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# Evaluation of Competition Between Turfgrass and Trees in the Landscape

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EVALUATION OF COMPETITION BETWEEN TURFGRASS AND TREES IN THE

LANDSCAPE


by


Christopher Hendrickson


A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

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ABSTRACT

Evaluation of Competition Between Turfgrass and Trees in the Landscape

by

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Utah State University, 2008

Major Professors: Dr. Kelly Kopp and Dr. Heidi Kratsch
Program: Plant Science (Plant Physiology)

Population growth in regions of the Intermountain West has resulted in rapid growth of residential neighborhoods. In Utah, the landscapes associated with these expanding neighborhoods consume vast quantities of treated water. This is a concern in all states of the Intermountain West, as water becomes increasingly scarce. Traditionally used turfgrasses, trees and other plants in Intermountain West landscapes require significant amounts of supplemental water considering the intense sunlight, dry winds and sparse rainfall typical of the region. Characterizing the interactions between turfgrass and tree species in these landscapes can aid in the identification of candidate species that consume less nutritional and water resources, while maintaining satisfactory appearance.

A study was conducted investigating the nature of interactions between tree and turfgrass species in a constructed landscape of the Intermountain West. An experiment was performed investigating differences in rooting length and volume between

combinations of two tree (*Robinia pseudoacacia* L., *Gleditsia triacanthos* var. *inermis* L.) and three turfgrass [*Poa pratensis* L., *Buchlöe dactyloides* (Nutt.) Engelm., *Festuca arundinacea* Schreb.] species.  A minirhizotron system was used to obtain root images at three times during the growing seasons of 2006 and 2007 at depths from 1-15 cm in each tree-turfgrass rooting zone.  Images were analyzed to determine combined total volume, length, and surface area of turfgrass and tree roots.  This research shows that root growth differences occur in turfgrass-tree combinations containing all three turfgrass species.  Buffalograss best resisted possible root growth inhibition, regardless of tree combination.  Further evidence shows that *Robinia* secondary growth is vulnerable to presence of turfgrass in proximity.

(75 pages)

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Christopher Hendrickson

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CHAPTER 1

INTRODUCTION

As the Intermountain West continues to expand in population, landscapes will continue to grow in number (Bartlett et al., 2000). These residential and commercial landscapes often require large amounts of supplemental water and fertilization to ensure species survival under conditions of extreme seasonal temperature fluctuations and small amounts of precipitation.

Plants placed in landscapes compete both above and below ground for the resources they need to survive and grow (Wilson, 1993). Above ground, light and space are sought to ensure they capture enough light for photosynthesis. Below ground, plants must compete for water and nutrients. In the Intermountain West these resources are often scarce. While the nature and severity of competition varies among plants, competition may result in proliferation of one species at the expense of another (Hardin, 1960).

Below ground competition among plants can have many sources, effects, and responses in plants. Nitrogen-fixing symbiotic root nodules and allelochemical production can be seen in response to below-ground competition. While these have been characterized in individual plants as general responses to competition, very little is known about how resource competition in the root zone affects landscape plants.

Characterizing and describing these effects and responses to below ground competition in the landscape is critical for developing management programs that aid in selecting better adapted plants requiring less supplemental water and nutrients.

The objective of this study was to determine the effects of root-zone competition between turfgrasses and trees in landscapes of the Intermountain West using a minirhizotron system for non-destructive sampling.

CHAPTER 2

LITERATURE REVIEW

Recent population growth in regions of the Intermountain West has resulted in rapid growth of residential neighborhoods (Bartlett et al., 2000). Properties have increased in number, and ornamental landscapes usually accompany these properties. This is a concern in Utah, as water becomes increasingly scarce. In a constructed or natural landscape, plants growing together in close proximity will compete for critical resources needed for growth and maintenance. In the root zone of a constructed landscape, this may occur as trees and turfgrasses compete for water and nutrients. Many plants have evolved morphological or biochemical adaptations to this competition above and/or below the ground (Watson, 2004). The effects of this competition and plant responses to them are poorly understood, and little work has been done to quantify and understand the impacts of competition on plants in constructed landscapes.

Traditionally used turfgrasses, trees and flowers often require significant amounts of supplemental water due to intense sunlight, dry winds and sparse rainfall. Over half of the treated municipal and industrial water for many cities in the high-elevation, arid western United States is applied to residential and commercial landscapes (Stewart et al., 2004). In the Intermountain West, often the greatest demand on municipal water resources is from residential customers (Smith, 2004; Cerny et al., 2002). In some cases, ordinances and laws have been issued mandating the implementation of landscapes that consume less water (Landscape Management, 2004).

The nature of competitive interactions between trees and turfgrasses comes from their shared location of roots in the soil profile. Requiring oxygen for respiration, the roots of both turfgrasses and trees grow mostly in the top 15 to 20 cm of the soil profile (Stewart et al., 2004). Less oxygen at greater soil depths limits tree root growth, though some tree roots can be found at depths exceeding one meter (Jackson et al., 1999). As turfgrass and tree roots approach and encounter one another, interactions that are both competitive and beneficial may occur. The effects of this competition and plant responses to them are poorly understood, and little work has been done to quantify and understand the impacts of competition on plants in constructed landscapes. Rooting density of both trees and turfgrasses decreases as depth in the soil profile increases, and is found mostly in the top 30 cm of the soil profile (Kozlowski and Pallardy, 1997; Stewart et al., 2005; Turgeon, 2002). It is in this area of the root zone that the most competition for water and nutrients occurs in landscapes. In areas where these resources are limited, competitive effects may be even greater (Wilson, 1993). Analyzing the effects of root-zone competition can provide an understanding of how turfgrasses and trees respond to limited resources.

Some root interactions result in production and release of allelochemicals, in which one species' roots inhibit the growth of another species (Bertin et al., 2003). These compounds can be exuded into the soil solution or exist as volatiles. Allelochemicals are a common and powerful means of gaining a competitive advantage in ecological communities. Interactions between plants with an allelopathic capacity and those subjected to the compounds, often result in significant inhibition of growth of the exposed plant (Appendix B).

Other research has found that among competition with herbaceous plants, tree root systems develop deeper root systems when compared to trees not under competition (Peek et al., 2005). Ecological and forestry studies have highlighted the reduced survival of tree seedling survival from the presence of grass cover (Bush and Van Auken, 1990; Wagner et al., 1999). In these studies, seedling survival probability was shown to significantly decrease from competing turfgrass cover. Honey mesquite seedlings were found to have reduced biomass when grown in the presence of herbaceous cover, when given equal light and water (Bush and Van Auken, 1990). Studies of grassland and prairie ecosystems showed that the increased mortality rates among tree seedlings under grass cover were not sufficient to induce total tree exclusion (Scholes and Archer, 1997). Analysis of root-zone interactions between tree and herbaceous species also shows that tree biomass decreases under competition with grass roots (Wilson, 1993). Notably, less biomass reduction was seen, in experimental trees, in competition with other tree roots of the same species (Wilson, 1993). Belsky (1994) showed that under intense rainfall, trees were not able to extend roots deeper into the soil profile, beyond the reach of herbaceous plant roots. Conversely, in low-rainfall sites, trees did not compete as intensely for water and nutritional resources, as they were able to extend their root systems deeper into the soil profile, beyond the reach of herbaceous species roots.

In nearly all residential and commercially landscape areas, placement of turfgrasses and trees in close proximity to one another is common. Often, the turfgrass and tree species are adapted to very different climates. Frequent use of non-native trees in landscapes of the Intermountain West is largely a function of the lack of suitable tree species, with nearly all requiring irrigation (R. Kjelgren, personal communication; M.

Kuhns, personal communication). Many of the commonly used tree species are not native to Utah (M. Kuhns, personal communication). Turfgrass species commonly used in landscapes have widely variable water and nutrient needs depending on genotypic and environmental factors (Turgeon, 2002).

