Birdsfoot Trefoil (*Lotus corniculatus*) Cultivars Adaption Concerning Drought and Heat Tolerance Enabling the Expansion of Geographic Adaptation to include Alabama with Disease Resistance to Fungi and Nematodes as well as Herbicide Injury

by

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Abstract

Lotus corniculatus L., birdsfoot trefoil (BFT), is a common flowering plant in the Fabaceae family, native to grasslands in temperate Eurasia and North Africa. BFT is a perennial herbaceous plant, similar in appearance to some clovers. The flowers are mostly visited by bumblebees and develop into small pea-like pods or legumes. BFT is often used as forage and is widely used as food for livestock due to its non-bloating properties. BFT can be used as a winter cover crop and help supply nitrogen to the soil the following season. A NIFA grant (No. 2013-67012-21408) was awarded to Auburn University to breed a BFT cultivar to extend the forage's geographic adaptation across the United States.

BFT is a non-bloating, cool-season forage legume and has potential to improve sustainability of pasture systems in the Eastern Transition Zone of the U.S. This research addresses pre-breeding, germplasm enhancement, and cultivar development. It combines the expertise of a plant breeder with that of forage management researchers and Extension specialists. Our eventual goal is to develop a trans-regional BFT cultivar with disease resistance with a wide geographic adaptation that has a longer stand life than existing cultivars.

Selection for persistence began improving the longevity of a stand by selecting individuals that survived drought/heat conditions. Diseases such as *Macrophomina phaseolina, Fusarium oxysporum, Rhizoctonia* solani, and *Meloidogyne incognita* need to be considered in a BFT production system. Clethodim, sethoxydim, glyphosate, quizalofop p-ethyl, and imazethapyr demonstrated acceptable herbicidal injuries to BFT while 2,4-DB and imazapic indicated unacceptable injuries. Clethodim can be recommended in a BFT-grass mixture and imazethapyr may be recommended in a BFT-broadleaf mixture.

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Background on Birdsfoot trefoil

Birdsfoot trefoil (*Lotus corniculatus* L.) is in the Fabaceae family. This flowering plant is considered a native to temperate areas of Eurasia and North Africa (Steiner et al., 2001). BFT is a perennial, herbaceous, flowering legume plant resembling clovers or alfalfa (Seaney and Henson, 1970). The plant is a nectar source of insects and is also used as food by larval species of Lepidoptera (Thomas, et al., 1999). Van Der Kooi, et al. (2015) reported bumblebees feed on the flowers. BFT has a well-developed branching taproot with side roots near the soil surface (Keoghan and Tossell, 1974). Most cultivars are erect and grow to a height of two to three feet (Keoghan and Tossell, 1974). The height can sometimes be taller when supported by other plants (Keoghan and Tossell, 1974). BFT stems are smaller in diameter and less rigid than alfalfa stems (Keoghan and Tossell, 1974). BFT can survive close grazing, trampling, and mowing. BFT is most often found in sandy soils. BFT flowers from June to September. The bloom is made up of a cluster of bright yellow flowers arranged in a whorl at the end of the flowering stems (Keoghan and Tossell, 1974). The stems are slender, well branched, and are moderately leafy (Keoghan and Tossell, 1974). Each stem produces 4 to 8 yellow flowers that produce one seedpod each (Keoghan and Tossell, 1974). When ripe, the brown seed pods extend outward at right angles from the stalk and look like a bird's foot hence the name 'birdsfoot' and the leaves are smooth and consist of five leaflets with the center three above the others thus the name 'trefoil' (Seaney and Henson, 1970). The plant remains green and tender during and after seed ripening (Seaney and Henson, 1970). BFT produces an average of 375,000 seeds per pound (Seaney and Henson, 1970). BFT is often used as forage and is widely used as food for livestock due to its non-bloating properties.

The plant's root system consists of a taproot with numerous lateral roots located mainly in the upper 15 inches of the soil profile (Seaney and Henson, 1970). The root system of BFT is more shallow and branched than alfalfa but with greater lateral spread (Frame, et al., 1998; Seaney and Henson, 1970; Smith et al., 1986); it is therefore more flooding- and heaving-tolerant but less drought-tolerant than alfalfa (Peterson, et al., 1992). BFT performs better on low phosphate soils than alfalfa (Blumenthal and McGraw, 1999), which could also be related to differences in root system morphology. This could be advantageous due to the soils in Alabama.

Lotus species are found throughout the world, but the greatest diversity occurs in regions with Mediterranean climates: the Mediterranean basin, the far western U.S., and northwestern Mexico (Kirkbride, 1999). BFT is one of the most widely distributed *Lotus* species and the most widely used (Blumenthal and McGraw, 1999). BFT is the major pasture species in Argentina and Uruguay, and is cultivated throughout North and South America, Europe, the British Isles, South Africa, New Zealand, Australia, Japan, and South Korea (Blumenthal and McGraw, 1999).

In North America, alfalfa is grown in preference to BFT on deep fertile soils, while BFT has proven to be better adapted on soils too acidic or limited in fertility, texture, or rooting depth for successful alfalfa production (Chevrette, et al., 1960; Heinrichs, 1970; Keeney, 1985; Seaney and Henson, 1970). Some BFT cultivars are also more saline-tolerant than alfalfa and white clover (*Trifolium repens*) (Jensen, et al., 1965; Maas, 1986; Schachtman and Kelman, 1991).

Cultivars of *Lotus* are best adapted to humid-temperate environments. BFT is not well adapted to high temperature (Nelson and Smith, 1969) or drought (Hoveland, 1994). Two reasons why *Lotus* is not usually grown below 30° N or S latitude: 1. long-day plants requiring 14-16 hours of daylength to flower (Forde and Thomas, 1966; Grant and Marten, 1985; McKee, 1963); at lower latitudes plants will not set seed and persist and 2. warm temperatures tend to promote foliar and crown-rotting diseases that restrict production and decrease stand persistence (Beuselinck et al., 1984).

BFT is used in agriculture as a forage plant, grown for pasture, hay, and silage. Taller growing cultivars have been developed for these purposes (Seaney and Henson, 1970). The concept of producing high-quality forage for livestock has been a problem on low output lands. Soils with few natural limitations are usually planted with alfalfa (Seaney and Henson, 1970). Soils with a low pH, poor drainage, or low fertility are not suitable for alfalfa production (Baliger et at., 1988; Charlton, 1983; Seaney and Henson, 1970). BFT is a forage legume that is more tolerant of these unfavorable production conditions. BFT is able to produce comparatively high yields and quality on land not suitable for alfalfa production (Seaney and Henson, 1970). BFT may be used as an alternative to alfalfa in poor soils. BFT is a perennial that acclimates well to production on poorly drained and/or low-pH soils (Baliger, et at., 1988; Charlton, 1983; Seaney and Henson, 1970). BFT is being grown more frequently in the northern United States and southern Canada where production of other forage legumes is limited. BFT has been used in grazing systems but there are now cultivars available that are suitable for hay production (Hoveland, et al., 1990; Marten and Jordan, 1979). BFT can reseed itself, is resistant to Phytophthora root rot and numerous alfalfa insects, responds well to fertilization, and does not cause bloat in animals (Illinois Extension).

The BFT normally used in forage production is a tetraploid with 2n=4x=24 chromosomes (Dalrymple, et al., 1984; Fjellstrom, et al., 2003). The generally tetrasomic inheritance (relating to a cell nucleus in which one chromosome is represented four times whereas all others are present in the normal number) suggests that it is an autotetraploid but certain traits such as tannin content seem to be inherited in a disomic manner (having one or more chromosomes present twice, but without having the entire genome doubled) (Dalrymple, et al., 1984; Fjellstrom, et al., 2003). Normally bivalent pairing has been observed during meiosis (Seaney and Henson, 1970). Favored pairing based on paternal and maternal source of the chromosomes is one explanation (Stift, et al., 2008). A thought is that most BFT accessions are highly allogamous, where cross-fertilization is enforced through a functioning self-incompatibility system (Stift, et al., 2008).

BFT is reported to be difficult to establish (Blumenthal and McGraw, 1999; Frame, et al., 1998; Seaney and Henson, 1970; Smith, et al., 1986) and to have poor competitiveness and persistence (Altier, et al., 2000; Barry, et al., 2003; Blaser, et al., 1956) in spite of its self-reseeding characteristic. BFT has a small seed size and poor seedling vigor, which will need careful management for successful establishment (McGraw, et al., 1986). Like other forage legumes, BFT will fix N_2 in symbiosis with the proper bacterium. Nodulation in BFT occurs in association with *Rhizobium lupini*, while alfalfa is inoculated with *Sinorhizobium meliloti*. Since these inocula are different species, a residual presence of alfalfa inoculum in the soil is of no value to BFT. BFT seed should be inoculated with *Rhizobium lupini* bacteria before planting. This will help with appropriate nodulation of the root system and enough atmospheric nitrogen fixation.

Depending on the source, BFT seed may be sold with the proper inoculum already applied, or fresh inoculum may be purchased separately and applied to non-inoculated seed. It is critical that fresh inoculum or properly inoculated seed be used in order for nodulation, and therefore N_2 fixation, to be effective. The group of rhizobia to which the inoculum for BFT belongs is well adapted to neutral or alkaline soils (Blumental and McGraw, 1999) so if BFT is to be seeded into acidic ($pH < 5.5$) soils, inoculated seed should also be pelleted with lime to aid in survival of the inoculum. Lime should be applied to low-pH soils as recommended by a soil test. Since BFT nodules senesce when herbage is removed, regrowth of grazed BFT should benefit from low levels of manure or fertilizer N while new nodules are developing (Blumental and McGraw, 1999).

BFT's small seed requires that it be placed no deeper than ¼ inch in the soil so it will attain maximum stands and yields (Blumental and McGraw, 1999). A smooth, firm seedbed will help the depth placement of the seed, which will allow for seed-to-soil contact (Blumental and McGraw, 1999). This will improve moisture uptake by the seed and improve the changes of germination and emergence. Early spring seedings are usually more successful than late summer seedings. Recommended seeding rates vary from as little as 1 lb./acre in complex mixtures while up to 8-10 lbs./acre are considered acceptable under normal conditions (Blumental and McGraw, 1999). Seed of BFT are small compared to some other legumes (half the weight of alfalfa seed), making calibration of seeding equipment somewhat challenging (Blumental and McGraw, 1999). BFT seedlings are less vigorous than alfalfa and less competitive with grass or weed species (Seaney and Henson, 1970). Therefore, overseeding an established stand of grass with BFT is not recommended unless the grass is chemically or mechanically suppressed (Blumental and McGraw, 1999), but successful frost seeding into established switchgrass (*Panicum virgatum*) stands has been reported (Gettle, et al., 1996). Certified seed should be used to ensure that seed is of the named cultivar and free of prohibited weed seeds. The pure live seed percentage reported on the seed tag, excluding hard seed, should be used to calibrate planters.

Fresh BFT contains cyanogenic glycosides. These release small amounts of hydrogen cyanide when separated. This is not poisonous to humans, because the dose is low and the cyanide is quickly metabolized (Scriber, 1978). Tannins are also found in *L. corniculatus*, which has been shown to increase the protein absorption of the small intestine (Barry, et al., 1999).

There are about 25 cultivars of BFT currently available in the United States and Canada (Haring, et al., 2008). These cultivars are characterized by growth pattern into two types, Empire and European (Haring, et al., 2008). Both types are called “broadleaf” trefoils.

Empire-type BFTs are adapted for grazing because they have fine stems, prostrate growth, and indeterminate growth (Undersander, et at., 1993). Empire types also grow slower during establishment and regrow slower following harvest than the European types (Undersander, et at., 1993). Dawn and Empire are high yielding Empire types that have done well (Undersander, et at., 1993).

