supports our findings (Table 6.1).

Williams (1984) found that delayed mowing of a cool-season pasture increased the weed seed bank. While the weed seed bank was not measured in this experiment, given the differences in viable seed production between mowing intervals, it is plausible that the infrequently mown plots deposited more seed into the seed bank. However, the influence of mowing frequency is not steadfast and can be weed species-specific. Fortnightly mowing removed field bindweed (*Convolvulus arvensis* L.) from warm-season turf but did not prevent dandelion colonization (Timmons, 1950).

No fertilizers or herbicides were applied to the experimental area over the course of the experiment, and a related research trial demonstrated that nitrogen fertilization reduces buckhorn plantain coverage (Chapter 5; Law, 2021) via increased turf competition. Thus, while frequent mowing will not control buckhorn plantain alone, combining frequent mowing with nitrogen fertilization and/or an effective herbicide application may provide the best outcome. Afterall, an integrated approach is most likely to provide optimal results (Christians et al., 2016). This research further demonstrates the weediness of buckhorn plantain.

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## CHAPTER 7. SUMMARY AND CONCLUSIONS

The aims of this research were to investigate the mechanism(s) of 2,4-D resistance in a resistant ecotype of buckhorn plantain (*Plantago lanceolata* L.), screen other potentially resistant ecotypes and measure them for fitness penalties, and identify effective turfgrass cultural control practices for managing buckhorn plantain.

In our most well-characterized 2,4-D resistant ecotype of buckhorn plantain (i.e., IN-GW), reduced translocation plays a role in the resistance mechanism, evidenced by the radiolabeled 2,4-D experiment. Reduced adsorption may contribute as well, but those differences were not as clear. In the 2,4-D dose-response experiment using malathion as a cytochrome P450 inhibitor, malathion pre-treatment did not fully revert the IN-GW back to susceptible. Considering the results from these two trials, the resistance mechanism in IN-GW is non-target-site in nature.

In total, this research identified four 2,4-D resistant buckhorn plantain ecotypes from Indiana and one from Ohio. Only one report of a failure to control buckhorn plantain was confirmed to be a susceptible ecotype. When compared to susceptible ecotypes in a garden study, no major fitness penalties were identified. These ecotypes originated from various turfgrass systems, but the common theme was that they were low maintenance areas.

Herbicide resistance forming in low maintenance turf is supported by other work performed as part of this dissertation, as nitrogen fertilization reduced buckhorn plantain coverage. Thus, low fertility areas are more likely to be infested with buckhorn plantain as well as be more reliant on an herbicide for control. Low mowing reduced buckhorn plantain, but low mowing also increased crabgrass and dandelion as well as reduced turf quality. Frequent mowing reduced viable seed production, which did not translate to a reduction in buckhorn plantain coverage after 2 yr.

This research demonstrates the complex mechanism of action of 2,4-D, as the resistance mechanism for buckhorn plantain was not fully elucidated. It also highlights the importance of utilizing best management practices for turf, including rotating herbicide chemistries, high and frequent mowing, and nitrogen fertilization. The identification of additional 2,4-D resistant ecotypes is a testament to the importance of herbicide resistance research as well as educating land managers on the topic. Lastly, this research further demonstrates the weediness of buckhorn plantain.