Plants produce allelochemicals to better compete for water and nutrient resources in the root zone. Allelochemicals are derived from modifications made to secondary metabolites present in plant tissues (Rice, 1984). These chemicals are produced by either decaying plant matter, volatilization from living plant tissues, or exudation from living tissues (Bonanomi et al., 2006; van Noordwijk, 1996). Allelochemicals are known to affect germination, growth, development, distribution, and reproduction of a number of plant species (Jose et al., 2006). Work has shown that a common trigger for production and release of these compounds into soils is insect herbivory (Thelen et al., 2005). Recent research has focused on the site of action for these allelochemicals. These physiological and biochemical approaches have sought to determine the mechanism(s) by which the compounds are hindering other plants' growth (Sanchez-Moreiras et. al, 2005). This study sought transcriptional responses to Arabidopsis plants subjected known allelochemical analogs. The up-regulation of these genes identified a novel set of genes that the plant used to combat and metabolize xenobiotic compounds. This work has shown that allelochemicals are found in the soil either from production by capable species root tissue, or by decomposing leaf litter. The compounds can be constantly produced, or produced due to predation by mammals or insects (Thelen et al., 2005). The effects of these compounds can be powerful, resulting in undesirable growth inhibition in vulnerable species (Rice, 1984). A well-known example of this is found in *Juglans nigra*

L. (black walnut). The tree exudes the allelochemical juglone from its roots and

decaying leaves (Rietveld, 1983) preventing the growth of some competing tree species

in close proximity.


*Minirhizotrons in Root Study*

A variety of techniques have been developed to study the rooting dynamics and

growth of trees and turfgrasses. However, virtually all of these techniques are destructive

to the plants. Destructive sampling can only offer data on the plants' root system at a

particular moment in time, which may not be representative of how the plants' root

systems behave through the course of a year. Rhizotron facilities allow the visual

monitoring of root growth through windows to study plant root systems, but these

facilities can be costly. Replication may be limited as a consequence. Addressing these

limitations, minirhizotron systems were developed, reducing experimental cost and

improving replication (McMichael and Taylor, 1987). The minirhizotron system has

been used with success in the study of root systems of plants for various purposes. This

nondestructive means of root imaging allows precise data to be extracted from dynamic

root systems over time. Root images captured with minirhizotron systems are analyzed

by software through which physical data can be assessed (Ingram and Leers, 2001).

Minirhizotron systems consist of a high-magnification camera inserted into a clear tube

placed in the soil of the root zone of plants to be studied. Visualized by connection to a

field computer, either video or still images of the plant roots are analyzed. Furthermore,

minirhizotron systems allow for frequent, non-destructive data collection from root

systems over time (Liu and Huang, 2002). Currently, available software allows for

analysis of the images collected using minirhizotrons and export of data for further work (Ingram and Leers, 2001). Programs such as WinRhizo™ and RooTracker™ allow for a computer operator to load images from the minirhizotron system for digitization. In this process, physical dimensions are calculated and assigned to all roots of all images. Further, these programs allow for the study of root architecture by tracking the growth of lateral roots off primary roots, designated by the operator. It is the combination of the minirhizotron system, with this software that has allowed for successful long-term study of root system dynamics.

Along with the camera and image collection computer, the minirhizotron system is also comprised of clear tubes through with the camera is inserted. Roots encountering these tubes are captured and analyzed. Tubes vary greatly in composition, orientation to target plants, length, shape and width/diameter. Research objectives can lead to different image collection practices. Intervals of 2, 5 or 10 cm could be used when collecting images with the minirhizotron system. To ensure maximum accuracy in repeated session imaging with minirhizotron systems, outsides of tubes are commonly marked with tape or etched marks of precise distance intervals. If images of identical locations over time diverge by even one or two millimeters, data analysis can be rendered inaccurate. This would lead to a data set that doesn't represent the actual dimensions of the root system.

Computation of root physical dimensions provides a sufficient description of rooting quantity and depth, which correlate to the plants' relative ability to extract water and nutrients from the soil profile (Ho et al., 2004).

*Turfgrass Roots*

Minirhizotrons have been used extensively in turfgrass studies since their introduction. Murphy et al. (1994) evaluated minirhizotron methods for measuring rooting of creeping bentgrass (*Agrostis stolonifera* L.) and annual bluegrass (*Poa annua* L.). They compared the method with destructive core sampling techniques for evaluation of root length and root weight densities and observed a seasonal pattern of turfgrass root development over time. The minirhizotron method was found to be suitable for studying root phenology and profile distribution of turfgrasses. The technique also gave a reasonable measurement of root quantity throughout the growing season (Murphy et al., 1994). Liu and Huang (2002) used the minirhizotron technique to study mowing effects on root production, growth, and mortality of creeping bentgrass. They found that low mowing height was found to decrease new root production and to increase root mortality for both cultivars, and low mowing increased the ratio of dead to new roots in length and number. The decreased root production and increased mortality caused by low mowing, in turn, had the potential to lead to water deficits and nutrient deficiencies (Liu and Huang, 2002). Further exploring effects of water deficit, Fu et al. (2003) evaluated root growth of tall fescue (*Festuca arundinacea* Schreb.) exposed to deficit irrigation during the summer with a minirhizotron system. Severe deficit irrigation applied to the turfgrass was found to increase total root number, illustrating the plants' compensation mechanisms (Fu et al., 2003).

*Tree Roots*

Tree rooting has been studied extensively in forest environments. Studies have characterized tree root growth as a result of changes in soil nutrient or gas levels of a forest ecosystem (Jose et al., 2006; Joslin et al., 2000; Kern et al., 2004). Work dating back over 10 years describes the forest nutrient flux in terms of carbon allocation and flow (Fogel, 1990; Jackson et al., 1999; Johnson et al., 2000; Thomas et al., 1996). Analysis of root dynamics with minirhizotrons was performed in forest settings, in which effects of elevated soil $CO_2$, nitrogen fertility and time have been examined on pine and fir forest rooting characteristics (Johnson et al., 2000). Other work has addressed the nutrient and water needs of trees in the urban and suburban environment. Kjelgren and Montague (1998) evaluated the effects of features in the urban landscape on commonly planted trees. The increased temperature and long-wave radiation associated with paved surfaces proved to have a substantial effect on *Acer platanoides* L. and *Fraxinus americana* L. water demand and water flux.

Tingey et al. (2003) used minirhizotrons to determine optimal sampling frequencies for evergreen and deciduous tree species. Using a minirhizotron system, Douglas fir (*Pseudotsuga menziesii* Mirb.) and littleleaf linden (*Tilia cordata* Mill.) were studied at sampling frequencies of 1, 2, 4 or 8 weeks. The least frequent sampling was found to underestimate production and mortality of roots. Thomas et al. (1996) used minirhizotrons to evaluate the seasonal root distribution of Monterey pine (*Pinus radiata* D. Don) grown at ambient and elevated carbon dioxide concentrations. Elevated carbon dioxide levels were found to increase root carbon density (88%) within 0.15 m radius of the trees compared with ambient rhizospheric carbon dioxide levels (35%). Root carbon

density, or root carbon dioxide flux density, measures respirative capacity per unit area of root tissue.

Turfgrasses and trees are not found growing together in significant numbers or sizes anywhere in nature, with a few exceptions (Watson, 2004). Some climatic, soil, and anatomical factors influence the effects of this competition for both plant types (Hernandez-Leos, 1998). Inevitably, the branches and leaves of trees growing near turfgrasses will intercept much of the light that otherwise would reach the turfgrass (Fry and Huang, 2004), creating a poor environment for turfgrass growth.

Nodulating trees have access to a larger pool of available nitrogen (N) than do non-nodulating trees. The N-fixing process occurring in the root nodules allows for trees to gain access to atmospheric N. If trees and turfgrass are planted together, competition for N may occur (Hernandez-Leos, 1998). With access to a larger source of N, nodulating trees would likely have a competitive advantage relative to non-nodulating trees, assuming supplemental N was not applied. For this reason, a nodulating tree species such as black locust (*Robinia pseudoacacia* L.) might gain a competitive advantage over other tree species either with or without turfgrass presence. The proliferation of the nodulating tree species black locust has been found to inhibit regeneration of pine forests in coastal sandy soils by outcompeting pines for space and N resources due to N fixation (Taniguchi et al., 2007). Black locust is also found thriving in large monocultures in Japan (Nasir et al., 2005). Black locust is an N-fixing tree, hosting *Rhizobium* bacterial symbionts in its root nodules. Black locust undergoes very rapid juvenile growth, and has a N-fixing capacity of 30-35 kg ha$^{-1}$ year$^{-1}$ (Boring and Swank, 1984). It is an early succession species, with the ability to form large stands that

rapidly out-compete nearby plants (Nasir et al., 2005). Honeylocust is not a nodulating tree (Dirr, 1998). While it lacks the ability to fix atmospheric N, it establishes well in most climates found in the continental United States and is commonly used in landscapes.