European-type BFTs are adapted for hay production because they are more erect, establish faster, and regrow faster after harvest (Undersander, et at., 1993). A European-type trefoil named Viking has been high yielding when produced for hay (Undersander, et at., 1993). Some newer cultivars such as Fergus, Norcen, and Tretana have production attributes similar to Viking (Undersander, et at., 1993). These tend to endure better under vigorous harvest management practices. This characteristic may allow three cuttings per year in some areas.

Harvesting BFT as hay: the first cutting should be taken at 1/10 bloom and a second cutting in mid- to late August (Smith, 1978). Allow time for regrowth between cuttings or grazing is suggested to maintain the stand (Sheath, 1975; Sheath 1980). Root reserves may not be adequate to initiate regrowth if the BFT plant is completely defoliated in midsummer because root reserves are low (Sheath, 1975; Sheath 1980).

Grass-BFT mixtures are used to achieve higher pasture yields than with a pure stand of BFT (Marten and Jordan, 1979; Sheaffer, et al., 1984). A grass-trefoil mixture can help reduce BFT lodging and curing time for hay. Heavy grazing maybe needed in the spring to reduce growth and allow BFT to compete well in a grass mixture. BFT competes better than alfalfa under continuous grazing. Close, continuous grazing is not suggested because its regrowth depends on energy supplied by top growth. BFT does not retain high levels of root reserves during the summer like alfalfa (Nelson and Smith, 1968b; Smith, 1962).

BFT grows new shoots from axillary buds located on the stems rather than from crown or basal buds as in alfalfa (Nelson and Smith, 1968a). Carbohydrate reserves needed for regrowth during the growing season are minimal within the root of BFT compared to other legumes (Nelson and Smith, 1968b; Smith, 1962). However, BFT is adapted to circumstances where defoliation is regular, such as grazed pastures, because it maintains a higher amount of residual leaf area near the soil surface than alfalfa. Forage managers since the early 1960s have used the saying "frequent but not close" for BFT and "close but not frequent" for alfalfa to differentiate among suitable defoliation methods and responses for these two species (personal communication with Dr. Edzard van Santen). In comparison with alfalfa, BFT can be defoliated more frequently, but because it does not store carbohydrates during the growing season, as does alfalfa, photosynthesizing tissues must be left to support the next regrowth cycle (Nelson and Smith, 1968; Smith and Nelson, 1967).

Harvesting or grazing between September 1 and the first killing frost is not suggested. This period is needed to for root reserves to achieve levels to help with winter survival and allow growth the following spring (Nelson and Smith, 1968; Smith and Nelson, 1967).

The quality of BFT is greater than that of alfalfa because of increased "bypass" proteins and smaller stems (Penn State Extension). The loss of quality with maturity is less noticeable with BFT than alfalfa (Penn State Extension). Leaf loss during hay making may be larger than alfalfa (Penn State Extension). BFT is more palatable than alfalfa and has greater average daily gains and meat yield per acre for heifers and sheep when it is grazed (Penn State Extension). Unlike alfalfa, grazed BFT does not cause animals to bloat.

Stockpiling is the practice of not harvesting BFT during the second half of the summer and grazing it in the fall after the first killing frost (Penn State Extension). BFT is appropriate for stockpiling because it is able to hold its leaves at maturity and after frosts (Penn State Extension). This allows it to retain a high level of quality. Stockpiling also allows root reserves to accumulate during the fall, which improves plant survival and spring growth.

<u>Drought/Heat Tolerance – Persistence (Trans-Regional Aspect)</u>

For the benefit of beef production, the "summer slump" (or period of slow mid-summer growth) of cool-season grass pastures can be offset with a more efficiently deep rooted (and thus, drought-resistant), high-quality, bloat-free legume than with a high productive but lower quality C4 grass. Farmers that have experience with BFT have found it difficult to maintain productive stands which provides a major component for this project. A perennial growth habit is a stated goal for forage legume improvement, but the more significant goal should be stand longevity. A forage legume plant is not perennial in the sense of an oak tree (*Quercus* spp.), which once it has survived the juvenile phase, may live for many decades. Stand longevity is a function of the plant population (personal communication with Dr. Edzard van Santen). Forage legume crop management purpose is to maintain a minimum number of plants, or shoots as in the case of alfalfa (Undersander, et al., 2011), for a productive stand over the desired stand life. Small increases in the persistence of individual members of that populations have an impact on the longevity of a stand, as does the recruitment of seedlings. There is no evidence for autotoxicity in BFT, as there is for alfalfa, but tall fescue has been found to be allelopathic toward BFT late in the growing season (Stephenson and Posler, 1988).

Drought tolerance means to the degree in which a plant is adapted to arid or drought conditions. Desiccation tolerance is an extreme degree of drought tolerance. Xerophytes are plants that are naturally adapted to dry conditions. Drought tolerant plants usually use the C4 carbon fixation or crassulacean acid metabolism (CAM) pathways to fix carbon during photosynthesis. These pathways are more energy efficient the C3 pathway. CAM is good for arid conditions because carbon dioxide can be taken up at night which allows the stomata to stay closed during the heat of the day and reduces water loss. Several adaptations for dry conditions are structural which include the following: the stomata to reduce water loss (such as reduced numbers or waxy surfaces), water storage in tender above ground parts or water filled tubers, the root system to increase water absorption, or trichomes (small hairs) on the leaves to absorb atmospheric water. During drought conditions the leaflets of BFT close around the petiole and stem. Arid conditions can lower the yield of many crops. Plant breeding programs for improved yield during drought conditions have economic importance.

Diseases

Diseases of *Lotus* are caused by pathogens dispersed among at least 19 fungal genera and they occur wherever *Lotus* is managed. All vegetative and reproductive parts of *Lotus* plants are vulnerable to at least one of these pathogens. For example, *Colletotrichum*, *Cercospora*, *Stemphylium*, and *Pseudopeziza* infect foliage of *Lotus* and other forage legumes. It is possible to find two or more diseases caused by these pathogens occurring simultaneously but the limited literature does not specify how often they occur alone or as complexes on *Lotus*. The probability of two or more pathogens co-infecting *Lotus* depends significantly on the microclimate of the forage canopy. For example, in Missouri during periods of high humidity and dew formation, it is common to find both foliar blight and leaf spot caused by *Rhizoctonia solani* and *S. loti*, respectively.

Less examination of the physiological characteristics of interactions between *Lotus* and foliar fungal pathogens have occurred. The most in-depth studies in this area to date have been those of Millar and Higgins (1970). These authors described the relationships of β-glucosidase production and cyanogenesis in *Lotus* infected with *S. loti*. In contrast to foliar diseases, fungal pathogens that infect plant crowns and roots, including *Fusarium* sp., *R. solani*, *Mycoleptodiscus terrestris*, and *Macrophomina phaseolina*, occur usually in the same tissues of individual plants (Altier, 1994; Chao, et al., 1994; Drake, 1958). It is unusual to find a single pathogen associated with roots of *Lotus*. It has been difficult to identify the role of any single organism to crown and root rot because the pathogens have specific environmental and host requirements for infections. Pettit et al. (1969) gave one of the only quantified studies of the impacts of pathogen of this crown rot complex. They described during controlled experiments that variable reductions in root biomass of BFT when inoculated with individual or combined pathogens including *M. terrestris*, *R. solani*, *F. oxysporum*, and *M. phaseolina*.

Crown and root rots are the most important diseases of BFT. Severe loss from these diseases is usually associated with warm weather and high humidity; crown and root rots are of greater importance in the South than in the northeastern or north central region of the U.S. (Drake, 1958).

Root rot is not caused by a single organism but is the result of a parasite-saprophyte complex, which may vary in different environments. Organisms that have been found in this complex are species of *Fusarium, Verticillium, Macrophomina*, *Mycoleptodiscus*, *Rhizoctonia*, and *Sclerotinia* (Kainski, 1959). Plants severely infected with root rot have widespread decay in the central portion of the upper taproot and crown and often fail to regrow after harvest. Entire fields may be destroyed after harvest when environmental conditions are optimum for development of the diseases. Differences in susceptibility and tolerance to root rots have been observed, and selection and breeding have been carried on to increase resistance (Henson, 1962). The cultivar, Dawn, developed cooperatively by the USDA and the Missouri station shows significant tolerance to root rots in Missouri when compared to Viking and Empire.

Several disease organisms attack stems and leaves of BFT. *Sclerotinia trifoliorum* Erikks. causes a rot of lower stems and crown, usually under heavy snow cover in the late winter or early spring (Kreitlow, 1962). Under warm, humid conditions *Sclerotium rolfsii* Sacc. and *Rhizoctonia solani* Kühn. attack the crown and lower foliage causing blight of the leaves and eventual death of the infected plants. *Rhizoctonia solani* is partially controlled by harvesting when plants first show symptoms of infection (Kreitlow, 1962). The most widespread foliar disease of BFT is caused by *Stemphylium loti*. This organism causes reddish brown stem and leaf lesions and results in premature leaf drop or death of individual stems. Immature seed pods may also be attacked which results in shriveling and discoloration of seed (Graham, 1953).

BFT is not as resistant as alfalfa is to *Fusarium*-type diseases. Individual BFT plants do not survive as long as alfalfa. English (1999) reported that Fusarium wilt caused stand decline in the northeastern U.S., and crown and root diseases limited the persistence of BFT in the eastern and central U.S. These and other diseases are more prevalent in warm, humid climates, also limiting the use of BFT in the southern U.S. It is essential to use a management strategy that allows the BFT to reseed itself to help maintain a stand.

Diseases that cause problems in BFT also include crown and root rots and foliar wilts caused by *Fusarium* spp. There are a limited number of BFT cultivars available and disease resistance remains a challenge. Pardee, a cultivar developed in Pennsylvania, has greatly improved disease resistance, but lacks adequate winter hardiness for Michigan conditions (Michigan State Extension). Avoid rapid and complete defoliation (leave some leafy stems at harvest), allow reseeding, avoid stockpiling past early bloom, avoid excessive shading by grasses, and allow plants to rest in fall to manage diseases in BFT.

The impacts of fungal plant pathogens on foliage, stem, and blossom growth and development are not well defined with few exceptions. Of the few reports in the literature, English (1992) stated that 90% or more BFT leaves produced per shoot each year could be damaged by foliar blight and leaf spot. Stewart et al. (1994) stated that yield losses could be up to 36% for BFT infected with *Colletorichum acutatum*.

Fusarium oxysporum, a fungal pathogen, causes a vascular wilt of *Lotus*. This disease has been described to severely reduce production of BFT in the northeastern U.S. (Murphy, et al., 1985). Recent research efforts have focused on selection of resistant germplasm for this disease (Zeiders and Hill, 1988).

Macrophomina phaseolina is a Botryosphaeriaceae plant pathogen fungus that causes damping off, seedling blight, collar rot, stem rot, charcoal, rot, basal stem rot, and root rot on many plant species (Babu, et al., 2007). *Macrophomina phaseolina* is a fungus that is able to infect approximately 500 plant species in more than 100 families (Babu, et al., 2007). It is a harmful seed and soil borne pathogen. Hosts include the following peanut, cabbage, pepper, chickpea, soybean, sunflower, sweet potato, alfalfa, sesame, potato, sorghum, wheat, and corn.

Macrophomina phaseolina affects the fibrovascular system of the roots and basal internodes of its host. This impedes the transport of water and nutrients to the upper parts of the plant which results in wilting, premature death, loss of vigor, and reduced yield (Khan, 2007).