Minirhizotrons have rarely been used, however, for the below-ground study of resource competition. Peek et al. (2005) used a minirhizotron system to study fine root mortality and distribution related to resource competition among two native grasses and Big Sagebrush in the Great Basin desert. While they did not directly compare species to one another, they did find that water availability did not correlate to fine root persistence in the species studied. Hernandez-Leos (1998) found that Norway maple (Acer platanoides L.) tree rooting was altered in the presence of turfgrass or barley competition. This study determined that maple root systems produced far more biomass at greater depths in the soil profile when there was herbaceous competition compared to trees in mulch-covered plots. These findings suggest a stress-response by the tree from the competition, possibly affecting tree resource acquisition in the soil profile.

Spatial and temporal rooting dynamics of two tree species and three turfgrass species grown in various combinations were evaluated with a minirhizotron system with the objective of determining the effect of tree and turfgrass combination on root growth.

CHAPTER 3

MATERIALS AND METHODS

*Plot Layout and Maintenance*

This study was conducted in 2006 and 2007 at the Utah State University

Greenville Research Farm in North Logan, Utah (41°7'N, 111°8'W).  The experiment

was arranged in a randomized, split-plot design with turfgrass species serving as the

whole-plot factor and tree species serving as the subplot factor.  Twenty plots measuring

6 × 6 m were installed on a Millville loam soil (a coarse-silty, mesic Typic Haploexeroll).

Whole-plot factors included tall fescue (*Festuca arundinacea* Schreb.), buffalograss

[*Buchlöe dactyloides* (Nutt.) Engelm.], Kentucky bluegrass (*Poa pratensis* L.) and

mulched plots with trees but no turfgrass cover.  Each main turfgrass plot was split into

two subplots containing one of two tree species, which served as the subplot factor

(Figure 1), including nodulating black locust (*Robinia pseudoacacia* L.) and non-

nodulating honeylocust (*Gleditsia triacanthos* var. *inermis* L.) trees.  Buffalograss and

Kentucky bluegrass plots were seeded and established in September 1998.  Tall fescue

plots were established from sod in April 2005.  In April 2005, one black locust (*Robinia

pseudoacacia* L.) and one honeylocust (*Gleditsia triacanthos* L.) tree was installed in

opposing corners of each plot (5 m from each other).  The 20 plots included eight

replications of buffalograss, four replications of tall fescue, four replications Kentucky

bluegrass, and four replications of mulch-covered plots.  Eight buffalograss replications

were included due to lack of space to include turfgrass controls, and to collect

additional data on the native buffalograss.

| | | | |
|---|---|---|---|
| TF | KBG | B | B |
| None | None | B | TF |
| B | TF | KBG | KBG |
| KBG | B | TF | B |
| B | B | None | None |

**N**

▼

Figure 1. The arrangement of the plots with letters to indicate whole plot factor. B,

'None', TF and KBG represent the whole plot factor, turfgrass coverage. 'B'-

Buffalograss; 'TF'-tall fescue, 'KBG'-Kentucky bluegrass, 'None'-plots covered only

with mulch. 'N'-North.

Two 90- × 5-cm minirhizotron tubes were installed at the base of each tree in each

plot for imaging of root systems of both tree and turfgrass species. Plots were hand-

weeded as needed and were mowed weekly at a height of 7.62 cm. Plots were fertilized

once annually with only 0.2441 kg N per 100 $m^2$ to encourage root nodule formation.

Plots were irrigated both years at 80% evapotranspiration (ET) replacement.

Minirhizotron (Bartz Technology, Carpinteria, CA) installation and use followed Murphy

et al. (1994). Tubes were installed April 2005, into the soil 20 cm from tree trunks in a

lateral orientation and at a 30° angle downward from the soil surface. Root-zone images

were captured at 1-cm increments down the first 30 cm of each tube using the

minirhizotron camera. Images were acquired at eight-week intervals in June, August, and

October (Sessions 1, 2, and 3, respectively) of 2006 and 2007. Turfgrass quality ratings

were assessed at each imaging session to quantify overall vigor and health, with 9 being

the best quality rating and 1 being the worst (Skogley and Sawyer, 1992).

*Minirhizotron System*

Two clear cylindrical CAB minirhizotron tubes (90-cm-long, 5-cm-diameter)

were inserted 20 cm from the base of the trunk of each tree into the soil using a metal

guide tube (Figure 2) to prevent scratching of tubes during insertion (Johnson et al.,

2001). Use of a guide tube also minimized soil compaction around the tube perimeter,

and established good soil-to-tube contact (Johnson et al., 2001). The tubes were placed at

30° angles into the soil surface, lateral to tree trunks 15 cm deep (Figure 2). This

placement was used to provide representative images of both primary and fine roots

originating from, and extending away from the trunk base. Open ends of all tubes at the

soil surface were covered between image-collection sessions with a polyvinyl chloride

(PVC) end-cap over the above-ground end of the tube. This was done to prevent

irrigation water, rain water and light from entering the tubes, as well as minimizing

interior condensation accumulation. Prior to each imaging session, tubes were cleaned of

any internal condensation or dirt with a 9.5-mm pile paint-roller (washed and cleaned

each time) placed on the end of a 2-m pole.  Upper ends of tubes were located

approximately one inch above the soil line.
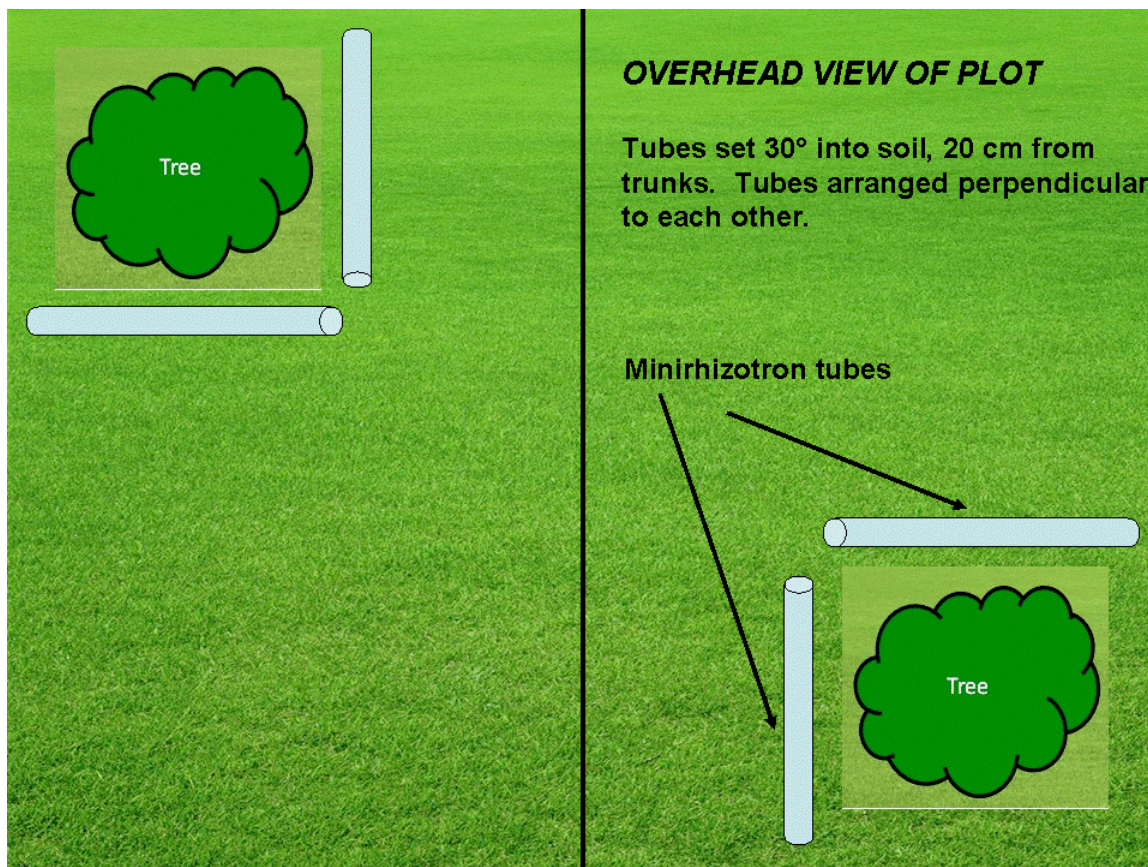


Figure 2. Placement of minirhizotron tubes in plots. Vertical line indicates split plot.

Tubes measure 40 × 5 cm, inserted 30° into soil.

Tubes were set at right angles to one another (Figure 2).  Top ends of access tubes

were 1 m apart.  The camera included with the minirhizotron system (BTC 100-X, Bartz

Technology Corp., Carpinteria, CA) was used to take still images of the roots.  Due to the

tube insertion angle of 30°, images taken at a tube length of 30 cm correspond to a soil

depth of 15 cm. With this tube insertion angle, imaging of soil at depths to one-half the length of the tube can occur. Deeper measurements would have required a greater angle of insertion.

For measurements, the camera was moved manually down the tube with an attached indexing handle. With the majority of competition between turfgrasses and trees expected in the top 30 cm of the soil profile (Kozlowski and Pallardy, 1997; Stewart et al., 2005; Turgeon, 2002), we chose to collect data in 1 cm intervals from the upper 15 cm of the soil profile. This interval could be varied based on the research question(s) being asked and the design of the experiment. Our measurements represented combined tree and turfgrass roots visible in each tube. Tree and turfgrass measurements were combined due to difficulty in distinguishing between tree and turfgrass species roots at all soil depths. Cellulose acetate butyrate tubes were chosen to improve root pigmentation, which can aid root species distinction (Withington et al., 2003), but this was not apparent in our study. We analyzed images to determine root physical dimensions using RooTracker™ v. 2.03 software (Duke University, Durham, NC).

*Root Dimensional Analyses*

Before analysis of minirhizotron images, RooTracker™ was calibrated by loading an acquired image of a metric ruler into the program. A diameter measurement was performed by inserting a user-drawn 1-cm-diameter circle on the ruler contained in the image. The program was calibrated to equate 66 pixels to 1 cm. This allowed RooTracker™ to assign dimensions to measured roots in acquired images (Figure 3). First we traced the root length with a series of connected lines along the center of each

root. The program then summed the pixel length of all traced lines for the root. We assigned a root diameter by tracing circles to the two-dimensional width of each root in each image.



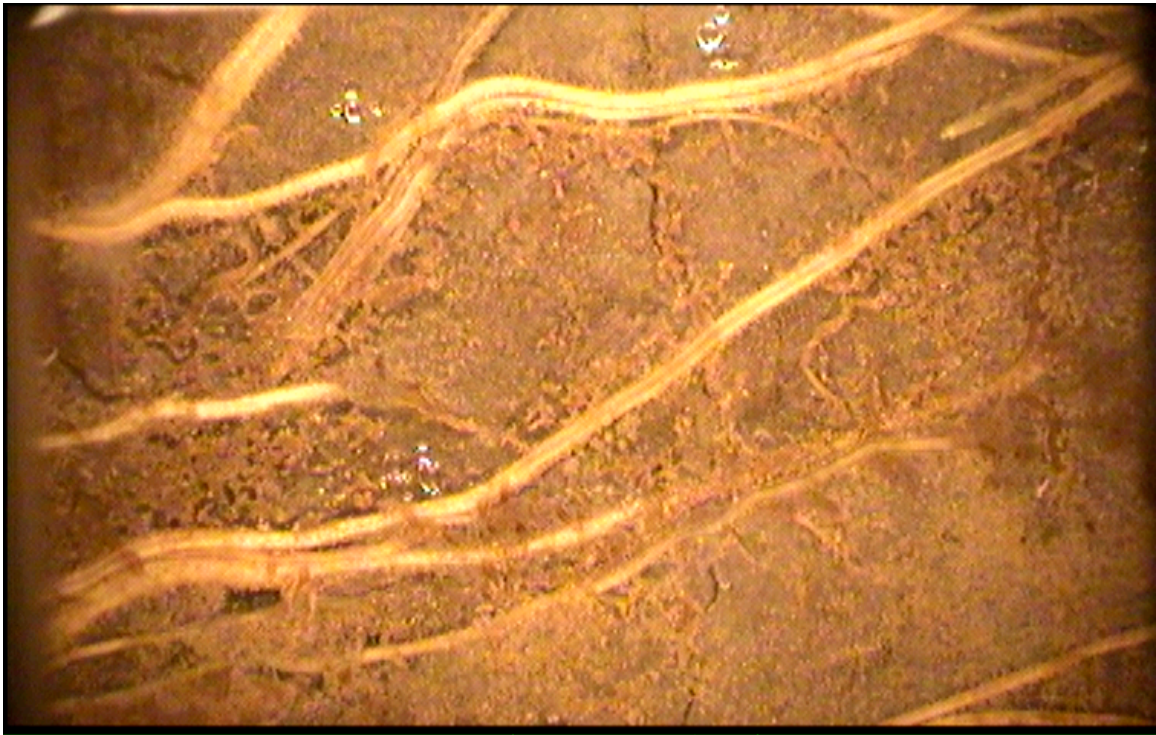Figure 3. A sample minirhizotron image, from a buffalograss-black locust plot, from 10 cm soil depth.

If the root changed diameter along its length, we traced multiple circles at each unique diameter section of the root. RooTracker™ then computed a differential diameter across root length from circle to circle. RooTracker™ computed root volume using the following formula:

$$Volume = \pi r^2 \times length$$

in which 'r' represents the root radius, 'length' represents the root length, and

'Volume' represents the computed root volume of an individual tree or turfgrass root.

To assess combined tree and turfgrass total root length (TRL) and volume (TRV), images were analyzed with RooTracker™ 2.03 digitizing software. Total root volume and TRL were acquired by summing all root volumes and lengths in each frame image. These values were then summed for all thirty frame images in each tube. These summed values represented combined TRV and TRL of both tree and turfgrass roots encountering the tubes. The TRV and TRL values were combined due to the difficulty in sufficiently distinguishing between tree and turfgrass species roots, particularly at depths exceeding 10 cm. Therefore, each frame image was treated as a composite of tree and turfgrass roots. Tree roots' total combined volume and length from mulched-plots were used to provide approximate maximum root content in turfgrass-covered plots.

Tree caliper measurements (cm) were recorded at diameter breast height (dbh) prior to each imaging session. Tree secondary growth (TSG) was determined by calculating the difference in tree caliper measurements from June 2006 to October 2007. Monthly mean maximum temperature and total monthly rainfall were recorded with an on-site weather station (Campbell Scientific ET 106, Campbell Scientific, Logan, UT).

*Statistical Analyses*

The experiment was statistically analyzed using PROC MIXED repeated measures analysis (SAS Inst., 2004). Turfgrass and tree species, as well as session of measurement were treated as fixed variables and replication was a random variable. The analysis was repeated by session of measurement. Data were log transformed to meet the

NID (equal variance and normal distribution of residuals) assumptions of analysis of variance.  Pair-wise comparisons of means were made using Saxton's 'pdmix800.sas' macro (Saxton, 1998). These analyses were repeated for tree secondary growth (TSG) measurements.

CHAPTER 4

RESULTS AND DISCUSSION

*Root Data*

Turfgrass species, tree species, and their interactions significantly affected TRV and TRL depending on session (Tables 1 and 2).  The interaction of treatment factors was significant for TRL, in June of 2006 and October of 2007 only.  The interaction of treatment factors was significant for TRV, in June and August of 2006 only (Tables 1 and 2).  Turfgrass species significantly affected TRL over the entire course of the experiment, but only significantly affected TRV in June and August of 2006.  Total root length and TRV was significantly lower in the mulch-covered plots than in all other plots, though not always significantly different (Figures 4 and 5).  In Kentucky bluegrass–honeylocust combinations, TRL and TRV fluctuated more than any other honeylocust-containing plots (Figure 4) in 2006.  Over the course of the experiment, TRL means for mulch-covered plots were significantly lower than those of tree-turfgrass plots (Table 3).  This was not the case for mulch-covered plot TRV means (Table 4).  Mean TRL and TRV differences between buffalograss and tall fescue in combination with both tree species were not statistically different in any session of measurement (Tables 3 and 4).  Tree secondary growth was significantly affected by tree species, turfgrass species and their interaction (Table 5).  All three turfgrass species affected lower secondary growth in black locust relative to mulched plots, while Kentucky bluegrass presence resulted in near-double the secondary growth inhibition of buffalograss and tall fescue (Figure 6 and 7).