Brown lesions may form on the hypocotyls or emerging seedling. Many symptoms can occur during or after flowering. This includes grey discoloration of the stem and taproots, shredding of the plant tissue in the stem and top of the taproot, and hallowing of the stem (Khan, 2007). Small black dots can form under the epidermis of the lower stem and in the taproot (Khan, 2007). When the epidermis is removed the small, black microsclerotia may be very abundant which can give a greyish-black hue to the plant tissue (Khan, 2007). Reddish-brown discoloration and black streaks can form in the pith and vascular tissues of the root and stem (Khan, 2007).

Macrophomina phaseolina has a monocyclic disease cycle. It has aggregates of hyphal cells which form microsclerotia within the taproots and stems of the host plants. The microsclerotia overwinter in the soil and crop residue which are the primary sources of inoculum in the spring. They have a survival rate of up to three years in the soil. They are black, spherical, or oblong structures that allow the fungus to survive under poor conditions such as low soil nutrient levels and temperatures above 30°C. The survivability of microsclerotia is lower in wet soils and surviving no more than 7 to 8 weeks. Mycelium cannot survive more than 7 days. Infected seeds can carry the fungus in their seed coats. These infected seeds either do not germinate or produce seedlings that die soon after emergence.

Macrophomina phaseolina is a heat and drought favoring disease that produces large quantities of microsclerotia under relatively low water capacities and high temperatures. When the conditions are positive the hyphae are able to germinate from the microsclerotia. The microsclerotia are able to germinate during the growing season when the temperatures are between 28 and 35°C. Microsclerotia germinate on the root's surface. The germ tubes located on the end of the microsclerotia form appressorium that are able to penetrate the host plant's epidermal cell walls using turgor pressure or through natural openings. The hyphae infect the roots of the host plant. The hyphae enter the cortical tissue and grow intercellularly which allows them to infect the roots and the vascular tissue. Mycelia and sclerotia are produced within the vascular tissue and plug the vessels. This causes the greyish-black color often observed in plants infected by *M. phaseolina*. It also prevents water and nutrients from being transported from the roots to the upper parts of the plant. The result of this systemic infection causes diseased plants to wilt and prematurely die.

There are several techniques used to manage *M. phaseolina* fungal infections. Fungicides are used to inhibit mycelial growth. These include thiram, iprodione, carbendazim, pyraclostrobin, fluquinconazol, tolyfluanid, and metalaxyl and penflufen + trifloxystrobin. There are options to fungicides such as a combination of soil solarization and organic amendments. Crop rotation can be an effective management practice. Tillage practices can reduce moisture in the soil and make the environment less favorable for the pathogen.

Nematodes

Nematodes that are pathogens of *Lotus* have received less attention than fungi. A larger part of the literature on nematodes has gone past survey and description and has focused on disease impacts and management (Thompson and Willis, 1970; Townshend and Potter, 1978; Willis and Thompson, 1972). Although numerous nematode species have been described, that are associated with the roots of *Lotus*, most reports have focused on two species, *Pratylenchus penetrans*, (Cobb, 1917) Filipjev & Schuurmans Stekhoven, the root lesion nematode, and *Meloidogyne hapla* Chitwood, the northern root knot nematode. Both of these nematodes adversely affect *Lotus* root growth and dry matter accumulation (Thompson and Willis, 1970; Townshend and Potter, 1978; Willis and Thompson, 1972). The soil environment influences the biology of these species. For example, Willis and Thompson (1969) stated that reproduction of *P. penetrans* on roots of BFT varied significantly in connection to soil moisture. These authors also found that the effects of nematode establishment on root growth and plant yield were significantly affected not only by soil moisture, but also by harvesting method. Townshend and Potter (1978) stated that *M. hapla* reduced stand yields by causing mortality at seedling establishment and at later stages of seedling recruitment. *M. hapla* reduced yields of BFT by reducing dry matter accumulation in surviving plants. The impacts of nematode infection on plant yield and stand persistence are confounded more by interactions with root-infecting fungal pathogens such as *Fusarium* sp. (Willis and Thompson, 1972). Interactions of this type are likely to occur with other pathogens.

Meloidogyne is listed to be a plant parasitic nematode that includes field crops, pastures, grasses, horticultural, ornamentals, and vegetable crops (Stirling, et al., 1992). There is currently a breeding program at the University of Florida devoted to determining nematode resistance of forages. Germplasm evaluations have been conducted on a range of forage legume and grass species (Quesenberry, et al., 2014). Progress from breeding and selection research included studies of the genetic basis of resistance (Quesenberry, et al., 2014). Data suggests that several species may contain genes that could be identified and transferred into forage legumes to help deliver root-knot nematode resistance (Quesenberry, et al., 2014).

Herbicidal injury

Weeds can be controlled during establishment by clipping or mowing. However, success of control depends upon the particular weed problem and relative growth stage of weeds and BFT. Under some conditions mowing broadleaf weed species allows weed grasses such foxtail, *Setaria* spp., and fall panicum, *Panicum dichotomilflorum* Michx., to grow and become more competitive than the original weeds (Kerr and Klingman, 1960).

Chemicals are available for control of both broadleaf weeds and grasses. EPTC or benefin incorporated into the soil's surface (5-7.5 cm) before planting can control some annual broadleaf weeds and nearly all annual grasses (Linscott and Hagin, 1968). EPTC is also effective in control of yellow nutsedge. When EPTC or benefin are used then companion grasses cannot be seeded with BFT (Linscott and Hagin, 1968). Broadleaf weeds are controlled by using post-emergence sprays of 2,4-DB or dinoseb (Linscott and Hagin, 1968). Under certain conditions, especially when growth rate is slow, BFT shows symptoms of injury when 2,4-DB is used (Linscott and Hagin, 1968). However, seedlings soon recover and under favorable growing conditions continue rapid growth (Linscott and Hagin, 1968). Studies have shown that BFT seedlings are more susceptible to injury from dinoseb than alfalfa, thus lower rates are recommended for BFT (Linscott and Hagin, 1968). Since dinoseb can cause severe injury if temperatures are high at the time of application, this herbicide is not recommended in all regions (Linscott and Hagin, 1968). A post-emergence spray of either 2,4-DB or dinoseb can be used in combination with a pre-plant treatment of EPTC to control both grasses and broadleaf weeds (Linscott and Hagin, 1968). The chemicals mentioned here are examples of herbicides that can be used for control of weed competition during establishment. Local recommendations should be reviewed each year to determine available herbicides and to ensure their proper use. Some common pasture herbicides include 2,4-D, dicamba, 2,4-D + dicamba, picloram + 2,4-D, aminopyralid + 2,4-D, triclopyr, 2,4-D + triclopyr, triclopyr + fluroxypyr, metsulfuron + chlorsulfuron, metsulfuron + nicosulfuron, and glyphosate.

Problem Statement

The goal of the proposed work is the development of a trans-regional birdsfoot trefoil (BFT; *Lotus corniculatus* L.) cultivar with disease resistance and wide geographic adaptation that has a longer productive stand life than existing cultivars. BFT is a cool season legume mainly used as a forage crop for grazing animals. High nitrogen (N) fertilizer prices in addition to the environmental preservation qualities of grassland-based agriculture have led to renewed interest in the use of N-fixing forage legumes as a source of feed for ruminant livestock. Enhanced BFT cultivars could add to increased resiliency and sustainability of pasture-based beef production systems. BFT has been used as a cool-season forage in North America for a considerable amount of time and shares a distributional range with alfalfa (*Medicago sativa* L.).

The Eastern Transition Zone (ETZ; Figure 1) is where considerable cattle production takes place on toxic endophyte-infected tall fescue [*Schedonorus arundinaceum* (Schreb.) Durmort.] pastures that cause losses in cattle health, weight gain, and reproduction. Currently cattle production in the ETZ relies on fescue pastures primarily with 'Kentucky 31'. There are almost 14-19 million hectares of tall fescue pasture in the U.S. with most being in the Southeast (Bouton, 2007; Young, et al., 2015). Most of the present land area in tall fescue is occupied by 'Kentucky 31' infected with the endophyte, *Neotyphodium coenophialum* (Morgan-Jones & Gams) Glenn, Bacon & Hanlin., even though endophyte-free and novel endophyte tall fescue cultivars have been introduced. This fungus grows symbiotically within the plant and produces ergot alkaloids, which have been connected to negative effects on grazing livestock. Decreased livestock performance caused by grazing endophyte-infected tall fescue, includes lower weight gain and reduced reproductive efficiency, and has a major economic effect with yearly loses of >$600 million (Fribourg and Waller, 2004).

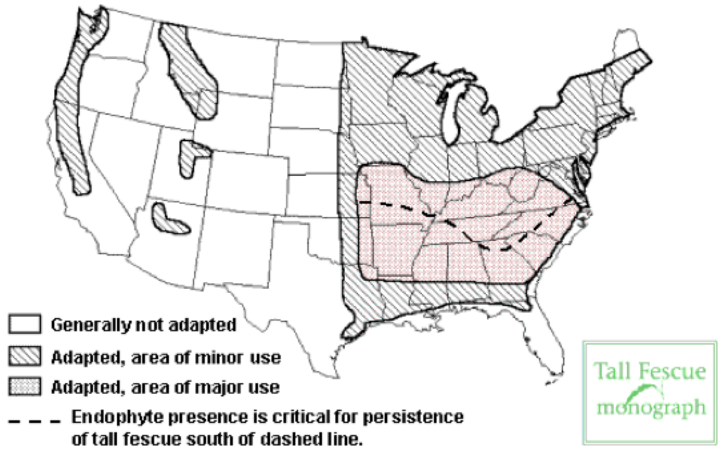


Figure 1. The areas of adaptation for tall fescue, including the ETZ, which is the pink speckled region on the map that stretches from Missouri and Arkansas on the west to the East Coast.

Approaches for managing endophyte-infected tall fescue have included the following such as reducing effects by avoiding grazing tall fescue pastures during times when alkaloid concentrations are highest, mitigating tall fescue with inter-planting grasses or legumes, or killing toxic tall fescue and switching it with novel endophyte or endophyte-free tall fescue cultivars or other suitable forage grasses or legumes (Lyman, et al., 2012; Owens, et al., 2012; Villalba, et al., 2011). Many producers in the ETZ depend on the first two tactics because the third option of substitution has shown to be expensive and time intensive. Whereas, other forage grass cultivar options for planting are not as tolerant of drought, pests, and grazing as endophyte-infected tall fescue (Lyman, et al., 2012; Owens, et al., 2012; Villalba, et al., 2011). This research suggests a fourth plan to develop a new legume cultivar with compatibility to tall fescue, with drought tolerance, and disease resistance. There is a lack of literature concerning the use of herbicides during establishment and within BFT.

Chapter 2: Selection for Persistence

<u>Introduction</u>

Birdsfoot trefoil (BFT; *Lotus corniculatus* L.) has been used as a cool-season forage in North America for a considerable amount of time and shares a distributional range with alfalfa (*Medicago sativa*). One difference is that BFT grows new shoots from axillary buds located on the stems rather than from crown or basal buds (Nelson and Smith, 1968b). Carbohydrate reserves needed for regrowth during the growing season is minimal within the root of BFT compared to other legumes (Nelson and Smith, 1968a; Smith, 1962). However, BFT is adapted to circumstances where defoliation is regular, such as grazed pastures, because it maintains a higher amount of residual leaf area near the soil surface than alfalfa. Forage managers since the early 1960s have used the saying "frequent but not close" for BFT and "close but not frequent" for alfalfa to differentiate among suitable defoliation methods and responses for these two species.

Farmers that have experience with BFT have found it difficult to maintain productive stands which provides a major component for this project. A perennial growth habit is a stated goal for forage legume improvement, but the more significant goal should be stand longevity. Stand longevity is a function of the plant population. Forage legume crop management purpose is to maintain a minimum number of plants, or shoots as in the case of alfalfa (Undersander, et., 2011), for a productive stand over the desired stand life. Small increases in the persistence of individual members of that population have an impact on the longevity of a stand, as does the recruitment of seedlings.

The ultimate goal is the development of a trans-regional BFT cultivar that has a longer productive stand life, which allows for wider geographic adaptation than existing cultivars. High nitrogen (N) fertilizer prices in addition to the environmental preservation qualities of grassland-based agriculture have led to renewed appeal in the use of N-fixing forage legumes as a source of feed for ruminant livestock. Enhanced BFT cultivars could add to increased resiliency and sustainability of pasture-based beef production systems.

The Eastern Transition Zone (ETZ; Figure 1) is where considerable cattle production takes place on toxic endophyte-infected tall fescue (*Schedonorus arundinaceum*) pastures which causes losses in cattle health, weight gain, and reproduction. There are almost 14 million hectares of tall fescue pasture in the U.S. with most being in the Southeast (Young, et., 2015). Most of the present land area in tall fescue is occupied by 'Kentucky 31' infected with the endophyte, *Neotyphodium coenophialum*, even though endophyte-free and novel endophyte tall fescue varieties have been introduced. This fungus grows symbiotically within the plant and produces ergot alkaloids, which have been connected to negative effects on grazing livestock. Decreased livestock performance caused by grazing endophyte-infected tall fescue, includes lower weight gain and reduced reproductive efficiency, has a major economic effect with yearly loses of >$600 million (Fribourg and Waller, 2004).

Alfalfa (*Medicago sativa*), red clover (*Trifolium pratense*), and white clover (*T. repens*) are the dominant forage legumes used in the U.S. Large commercial companies carry out the breeding of these species. The same companies have shown little interest in the breeding of legumes that do not cause bloat. These alternative perennial forage legumes have not shown a proven value for increasing ruminant weight gain while reducing the negative environmental impacts associated with livestock production (Waghorn, et., 2002; Waghorn, 2008). There is a large community of beef producers in the ETZ who would benefit economically from enhanced production on endophyte-infested tall fescue pastures. A public plant breeding program can respond to the need for a new cultivar of BFT that is adapted to the ETZ.

We proposed to increase the survivability of BFT by developing birdsfoot trefoil germplasm from which cultivars with improved stand endurance could be developed. The hypothesis of our study was that a productive, persistent moderate to high tannin BFT with disease resistance planted in pastures with endophyte-infected tall fescue would increase the productivity of ruminants.

The research objectives were as follows: 1) obtained seed from current commercial cultivars (purpose is to acquire genetic resources related with persistence under hot, humid environmental conditions in the ETZ: trans-regional scope), 2) tested persistence in the ETZ (AL), and 3) created synthetic populations. The hypotheses for the objectives were as followed: 1) breeding techniques would increase persistence of BFT and 2) breeding techniques would increase knowledge about the characteristics of BFT accessions.

Materials and Methods

A cultivar must have the broadest possible adaptation for commercial success; for this reason, Auburn University's Plant Breeding Unit (PBU) in Tallassee, AL in the Eastern Transition Zone (ETZ) was used. Populations from three commercial cultivars ('Empire', 'Norcen', and 'Pardee') were chosen as these populations were expected to exhibit a longer stand life. This material was used for cultivar development through four to five cycles of recurrent selection based on selectively introgressed into developed populations.

Cultivars are generally characterized by growth habit into two types; Empire (prostrate growth that is grown for grazing) and European (erect growth that is grown for hay production). Empire was developed in New York and the first BFT cultivar in North America. It has a semierect growth habit and flowers 10-14 days later than European types. It is moderately winter hardy and selected for persistence. Its PI is G22518. Norcen was developed in Missouri. It is a broad-leaved, intermediate type that has good forage yield and winter-hardiness. It contains a diverse genetic background, which provides resiliency to different environments within the north central U.S. Its PI is 592427 and/or NSL 174058. Pardee was developed by Cornell University to have resistance against Fusarium wilt. It blooms about 10 days earlier than Norcen. Its PI is 964338.

In 2016, we initiated evaluations of cultivars in spaced plant nurseries established from greenhouse plants at PBU. The nursery had a residual soil pH and fertility level characteristic for the region. The soil type at PBU is Kalmia loamy sand (10% clay, 10% silt, and 80% sand). We expected the resulting pre-breeding populations would be similar because selections were made based on within evaluation site only. The experimental design was a randomized complete block (r = 3) with 3 plots per block and 48 plants per plot. Plants were on a three feet equidistance spacing, where 8 plants long and 6 plants wide arrangement of plots lead to approximately square blocks. We compiled data to create a selection index based on 20% survivability of all plants across cultivar types. This was accomplished by mass selection. We collected seeds from our initial stands produced by the three commercial cultivars, which began the selection breeding process immediately without conducting multiple rounds of random mating.

Remaining plants were transplanted at Blue Moon Farms in Lebanon, Oregon for seed production. These plants were allowed to intermate and produce seeds. A sufficient number of beehives were placed in the area to ensure an adequate number of pollinators were available. Seed maturation was monitored and each plant was threshed, and the seeds cleaned, conditioned, and weighed. The half-sib families were used in the next cycle in the breeding process. This was accomplished by recurrent selection.

In year two, these half-sib families were planted to establish the next selection at PBU. The experimental design was identical to year 1. We expected these populations to be different among cycles and produce a homogenous cultivar with longer stand life.

Results and Discussion

During the first year (2016-17) we began with equal numbers of individuals in both size categories to select from and reduced the numbers to approximately 20% of their original numbers (Figure 2). After the environmental selection process the numbers were similar for both cultivar and size (Figure 2). The following season (2018) saw a decrease in the number of small Empire plants since they were subjected to mass selection based on plant size in the previous generation (Figure 3). Figures 4 and 5 demonstrate a stand count progression through time at 17 intervals. We saw a similar trend in both figures; when the temperature is high during the summer months more individuals succumb to the environmental pressure. Figure 4 illustrates this trend going from summer to fall and eventually winter; Figure 5 indicates this trend by going from early spring to summer. Visual observations of root depth increased from 2016 to 2018, which is believed to be attributed to the plants being selected for drought tolerance. BFT's responses to extreme temperatures included evaporative cooling, turgor response, and organic anti-freeze. Exposure to heat and light causes the stomata of the plant to open; this allows the water inside to evaporate. As a result of this water's evaporation, the plant's internal temperature is reduced, and the plant runs the risk of dehydration. BFT can respond by altering the turgor pressure, causing the leaves to wilt, which allows them to reduce the surface area of the leaves exposed to the sun. Another observation that was made was the leaves folded around the stem and became discreet, often giving the effect of excessive leaf loss, this occurred more prevalently during the 2018 season.

Legumes play an important role in forage production since they add protein to animal diets and supply nitrogen to plants by symbiotic nitrogen fixation. The important genus, *Lotus*, contains many species, some of which have been used for agricultural production. The future potential for *Lotus* is promising with continued development of new cultivars and management practices.

Much work has been completed to assist in establishing a role for *Lotus* in low input grazing and even hay making systems throughout the world. The adaptation of *Lotus* to acid, low fertility soils, tolerance of high levels of Al, bloat safety, and provision of by-pass protein to ruminant animals makes the species an attractive proposition in low input grazing and hay making systems. The three commercially used *Lotus* species have small seeds that have presented particular problems in pasture establishment. Problems relating to establishment have been met by a combination of plant breeding and selection for increase seed size, seed pelleting technology, and development of appropriate sowing technique. Grazing management for persistence now presents the greatest challenge to agronomists and farmers if *Lotus* is to be grown more widely. The more prostrate and even rhizomatous selections of BFT being developed in the US and Australia may help to improve the persistence of this species in more intensive livestock systems.