Table 1. Summary of analyses of variance indicating significant source effects on combined turfgrass and tree total root length (cm) differences by session.

| | | 2006 | | | 2007 | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| source | df | session 1 | session 2 | session 3 | session 1 | session 2 | session 3 |
| turfgrass | 3 | *** | *** | *** | *** | *** | *** |
| tree | 1 | *** | NS† | NS | NS | NS | NS |
| turf × tree | 3 | *** | NS | NS | NS | NS | * |

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

† NS = not significant.

Table 2. Summary of analyses of variance indicating significant source effects on combined turfgrass and tree total root volume ($cm^3$) differences by session.

| | | 2006 | | | 2007 | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| source | df | session 1 | session 2 | session 3 | session 1 | session 2 | session 3 |
| turfgrass | 3 | * | *** | NS† | NS | NS | NS |
| tree | 1 | NS | ** | NS | NS | NS | NS |
| turf × tree | 3 | * | *** | NS | NS | NS | NS |

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

† NS = not significant.

Table 3. Turfgrass total root length (cm) means by session of measurement.

|  | 2006 |  |  | 2007 |  |  |
|---|---|---|---|---|---|---|
| turfgrass | session 1 | session 2 | session 3 | session 1 | session 2 | session 3 |
| B‡ | $270.671^{B}$ | $626.662^{A}$ | $522.546^{A}$ | $569.416^{A}$ | $649.934^{A}$ | $696.592^{A}$ |
| KBG† | $860.067^{A}$ | $559.937^{A}$ | $488.501^{A}$ | $581.923^{A}$ | $689.226^{A}$ | $811.084^{A}$ |
| TF† | $232.403^{B}$ | $527.937^{A}$ | $545.297^{A}$ | $585.741^{A}$ | $669.292^{A}$ | $747.170^{A}$ |
| None (Mulch)† | $9.595^{C}$ | $21.119^{B}$ | $14.636^{B}$ | $17.825^{B}$ | $20.530^{B}$ | $25.853^{B}$ |

† *N*= 4, unless otherwise indicated.

‡ *N*= 8.

Note: All means of tree / turfgrass combinations followed by the same letter within each session group, are not significantly different at *p* =0.05 according to Fisher's least significant difference test.

HL–honeylocust, BL-black locust, B-buffalograss, KBG-Kentucky bluegrass, TF-tall fescue, None-mulch-covered

*Tree Caliper Measurements*

There was a significant interaction among tree × turfgrass species on TSG over the course of the experiment (Table 7). All black locust trees in the mulch-covered plots had greater TSG than all black locust trees in plots containing turfgrass (Figure 3). Honeylocust TSG was significantly inhibited in competition with buffalograss relative to mulched control plots. Some physical damage occurred to black locust trees in both 2006 and 2007 due to wind damage and predation by the locust borer (*Megacyllene robineae* Forster).

Table 4. Combined tree and turfgrass total root volume ($cm^3$) means by session of measurement.

| Turfgrass / tree combination[†] | 2006 | |
|---|---|---|
| | session 1 | session 2 |
| HL-Mulch | 0.849[B] | 3.098[CD] |
| HL-B[‡] | 6.473[B] | 9.375[BCD] |
| HL-TF | 14.336[B] | 15.813[B] |
| HL-KBG | 40.633[A] | 43.553[A] |
| BL-Mulch | 0.316[B] | 1.378[D] |
| BL-B[‡] | 9.253[B] | 12.211[BC] |
| BL-TF | 11.128[B] | 14.865[B] |
| BL-KBG | 7.801[B] | 7.65[BCD] |

† $N$= 4, unless otherwise indicated.

‡ $N$= 8.

Note: All means of tree / turfgrass combinations followed by the same letter within each session, are not significantly different at $p$ =0.05 according to Fisher's least significant difference test.

HL–honeylocust, BL-black locust, B-buffalograss, KBG-Kentucky bluegrass, TF-tall fescue, Mulch-mulch-covered.
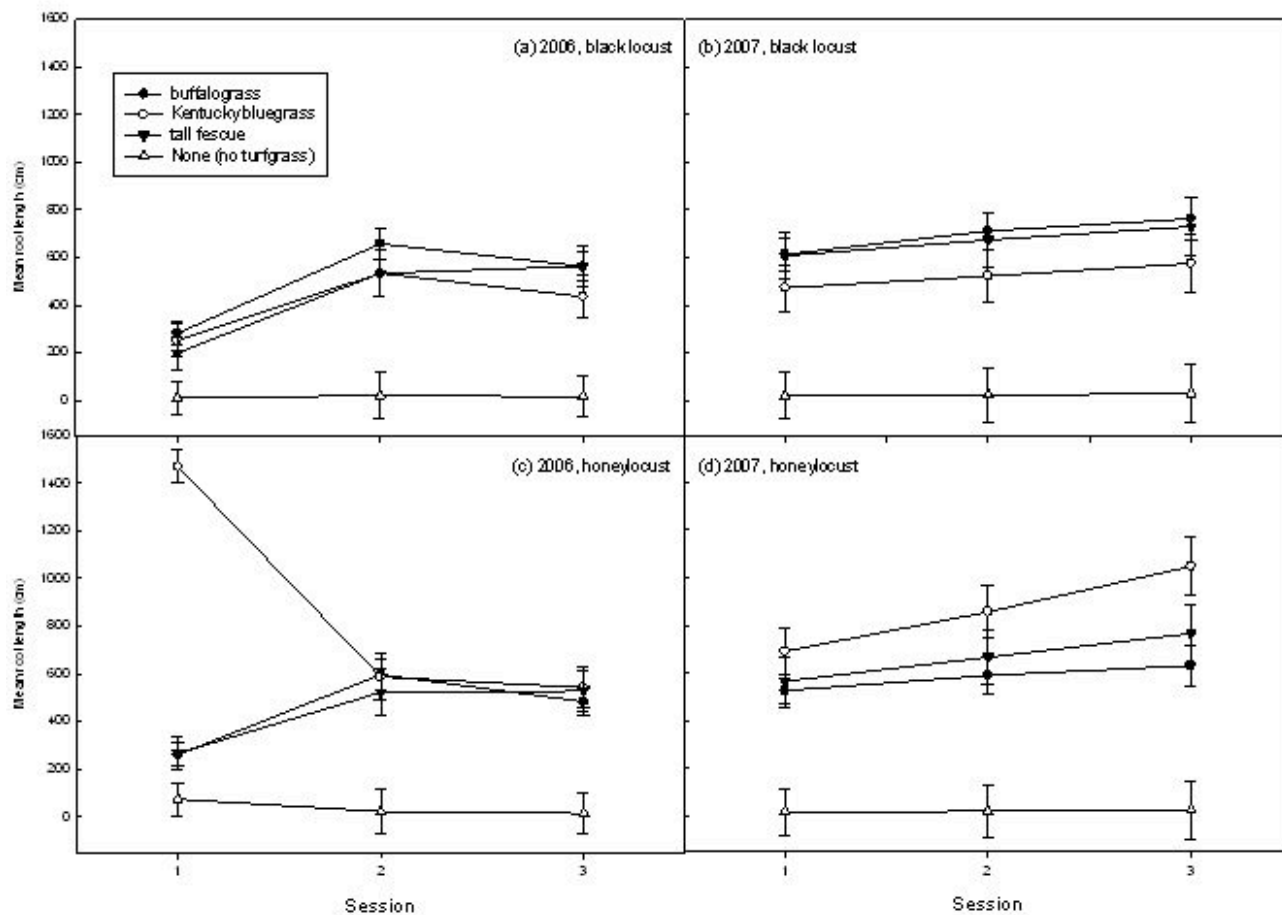
Figure 4**.** Combined turfgrass and tree total root length (TRL) means by session for 2006 and 2007 (cm).  Vertical bars represent standard error of means.
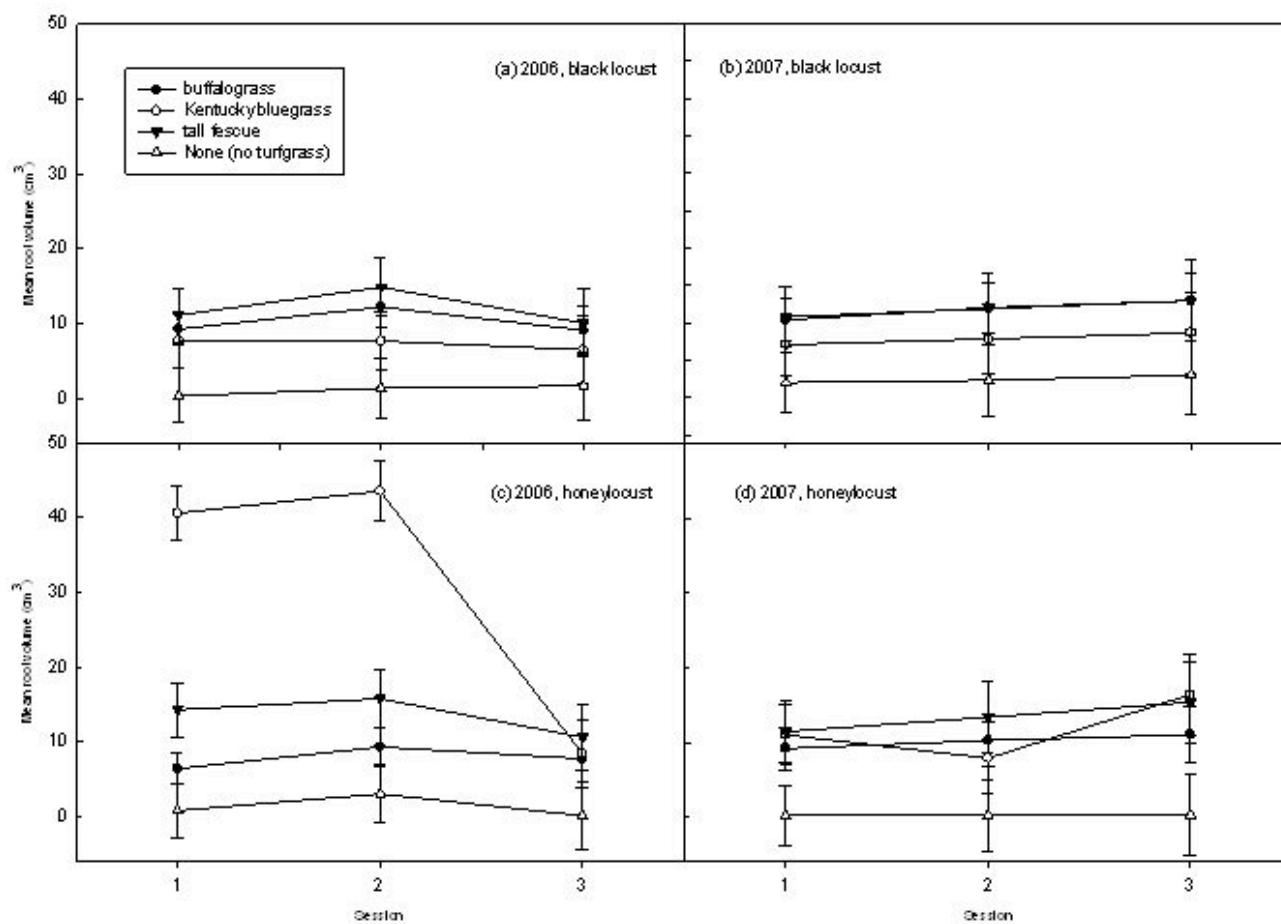
Figure 5. Combined turfgrass and tree total root volume (TRV) means by session for 2006 and 2007 ($cm^3$). Vertical bars represent standard error of means.