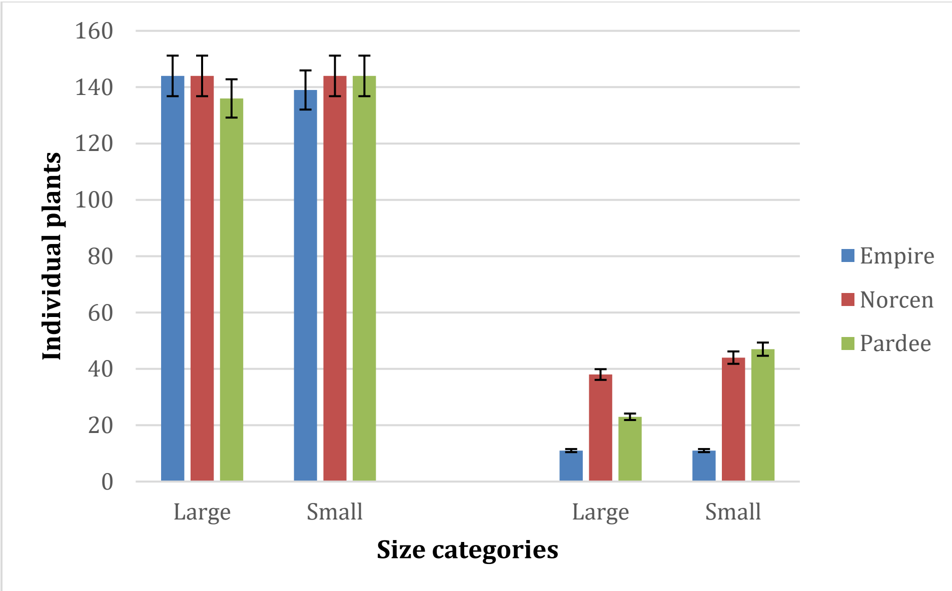


Figure 2. BFT selection based on size for 2016-17

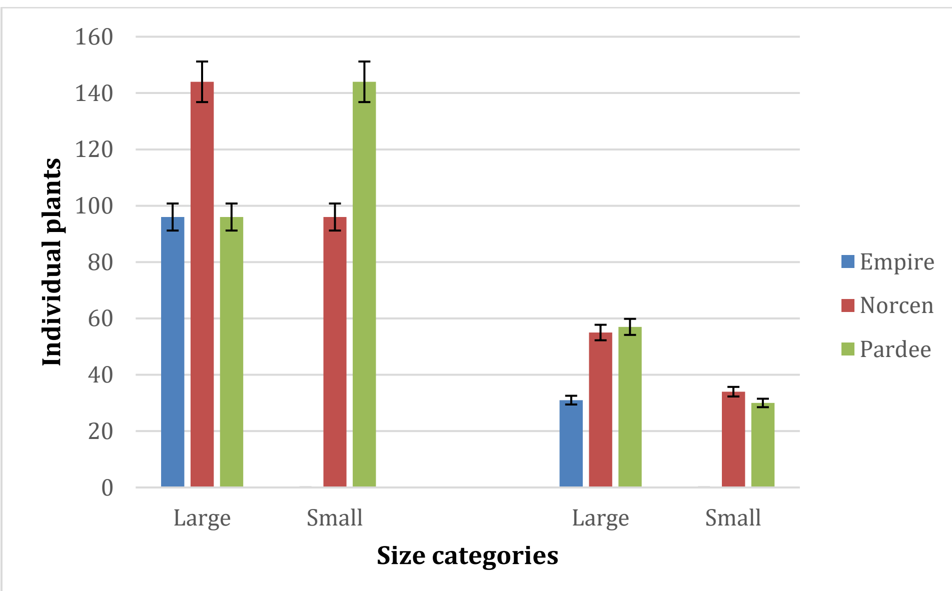


Figure 3. BFT selection based on size for 2018

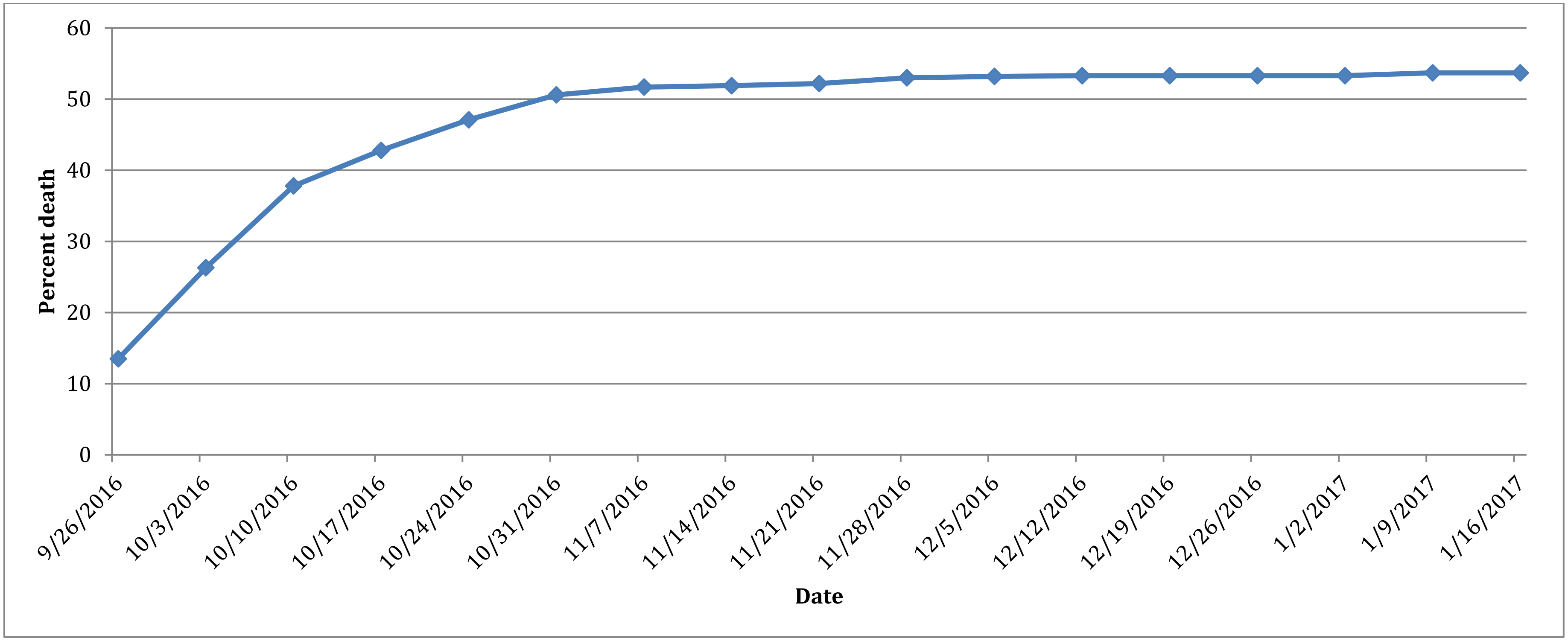


Figure 4. BFT plant decline and death progression over time for 2016-17

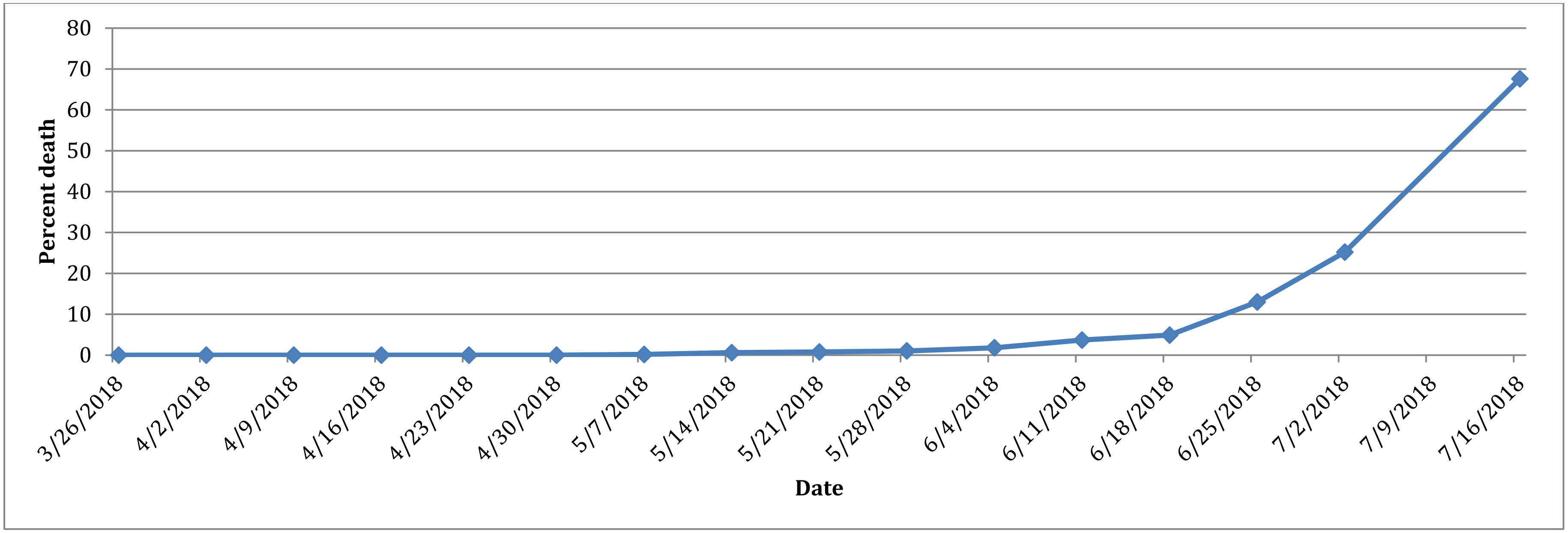


Figure 5. BFT plant-death progression over time for 2018

Chapter 3: Diseases

Macrophomina phaseolina

First Report of *Macrophomina phaseolina* on Birdsfoot Trefoil (*Lotus corniculatus* L.) in Alabama

Lotus corniculatus L. (birdsfoot trefoil) is a perennial herbaceous forage plant in the pea family Fabaceae and is grown for hay as an alternative to alfalfa on poor soils. A NIFA grant (No. 2013-67013-21408) was awarded to Auburn University to breed a birdsfoot trefoil cultivar to extend the forage's geographic adaptation across the United States. Stand decline disease of the birdsfoot trefoil cultivars and breeding lines was observed at the Plant Breeding Unit of the E.V. Smith Research Center in Tallassee, Alabama. Symptoms included wilting of the plants, discoloration of the roots and hypocotyl at the soil line, and eventually plant death. Entire symptomatic plants were collected from September through October in 2015 from half-sib individuals. Hypocotyl and root sections were diced into 0.5 cm pieces, surface sterilized, and aseptically placed onto water agar and acid potato dextrose agar medium. Cultures were incubated at 22 – 24°C for 5 to 7 days. An abundance of black, oval shaped sclerotia were produced on both media types typical for *Macrophomina phaseolina*, a soilborne fungus causing charcoal rot as described by Tassi and Goid (1947). Fungal colonies displayed limited mycelial growth with a black reverse when the bottom of the petri dish was examined. No pycnidia were produced. One hundred sclerotia of *Macrophomina phaseolina* were measured using the Nikon Eclipse Ti-U microscope; diameters ranged from 53.93-127.84 µm (average of 83.07 µm). Molecular *M. phaseolina* species identification was performed. DNA was isolated using the DNeasy® Plant Mini Kit from Qiagen, Inc. (Maryland, USA). The universal primers ITS1 and ITS4 (White et al., 1990) were used to amplify the internal transcribed spacer (ITS) region (approx. 500 bp). Amplicons were sequenced (Eurofins Genomics Louisville, KY) and results were cross-referenced with NCBI GenBank. Mega BLAST showed 100% shared identity with sequences of three *M. phaseolina* isolates (GenBank accession numbers: HM990163.1, HQ649832.1, and FJ643531.1).

Koch's Postulates were performed using *M. phaseolina* cultures in greenhouse tests. Three birdsfoot trefoil cultivars (Empire, Norcen, and Pardee) were evaluated for *M. phaseolina* susceptibility. Cultivars were planted with and without the addition of *M. phaseolina*. A randomized complete block design was established with three replications per cultivar. Two complete experiments were conducted in different greenhouses for a total of 128 experimental units (each pot containing 1-10 plants). Disease symptoms and plant ratings (1=healthy to 5=dead) were recorded weekly. Higher disease symptom ratings were observed with a significant ($P = 0.05$) increase of seedling root and hypocotyl necrosis compared between infected plants and controls (average disease rating of 2.75 for inoculated plants versus 0.83 for non-inoculated controls). All plant cultivars were equally susceptible to disease with an average 2.5, 3.0, and 2.8, respectively, at 29 days after planting. *M. phaseolina* was successfully re-isolated from hypocotyls collected from symptomatic plants but not from control plants. Koch's Postulates confirmed that *M. phaseolina* is the causal agent of birdsfoot trefoil stand decline and root necrosis in Alabama. *M. phaseolina*, a species in the *Botryosphaeriaceae*, has been previously identified to infect birdsfoot trefoil in Maryland (Drake, 1958). This pathogen causes stand decline, root rot, and charcoal rot in more than 500 crop and non-crop host plants (Smith and Carvil, 1977). It is an important pathogen of crops and wide spread in Alabama and, thus, must be considered in birdsfoot trefoil productions systems.

Fusarium oxysporum

First Report of *Fusarium oxysporum* on Birdsfoot Trefoil in Alabama

Lotus corniculatus (birdsfoot trefoil) is a common flowering plant in the Fabaceae family and native to Eurasia and North Africa. Birdsfoot trefoil is a perennial herbaceous plant, which is similar in appearance as some clovers. It is used in agriculture as a forage plant and also grown for pasture, hay, and silage due to its non-bloating properties. Birdsfoot trefoil may be used as an alternative to alfalfa in poor soils. Many strains within the *Fusarium oxysporum* complex are pathogenic to plants especially in the agricultural settings. Fusarium wilt is a common vascular wilt fungal disease. Progression of the fungus into the vascular tissue may elicit a delayed response that reduces the water conducing capacity of the plant to induce wilting (Ploetz, 2006). *Fusarium* sp. has been previously identified to infect birdsfoot trefoil in Maryland, North Carolina, and Virginia (Drake, 1958) and also in New York (Roberts, 1956). This research involves the relationship between fungal diseases and early stand decline in birdsfoot trefoil. The objective is to produce a birdsfoot cultivar with high tannin concentration and an extensive geographic adaptation with extended stand life than current cultivars. Entire plants were collected in September from symptomatic plants of half-sib individuals. Stems and roots were diced into 1 cm pieces; surface sterilized, and aseptically placed onto water agar and acid potato dextrose agar medium, with ten diced stems or roots per petri dish. Cultures were incubated at 22.2 – 24.4°C for 5 to 14 days when aerial whorled conidiophores with conidia appeared allowing for identification to a *Fusarium* species. *F. oxysporum* was identified by observing morphological characteristics similar to those of Snyder and Hansen (1940). Microconidia for the *F. oxysporum* species ranged between 4.03-8.04 µm in length with an average of 5.82 µm x 2.23-4.69 µm in width with an average of 3.01 µm. Macroconidia for the

F. oxysporum species ranged between 24.94-50.01 µm in length with an average of 38.60 µm x 2.23-3.89 µm in width with an average of 2.95 µm. DNA sequencing was also used to confirm *F. oxysporum*. DNA was isolated using the DNeasy® Plant Mini Kit from Qiagen, Inc. (Maryland, USA). The primers ITS1 and ITS4 (White et al., 1990) were used to amplify the internal transcribed spacer (ITS) region (approx. 500 bp), which was sequenced by Eurofins MWG Operon LLC, a Eurofins Genomics company in Louisville, KY. Sequence results were cross referenced with NCBI GenBank. GenBank Mega BLAST showed 100% shared identity with *F. oxysporum* (GenBank accession number: KU059956.1).

Koch's Postulate was performed using *F. oxysporum* cultures in greenhouse tests. Pots were located inside of the greenhouse. Three varieties of birds-foot trefoil were tested: Empire, Norcen, and Pardee. Pots were planted in mid December and stand counts were taken every two weeks for three months. Fungus culture had been grown for approximately three weeks before inoculation. 15 mL of fungal suspension was added to each pot; 5 ml was added to each pot then seeds were covered with sand then 10 ml was applied to each pot. After the initial stand count extra plants were selected/removed to reduce the numbers down to 10 per pot for standardization. The experiment was a randomized complete block design and replicated 3 times. Disease symptoms were observed with plant ratings (1=healthy to 5=dead) recorded. Lower stand counts showed a significant difference (5.33 infected individuals verses 6.83 non-infected individuals; p-value = 0.05) compared between the infected plants and control plants at 29 days after planting. Higher disease ratings showed a significant difference (2.83 for infected plants verses 0.83 for non-infected plants; p-value = 0.05) between infected plants and control plants at 29 days after planting. Stems and roots collected from symptomatic plants confirmed the presence of *F. oxysporum* by re-isolation. This indicates *Fusarium oxysporum* is a seedling disease of bird’s foot trefoil. To the best of our knowledge, this is the first report of *F. oxysporum* infecting birdsfoot trefoil in Alabama.

Rhizoctonia solani

First Report of Binucleate *Rhizoctonia* AG-G on Birdsfoot Trefoil (*Lotus corniculatus* L.) in Alabama

Lotus corniculatus L. (birdsfoot trefoil) is a perennial herbaceous forage plant in the Fabaceae family and is grown for hay as an alternative to alfalfa on poor soils. A NIFA grant (No. 2013-67013-21408) was awarded to Auburn University to breed a birdsfoot trefoil cultivar to extend the forage's geographic adaptation across the United States. Stand decline disease of the birdsfoot trefoil cultivars and breeding lines was observed at the Plant Breeding Unit of the E.V. Smith Research Center in Tallassee, Alabama. Entire symptomatic plants were collected from September through October in 2015 from half-sib individuals. Hypocotyl and root sections were sectioned into 0.5 cm pieces, surface sterilized, and aseptically placed onto water agar and acid potato dextrose agar (APDA) medium. Cultures were incubated at 22 – 24°C for 5 to 7 days. Isolates exhibited typical morphology described for binucleate *Rhizoctonia* taxa (Roberts, 1999) Tan mycelium with right-angle branching and 1 – 2 mm Sclerotium were produced on both media types.

Two internal transcribed spacer regions of DNA were amplified using the primers ITS-1 and ITS-4 provided by Eurofins Genomics (Louisville, Kentucky, USA). DNA thermocycler amplification procedure was initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. After amplification, PCR products were sequenced by Eurofins Genomics. The sequences for four isolates were analyzed using the National Center for Biotechnology Information (NCBI) BLAST and shared 100% identity with binucleate *Rhizoctonia* AG-G (Accession: number to be determined). These sequences were submitted to GenBank and can be accessed as Accessions number to be determined.

Koch's Postulates were performed using the binucleate *Rhizoctonia* AG-G cultures to test three birdsfoot trefoil cultivars (Empire, Norcen, and Pardee). Cultivars were planted with and without the addition of *Rhizoctonia* AG-G. In a RCBD with five replications per cultivar. Two complete experiments were conducted for a total of 60 experimental units (each pot containing 1-10 plants). Plant emergence was reduced indicating a significant (p = 0.05) increase of seedling disease within the infested plants compared to the controls (average emergence of 21.3% for non-inoculated controls versus 4.7% for inoculated plants). All three cultivars were equally susceptible to pre-emergence damping off caused by the binucleate *Rhizoctonia* AG-G. The birdsfoot trefoil plants that survived for 60 days did exhibit a reduction of 83.6% fresh shoot mass (p = 0.05) in the binucleate *Rhizoctonia* AG-G inoculated plots compared to the controls. Binucleate *Rhizoctonia* AG-G was successfully re-isolated from hypocotyls collected from symptomatic plants but not from control plants. Koch's Postulates confirmed that this binucleate *Rhizoctonia* AG-G is the causal agent in the reduction of birdsfoot trefoil plant biomass/yield in Alabama. *Rhizoctonia solani* has been previously identified to infect birdsfoot trefoil in Kentucky, Maryland, North Carolina, Tennessee, Virginia, and West Virginia (Drake, 1958). It is an important pathogen of crops and wide spread in Alabama and, thus, must be considered in birdsfoot trefoil productions systems.

Meloidogyne incognita

First Report of the Root-knot Nematode (*Meloidogyne incognita*) on Birdsfoot Trefoil (*Lotus corniculatus* L.) in the Southern United States

Lotus corniculatus L. (birdsfoot trefoil) is a perennial herbaceous forage plant grown for hay as an alternative to alfalfa on poor soils. A NIFA grant (No. 2013-67013-21408) was awarded to Auburn University to breed a birdsfoot trefoil cultivar to extend the forage's geographic adaptation across the United States. As part of this project, birdsfoot trefoil cultivars and breeding lines were observed with symptoms including chlorosis, stunting, and root galling. Symptomatic plants were collected and root systems exhibiting numerous galls, typical of *Meloidogyne* infection were subjected to NaOCl-extraction (Hussey and Barker, 1973) and nematode eggs enumerated.

Eggs were hatched for species identification. Individual juveniles (10 per sample) were picked out of the population, crashed and immediately used for PCR (Harris and Powers, 1993). The juveniles DNA was amplified via PCR using primers IncK-14F and IncK-14R that are specific for amplification of *Meloidogyne incognita* (Randig et. al., 2002). The amplified PCR product was run on a 1% agarose gel and a 400 base pair fragment was observed under a UV light, confirming the population to be *M. incognita* (Randig et. al., 2002).

Meloidogyne incognita eggs were used to perform Koch's postulates in a greenhouse pathogenicity test. Three cultivars of *L. corniculatus* (Empire, Norcen, and Pardee) were evaluated for *Meloidogyne* susceptibility. The cultivars were sown in a 15 cm polystyrene pots containing a 1:1 mixture of pasteurized sand and Kalmia loamy sand. Each pot was inoculated 30 days after planting with 100,000 *Meloidogyne incognita* eggs or not inoculated as a control. The test was arranged in RCBD with 5 replicates and the entire test was repeated. Nematode populations were determined 60 days after planting. Data were analyzed using SAS 9.4 PROC GLIMMIX, student panels were generated to determine the normality of the residuals, and LS-means compared using Tukey-Kramer's method at $P \leq 0.05$.

At 30 days after inoculation, *M. incognita*-inoculated birdsfoot trefoil cultivars Empire and Pardee exhibited a significant ($P \leq 0.05$) reduction in average plant fresh biomass of 19.8% and 17.5% as compared to the control, respectively. Nematode population densities on Empire, Norcen, and Pardee ranged from 3009-8425, 3419-8719, and 6888-23251 eggs per gram of root, respectively. The reproductive factor (the final nematode population divided by the initial inoculum density) was 1.17 for Empire, 1.49 for Norcen, and 1.43 for Pardee. The three cultivars of birdsfoot trefoil were susceptible to *M. incognita* and increased *M. incognita* population densities. The nematode also reduced birdsfoot trefoil biomass reducing the forage production potential of this crop. To our knowledge, this is the first report of *Meloidogyne incognita* infecting *Lotus corniculatus* in Alabama and the southern region of the USA.

Chapter 4: Herbicide Injury

Introduction

The intent in using herbicides is to kill unwanted plants in order to enable desired crops to establish and thrive but sometimes the use of herbicides has unintended consequences such as injuring non-target plants. Herbicide damage on non-target plants may cause slight to serious injury symptoms and occasionally economic damage. Herbicide chemistry and physical properties usually determine how herbicides interact with the biological and physical systems of the plant. Factors determining herbicide efficacy and crop safety are complex and include plant species, plant size, stage of growth, soil chemistry and physical properties, soil moisture, temperature, and relative humidity.

Weed control research in BFT and greater lotus (*Lotus uliginosus* Schkuhr) has centered on seed crops (Fairey, 1994). Ethofumesate (group 16; unknown mode of action) has proven to be the most effective in removing white clover from Maku seed crops (Brock, 1973; Brock and Henderson, 1976; Brock, et at., 1989, 1990). More recently chlorimuron (group 2; ALS/AHAS inhibitors) has shown potential for white clover, yarrow, and suckling clover control (Hare and Rolston, 1990). Work in Australia by Loch and Harvey (1988) showed that Sharnae seedlings had a high degree of tolerance to imazethapyr (group 2; ALS/AHAS inhibitors) and 20% imazaquin (group 2; ALS/AHAS inhibitors), which is recommended for use with Maku greater lotus in New Zealand, severely effects Sharnae. Commonly used pre-emergent herbicides such as Tri- and Benfluralin (group 3; microtubule assembly inhibitor) appear to be effective in controlling grasses and some broadleaf weeds when BFT and greater lotus are established in a prepared seedbed (Loch and Harvey, 1988). The tolerance of BFT to glyphosate compared with most annual weeds suggested that this may be used to manipulate pasture composition

(Boerboom, et al., 1990). To further knowledge concerning herbicide use in BFT production, seven herbicides were evaluated based on literature showing potential promise in their use.

Materials and Methods

Treatments

These studies were conducted at the Plant Research Science Center at Auburn University in both the greenhouse and microplots located outside. Three BFT cultivars, Empire, Norcen, and Pardee, were planted in 4-inch pots (27.95 cubic inches) filled with 60% standard potting mix and 40% sand. Ten seeds per cultivar were planted. Seven herbicides were arranged in a randomized complete block design with four replications consisting of quizalofop p-ethyl (Assure II) at 12 oz./A; 876 mL/ha, 2,4-DB (Butyrac 200) at 64 oz./A; 4674 mL/ha, ammonium salt of imazapic (Panoramic) at 4 oz./A; 292 mL/ha, sethoxydim (Poast) at 24 oz./A; 1753 mL/ha, imazethapyr (Pursuit) at 4 oz./A; 292 mL/ha, glyphosate (Roundup) at 8 oz./A; 584 mL/ha, and clethodim (Select Max) at 16 oz./A; 1169 mL/ha (Table 1). After approximately two months plants were transplanted to 8-inch pots (186.85 cubic inches) for the greenhouse trial or to microplots (25 L), which contained a Kalmia loamy sand (80%, 10% silt, and 10% clay). Container, or pot size, effects plant growth caused by either an increase or reduction of photosynthesis per unit leaf area (Poorter, et al., 2012).