Table 5. Summary of analyses of variance indicating significant source effects on tree secondary growth (cm) (TSG) from 2006 to 2007.

| source | df | TSG |
| --- | --- | --- |
| tree | 1 | *** |
| turfgrass | 3 | ** |
| tree × turfgrass | 3 | * |

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

Tree and turfgrass species and tree × turfgrass interactions impacted tree secondary growth in this experiment (Table 7).  Growth among the three turfgrass species reduced secondary growth in black locust (Figure 6).  This is consistent with previous findings by Stewart et al. (2005) who cited that both buffalograss and Kentucky bluegrass inhibited successful establishment of littleleaf linden trees (*Tilia cordata* Mill.).  Due to shared deep rooting characteristics with buffalograss (Turgeon, 2002), tall fescue may have had the same effects on tree establishment and subsequent growth in this study. Hernandez-Leos (1998) also found that Kentucky bluegrass inhibits tree root growth and establishment.  Only buffalograss competition inhibited secondary growth of honeylocust (Figure 7).  Of all black locust trees, those grown in control plots without a turfgrass cover achieved the most secondary growth (Figure 6).  Of all honeylocust trees, only those in competition with buffalograss exhibited significantly reduced TSG relative to the

mulched-plots (Figure 7). The higher growth rate of black locust trees in mulch-

covered plots suggests the presence of competitive effects, though this cannot be proven.
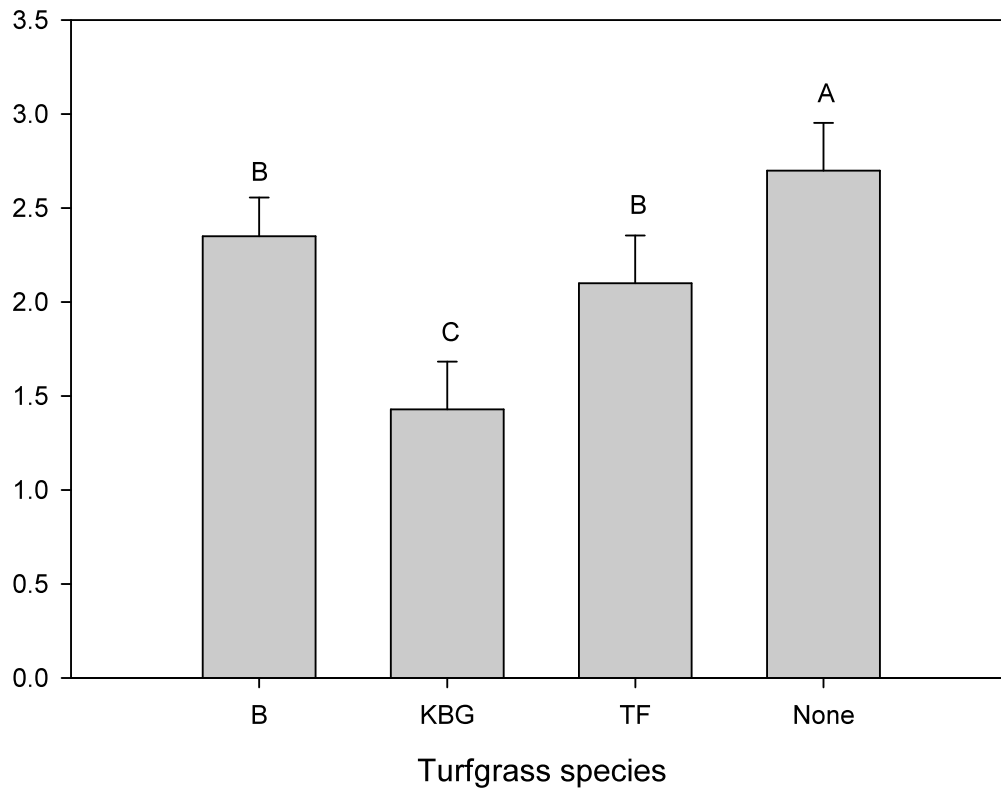


Figure 6. Mean secondary growth (cm) of black locust trees in combination with

turfgrasses from June 2006 to October 2007.