The greenhouse herbicide treatments were applied via a DeVries Manufacturing® spray chamber with the speed setting on setting 78 to produce 15 gpa at 3 mph speed. The average temperature in the greenhouse was 78.6 ºF for the greenhouse trial with an average relative humidity of 89.9%. The average temperature in the greenhouse was 72.8 ºF with an average relative humidity of 65.5% for the microplot plants before transplanting them. For the microplots, a CO_2 backpack sprayer with a four nozzle boom measuring 6.33 feet and a 19 inch nozzle spacing. The sprayer applied the herbicide treatments at a height of 24 inches and at 38 psi to produce 15 gpa at 3 mph. The nozzles used in both the chamber and backpack applications were Greenleaf TDXL 110015. The weather conditions for application were an average temperature of 80 ºF (range: 69-91 ºF), no precipitation, max wind speed of 8 mph (range: 3-8 mph), and average humidity of 54% (range: 40-74%).

Data Collection

Visual evaluations were made on a scale of 0-100% with 0 = no crop injury and 100= crop death to determine the injury of herbicide treatment on BFT growth (Frans, 1986). BFT injury was assessed on 7, 14, 21, 28, and 56 days after herbicide treatments (DAT). Biomass was recorded 56 DAT.

The experimental design was an RCBD and statistical analysis was conducted using SAS 9.4 PROC GLIMMIX with means compared using Tukey-Kramer method at $P \leq 0.05$ was used.

Results and Discussion

A location effect was observed for the days after treatment due to the controlled environment of the greenhouse compared to the microplots which can be explained by the varying temperatures, precipitation, and wind that those plants received (Table 2). The 56 days after treatment was seen to have a location by cultivar and fresh shoot mass interaction likely do to the extended time to be affected by the herbicide treatment (Table 2). Injurious herbicides had a negative effect on the BFT plants no matter where the plants were located as seen in Tables 2 and 3; which is expected due to the individual herbicide's chemistry in disrupting a plant's metabolic pathway. A cultivar by herbicide effect was observed only for days 21 and 56 after treatment in the microplot data (Table 3). Pardee was the only cultivar affected by herbicide application in the greenhouse trial which could be an interaction between Pardee and the herbicides (Table 4 and Figure 6). 2,4-DB and imazapic significantly reduced the total biomass of cultivars in greenhouse tests but clethodim, sethoxydim, glyphosate, quizalofop p-ethyl, and imazethapyr did not reduce the cultivars' biomass (Figure 7). 2,4-DB is in the phenoxy family (group 4) which is the synthetic auxins; affects cell wall plasticity and nucleic acid metabolism. Imazapic is in the ALS or AHAS inhibitors family (group 2) which results in plant death due to ALS inhibition and low branched-chain amino acid production (LaRossa and Schloss, 1984). In microplot trials, cultivars Empire and Norcen were not significant in fresh shoot biomass nor were cultivars Norcen and Pardee, but Empire and Pardee illustrated a significant difference in their respected biomasses (Table 5 and Figure 8). None of the herbicides demonstrated a significant difference in fresh shoot biomass for the microplot trial; however, sethoxydim, imazethapyr, and 2,4-DB did result in lower biomasses compared to the control (Figure 9). Only two treatments (the control and imazapic) showed a positive linear regression in the greenhouse trial which indicates the BFT plants demonstrated increased injury over time (Figure 10). All the treatments showed a positive linear regression in the microplot trial which indicates the BFT plants demonstrated increased injury over time (Figure 11). Clethodim can be recommended in a BFT-grass mixture and imazethapyr may be recommended in a BFT-broadleaf mixture. We hypothesized that BFT converts 2,4-DB to 2,4-D because of beta-oxidation which occurs much slower in legumes than other plants but due to BFT's metabolism this process of degrading long carbon chains such as the carbon tail of 2,4-DB occurs quickly (Figure 12).

Table 1. Groups and chemical families of herbicides used in greenhouse screening and microplot trial.

Group/MOA	Chemical family
1: ACCase inhibitors	Cyclohexanedione "DIMs"
2: ALS inhibitors	Imidazolinone "IMIs"
4: Growth regulators	Phenoxy-carboxylic acid
9: Aromatic amino acid synthesis inhibitors	Glycine

Table 2. ANOVA table demonstrating location, cultivar, herbicide, and their interactions.

Effect	df	DAT7	DAT14	DAT21	DAT28	DAT56	FSM
Location	1	0.793	0.001	<.0001	<.0001	<.0001	<.0001
Cultivar	2	0.655	0.237	0.740	0.403	0.046	0.011
Location*Cultivar	2	0.572	0.400	0.655	0.751	0.332	0.050
Herbicide	7	<.0001	<.0001	<.0001	<.0001	<.0001	0.633
Location*Herbicide	7	0.000	<.0001	<.0001	0.023	0.826	0.726
Cultivar*Herbicide	14	0.625	0.839	0.079	0.567	0.205	0.192
Location*Cultivar*Herbicide	14	0.745	0.786	0.136	0.187	0.101	0.227

Table 3. ANOVA table demonstrating greenhouse and microplot herbicide injury ratings by dates.

	Greenhouse					Microplot				
	DAT7	**DAT14**	**DAT21**	**DAT28**	**DAT56**	**DAT7**	**DAT14**	**DAT21**	**DAT28**	**DAT56**
Effect			**p-value**					**p-value**		
Cultivar	0.2626	0.3446	0.7350	0.7740	0.4132	0.9820	0.5828	0.9815	0.3929	0.1562
Herbicide	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Cultivar*Herbicide	0.2318	0.3889	0.1398	0.3934	0.1174	0.8877	0.8981	0.0136	0.2316	0.0140

Table 4. ANOVA demonstrating effects of the herbicides on BFT cultivars' total biomass and herbicide without an interaction between the two in the greenhouse trail.

Effect	**df**	**p-value**
Cultivar	2	<.0001
Herbicide	7	<.0001
Cultivar*Herbicide	14	0.6947

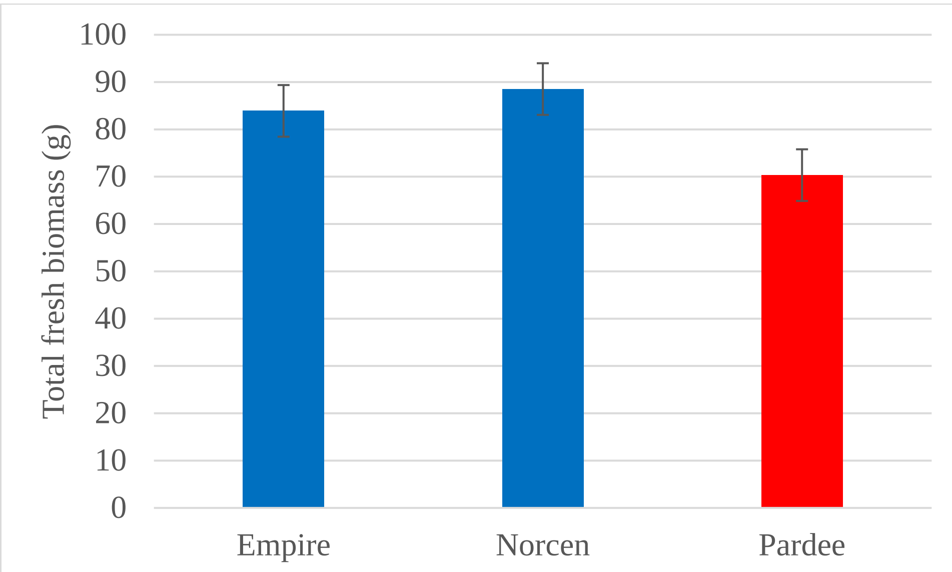


Figure 6. Greenhouse herbicides demonstrate difference between cultivars' total fresh biomass after herbicide applications.

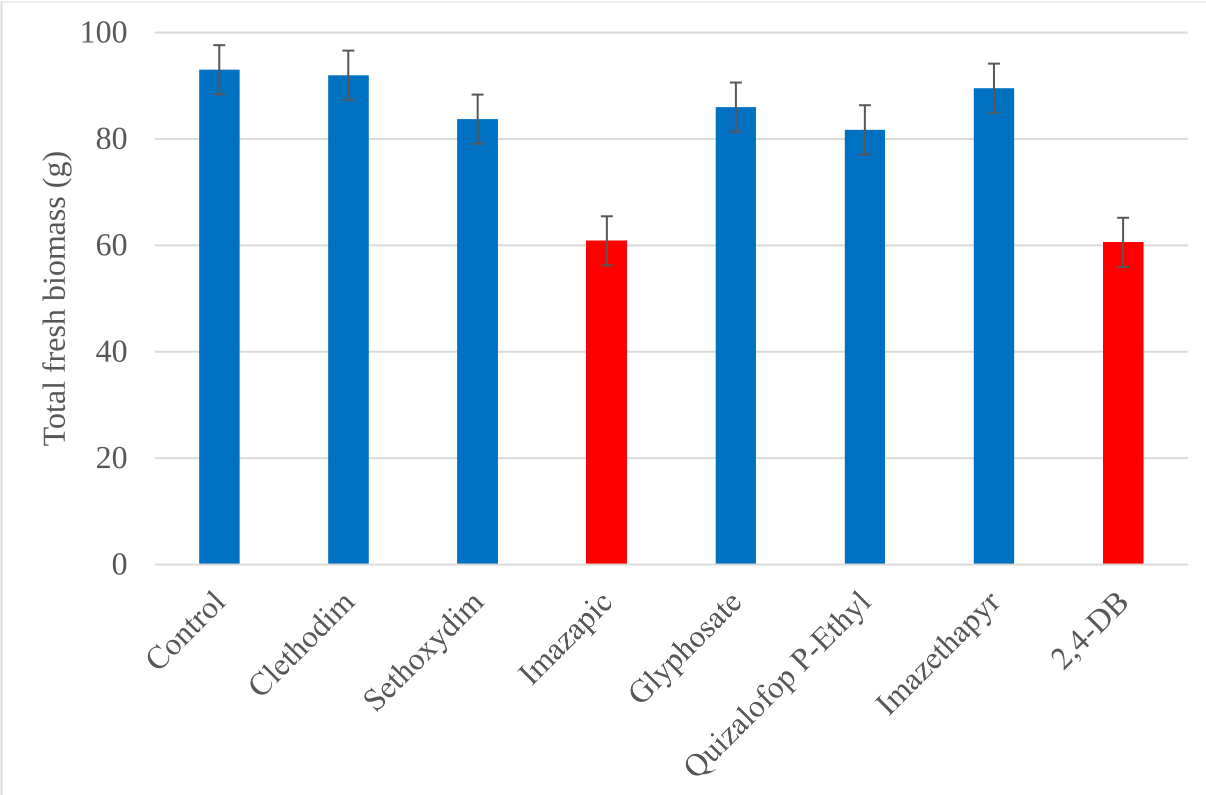


Figure 7. Greenhouse herbicide effects on BFT cultivar total fresh biomass.

Table 5. ANOVA demonstrating effects on biomass of cultivars and interaction between cultivars and herbicides in the microplot trial.

Effect	df	p-value
Cultivar	2	0.0079
Herbicide	7	0.4942
Cultivar*Herbicide	14	0.0591

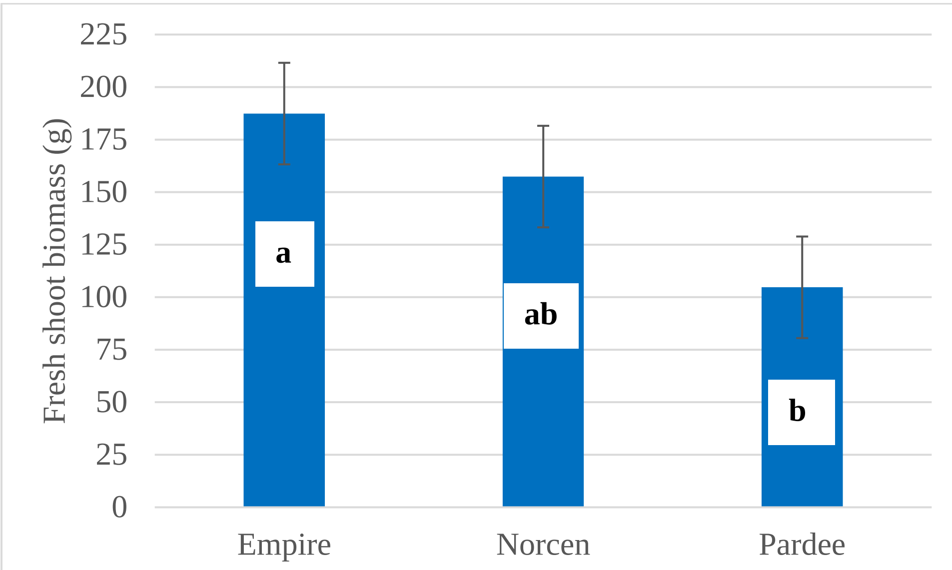


Figure 8. Microplot herbicide effects on cultivars' fresh shoot biomass.

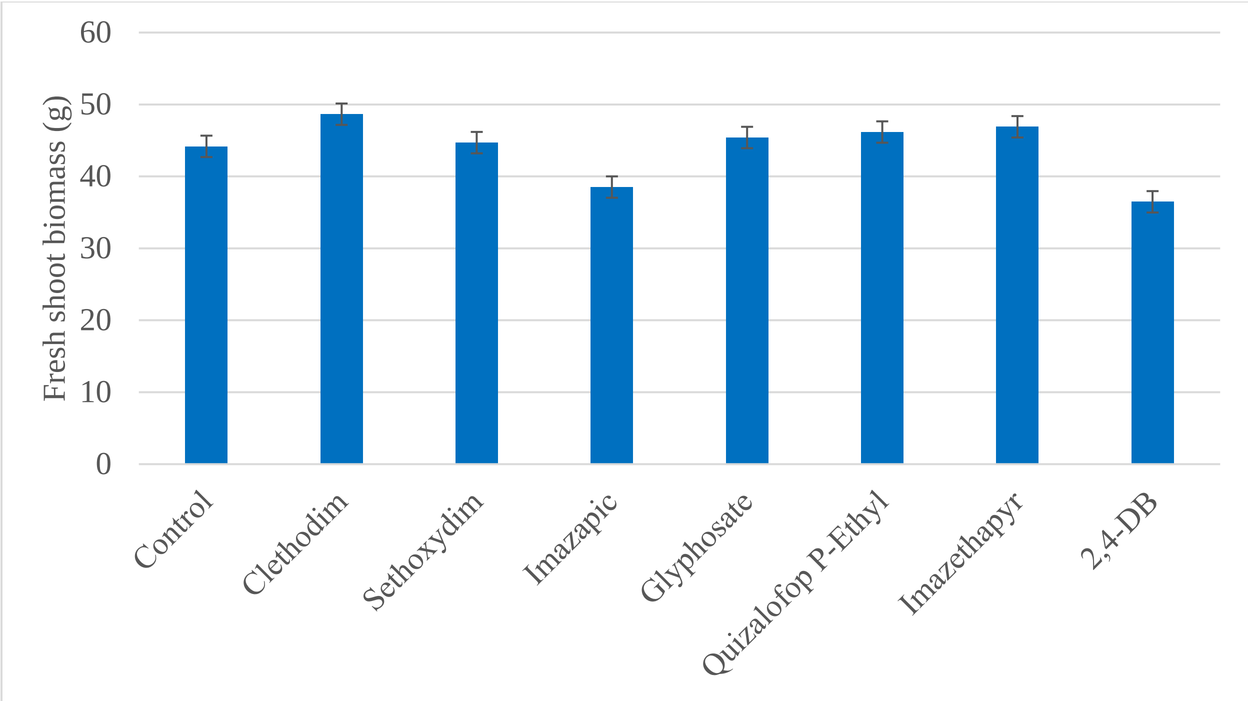


Figure 9. Microplot herbicide effects on BFT cultivar fresh shoot biomass.

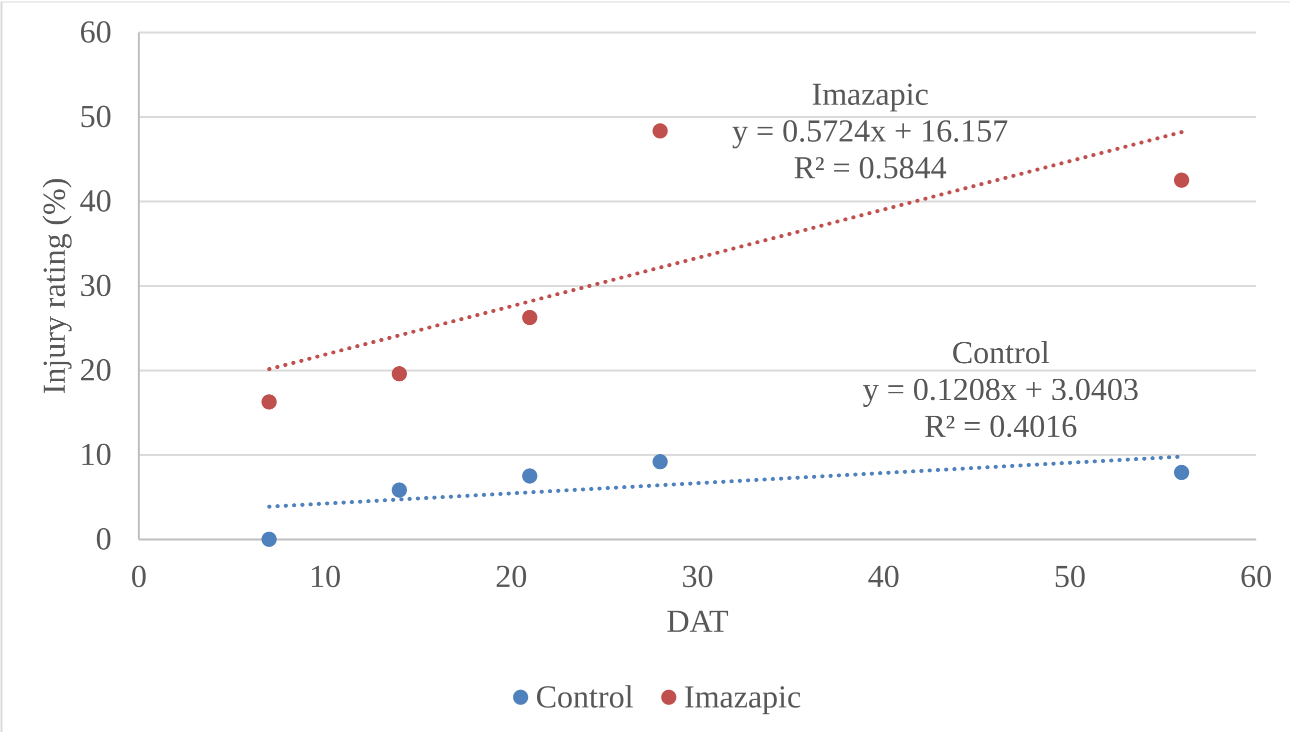
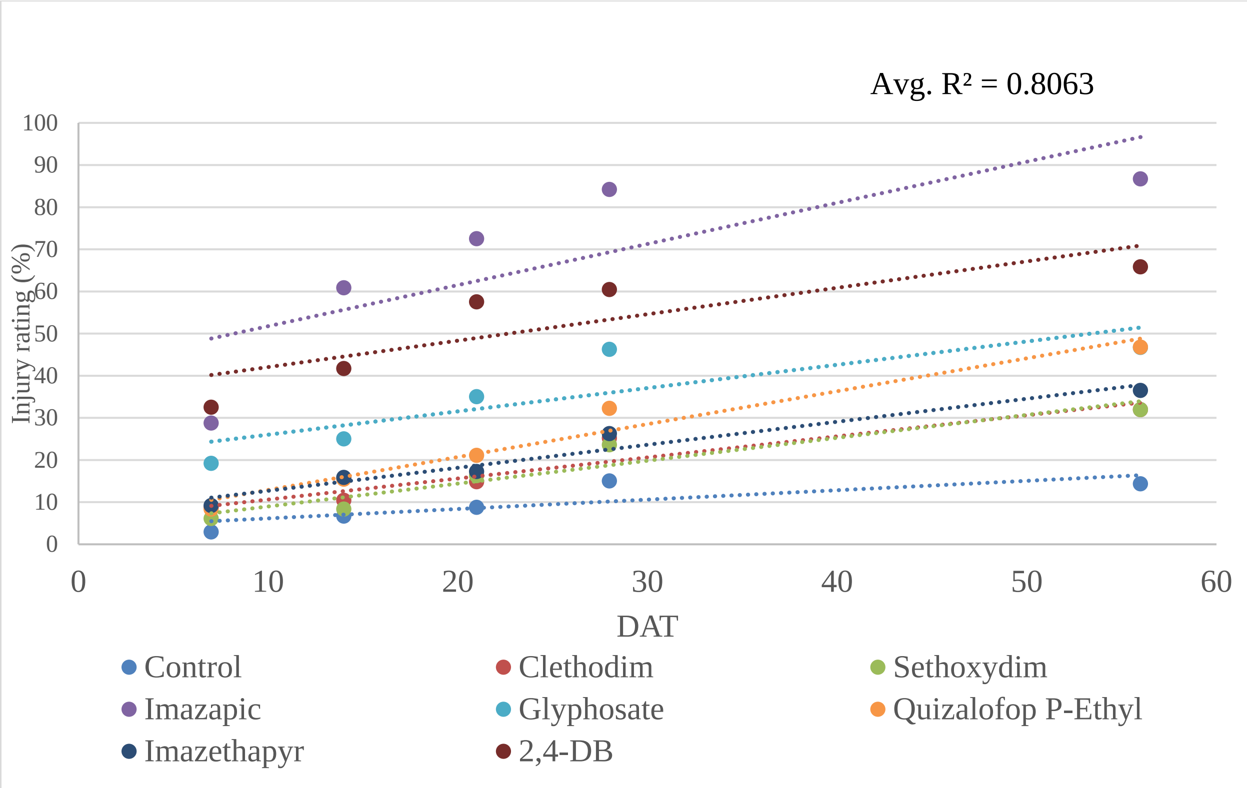
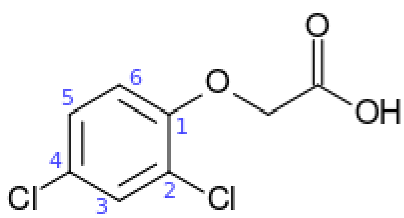


Figure 10. Greenhouse injury ratings by days after herbicide application.



Treatment	**linear equation**	**R^2**
control	y = 0.2228x + 3.9197	0.6735
Clethodim	y = 0.5x + 5.6185	0.8987
Sethoxydim	y = 0.543x + 3.5478	0.916
Imazapic	y = 0.9757x + 41.997	0.6157
Glyphosate	y = 0.5529x + 20.486	0.715
Quizalofop P-Ethyl	y = 0.7809x + 5.0866	0.9585
Imazethapyr	y = 0.5459x + 7.2436	0.9506
2,4-DB	y = 0.6269x + 35.785	0.7224

Figure 11. Microplot injury ratings by days after herbicide application.



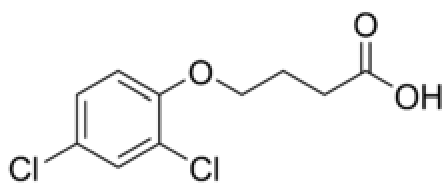


Figure 12. Chemical structures of 2,4-D (left) and 2,4-DB (right).

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