B-buffalograss, KBG-Kentucky bluegrass, TF-tall fescue, None- mulch-covered

Note: All means of tree / turfgrass combination followed by the same letter are not significantly different ($p = 0.05$) according to Fisher's least significant difference test.
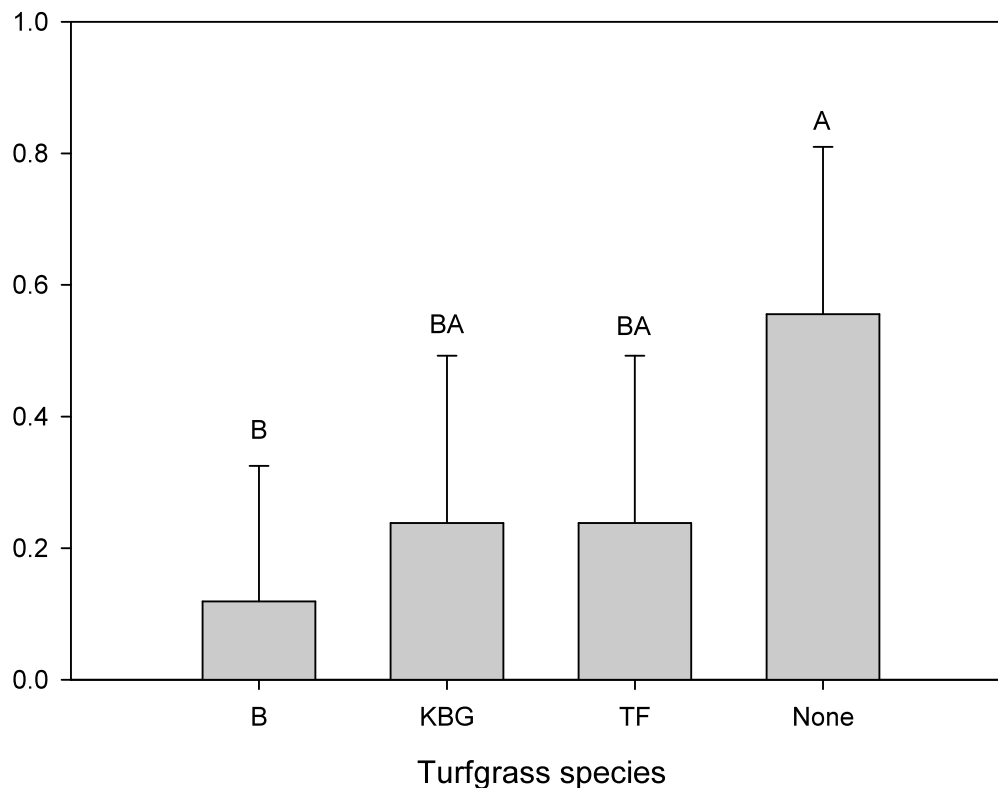
Figure 7.  Mean secondary growth (cm) of honeylocust trees in combination with

turfgrasses from June 2006 to October 2007.

B-buffalograss, KBG-Kentucky bluegrass, TF-tall fescue, None- mulch-covered

Note: All means of tree / turfgrass combination followed by the same letter are not significantly different ($p = 0.05$) according to Fisher's least significant difference test.

*Climate*

June and July of 2006 did not have as much rainfall as June and July of 2007

during peak heat stress (Figure 8).  Monthly mean maximum temperatures were nearly

identical, peaking at 33-34°C in July of both years.
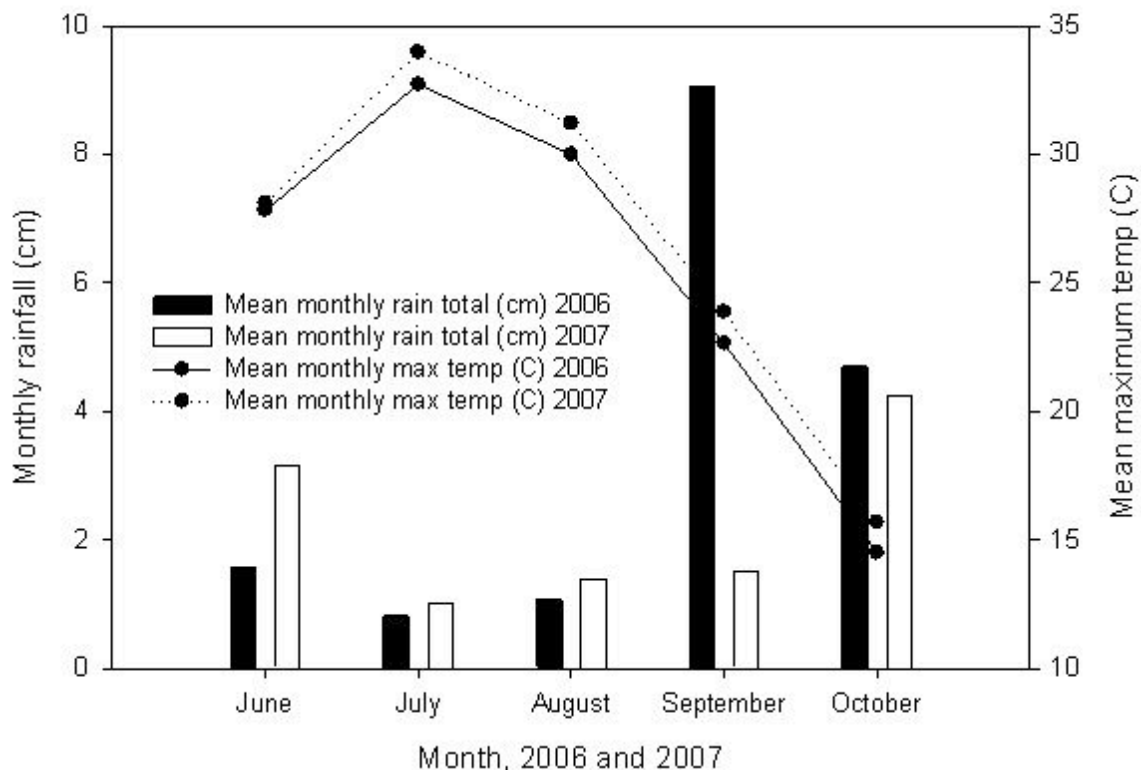
Figure 8. Mean monthly maximum temperature (°C) and total monthly rainfall (cm) for the 2006 and 2007 growing season. Mean temperature calculated as average of high temperature of each 24-hour period per month.

*Visual Responses*

Differences observed in TRV and TRL did not manifest above ground for the turfgrasses, as turfgrass ratings were consistent with expected seasonal declines and recoveries. Ratings were performed following the procedures described in Skogley and Sawyer (1992).

Due to low amount of tree roots identified in all plots, conclusions regarding the presence and effects of competition between the trees and turfgrasses could not be made. However, some indirect data suggests that competitive interactions could have occurred

between some of the turfgrasses and trees in our experiment. Kentucky bluegrass

rooting was inhibited by presence of black locust trees. Further, tall fescue and

buffalograss exhibited consistent root length and volume. Finally, buffalograss only

inhibited secondary growth of honeylocust, while all turfgrass presence inhibited

secondary growth of black locust relative to trees in mulch covered plots.

Total root volume and TRL in plots containing Kentucky bluegrass was lower in

the presence of black locust trees relative to honeylocust trees (Figure 4 and 5), though

not always significantly different. Sullivan et al. (2000), found that Kentucky bluegrass

root systems consisted of >80% fine roots (<0.2 mm diameter), and these roots were

positively correlated to the plants' total N uptake rate. Our results are consistent with

these findings, given the presence of competing roots from black locust trees. Evidence

for black locust root presence was found in mulch-covered plots. Mulched plot TRL

means were nearly 15 cm in October 2006 and 26 cm in October of 2007 (Figure 4). The

N-fixing ability of black locust may have allowed for more N uptake, and consequent

growth (Boring and Swank, 1984) compared to Kentucky bluegrass. Secondly, reduction

in TRV and TRL of black locust and Kentucky bluegrass plots, compared to buffalograss

and tall fescue, may be a function of Kentucky bluegrass's native climate. A native grass

of Europe and northern Asia (Casler and Duncan, 2003), Kentucky bluegrass is uniquely

adapted to climates with higher annual precipitation and lower mean monthly summer

temperatures than those of the 2006 and 2007 growing seasons (Figure 8). This may

exacerbate growth inhibition of roots by nodulating black locust roots. Taking advantage

of its adaptation to higher soil moisture content from more frequent rain events, Kentucky

bluegrass developed a highly dense system of fine roots. Such a shallow network of fine

roots has been shown to undergo severe dieback during heat stress (Bonos and Murphy, 1999; Turgeon, 2002). The Kentucky bluegrass in this experiment was exposed to stress-inducing temperatures both 2006 and 2007.

Total root volume and TRL of buffalograss and tall fescue were more consistent over time than with Kentucky bluegrass in the presence of black locust trees (Figure 4). Klingenberg (1992), found that buffalograss frequently produces fine root mass at depths up to and exceeding 0.9 m. Fu et al. (2007) found that tall fescue produces significantly more root length and surface area at up to 18 cm soil depth under a 20% ET replacement irrigation regime, compared to 60 and 100% ET irrigation regimes. The superior rooting depth potential of tall fescue and buffalograss, may explain our consistently greater TRL and TRV across all sessions in these plots (Klingenberg, 1992; Qian et al., 1997; Weaver, 1958). Huang (1999) found that buffalograss produced roots well below 30 cm in depth even under localized soil drying. Our results were consistent with these findings, in that tall fescue and buffalograss may have avoided the effects of competition with the nodulating tree because their root systems can often extend to a depth below many of the black locusts' fine roots (Huang, 1999; Qian et al., 1997), in contrast to mulch-covered plots. Differences in TRL versus TRV and their significance in the same imaging sessions suggest the presence of tree roots (in the amounts measured in mulch-covered plots). With larger diameters observed than turfgrass, tree roots would maintain a high TRV as TRL drops. This could result from turfgrass root dieback during peak summer heat stress. Mulch-covered plots offered an approximation of maximum tree root length and volume without the competitive effects of turfgrass presence. While still present,

tree root length and volume in turf-covered plots were likely reduced, to different

extents, given the effects of competition on each tree from the turfgrass cover.

CHAPTER 5

CONCLUSIONS

Competitive effects may have existed between the turfgrass and tree species in this experiment, but this cannot be proven.  Provided a consistent means of species identification, our system may be used to further study competitive interactions in the root zone of constructed landscapes.  Despite this difficulty with this, we cannot conclude that there were homogenous root growth responses among the tree-turfgrass combinations.  Though our objective was not satisfied, additional research in which roots could be distinguished should still be pursued.  Additionally, research with other landscape plant species would also be useful.

This study encountered some difficulties that made the achievement of experimental objectives quite challenging.  While our null hypothesis was not accepted (in Chapter 3) we were unable to adequately meet experimental objectives.  Further study of below-ground interspecific competition will require a consistent and uniform means of root species distinction for definitive conclusions to be made using our system.  Analysis of turfgrass and tree combinations do show distinct differences among both tree caliper growth and rooting extent (Tables A-1, A-2).  With such economically important consequences, this area of research necessitates further investigation.

In study of below-ground competition and its effects, perhaps the most important requirement is a complete and thorough understanding of minirhizotron technique.  By applying the benefits, and minimizing minirhizotrons' drawbacks experiments can be

designed that capture the subtle effects of below-ground competition among trees and turfgrasses.

Research into resource competition among plants remains a critical area of research for biochemical, ecological, agronomical, and horticultural concerns. Some of the root growth inhibition seen in our rhizotron study suggested the presence and effects of allelochemical exudation. For this reason, we explored the chemical families present in fine root tissues of our field-grown black locust specimens (Appendix B).

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APPENDICES

Table A-1. Black locust tree caliper measurements (at dbh) for 2006- 2007

**Calipers (cm) for sessions 1-6 from 2006-2007**

| Whole-plot turfgrass | Tree Number | Session 1 | Session 2 | Session 3 | Session 4 | Session 5 | Session 6 |
|---|---|---|---|---|---|---|---|
| Buffalograss | 1 | 5.08 | 5.715 | 6.35 | 6.6675 | 6.985 | 7.3025 |
| Kentucky bluegrass | 3 | 5.3975 | 5.3975 | 5.715 | 6.0325 | 6.35 | 6.35 |
| Buffalograss | 5 | 8.335 | 10.97 | 10.97 | 11.97 | 14.605 | 15.3025 |
| None (Control) | 7 | 9.525 | 10.16 | 10.4775 | 11.43 | 11.7475 | 12.065 |
| Tall fescue | 9 | 5.08 | 5.715 | 5.715 | 6.35 | 6.985 | 7.3025 |
| Kentucky bluegrass | 11 | 6.985 | 6.985 | 7.3025 | 7.62 | 7.9375 | 8.5725 |
| None (Control) | 13 | 8.065 | 10.7 | 13.335 | 13.6525 | 13.97 | 14.605 |
| Tall fescue | 15 | 8.255 | 8.5725 | 9.2075 | 9.6675 | 9.985 | 10.3025 |
| Buffalograss | 17 | 3.81 | 4.445 | 5.3975 | 6.35 | 6.6675 | 6.985 |
| Buffalograss | 19 | 7.62 | 7.62 | 8.255 | 8.255 | 8.5725 | 8.89 |
| None (Control) | 21 | 8.89 | 10.16 | 10.795 | 10.795 | 11.43 | 12.7 |
| Tall fescue | 23 | 6.35 | 6.6675 | 6.985 | 7.62 | 7.62 | 8.5725 |
| Kentucky bluegrass | 25 | 5.715 | 6.0325 | 6.35 | 6.35 | 6.35 | 7.62 |
| Buffalograss | 27 | 6.35 | 6.985 | 7.3025 | 7.9375 | 8.255 | 9.2075 |
| Buffalograss | 29 | 5.715 | 6.35 | 6.35 | 7.62 | 8.5725 | 9.525 |
| Buffalograss | 31 | 7.62 | 7.62 | 8.255 | 8.255 | 8.89 | 9.8425 |
| Tall fescue | 33 | 5.715 | 5.715 | 6.35 | 6.35 | 6.985 | 7.62 |
| Kentucky bluegrass | 35 | 5.08 | 5.08 | 5.3975 | 5.3975 | 5.715 | 6.35 |
| Buffalograss | 37 | 4.445 | 4.445 | 4.7625 | 5.08 | 5.08 | 5.715 |
| None (Control) | 39 | 12.7 | 13.335 | 13.6525 | 13.6525 | 13.97 | 14.605 |

Table A-2. Honeylocust tree caliper measurements (at dbh) for 2006 and 2007

**Calipers (cm) for sessions 1-6 from 2006-2007**

| Whole-plot turfgrass | Tree Number | Session 1 | Session 2 | Session 3 | Session 4 | Session 5 | Session 6 |
|---|---|---|---|---|---|---|---|
| Buffalograss | 2 | 1.905 | 1.905 | 1.905 | 1.905 | 1.905 | 1.905 |
| Kentucky bluegrass | 4 | 3.175 | 3.175 | 3.175 | 3.175 | 3.175 | 3.4925 |
| Buffalograss | 6 | 2.54 | 2.54 | 2.54 | 2.54 | 2.54 | 2.54 |
| None (Control) | 8 | 5.715 | 5.715 | 5.715 | 5.715 | 6.35 | 6.35 |
| Tall fescue | 10 | 1.905 | 1.905 | 1.905 | 1.905 | 2.54 | 2.54 |
| Kentucky bluegrass | 12 | 2.54 | 2.54 | 2.54 | 2.54 | 2.8575 | 2.8575 |
| None (Control) | 14 | 6.35 | 6.35 | 6.35 | 6.35 | 6.6675 | 6.6675 |
| Tall fescue | 16 | 1.905 | 1.905 | 1.905 | 1.905 | 1.905 | 1.905 |
| Buffalograss | 18 | 6.6675 | 6.6675 | 6.6675 | 6.6675 | 6.985 | 6.985 |
| Buffalograss | 20 | 2.2225 | 2.2225 | 2.2225 | 2.2225 | 2.2225 | 2.2225 |
| None (Control) | 22 | 7.62 | 7.62 | 7.9375 | 7.9375 | 7.9375 | 8.255 |
| Tall fescue | 24 | 1.905 | 1.905 | 1.905 | 1.905 | 2.2225 | 2.2225 |
| Kentucky bluegrass | 26 | 2.54 | 2.54 | 2.54 | 2.54 | 2.54 | 2.54 |
| Buffalograss | 28 | 2.54 | 2.54 | 2.54 | 3.175 | 3.175 | 3.175 |
| Buffalograss | 30 | 1.905 | 1.905 | 1.905 | 1.905 | 1.905 | 1.905 |
| Buffalograss | 32 | 2.54 | 2.54 | 2.54 | 2.54 | 2.54 | 2.54 |
| Tall fescue | 34 | 1.905 | 1.905 | 1.905 | 1.905 | 1.905 | 1.905 |
| Kentucky bluegrass | 36 | 2.54 | 2.54 | 2.54 | 2.54 | 2.54 | 2.8575 |
| Buffalograss | 38 | 1.905 | 1.905 | 1.905 | 1.905 | 1.905 | 1.905 |
| None (Control) | 40 | 6.0325 | 6.0325 | 6.35 | 6.35 | 6.35 | 6.6675 |

APPENDIX B

INVESTIGATION OF ALLELOCHEMICAL PRESENCE IN FINE ROOT TISSUE OF

BLACK LOCUST (*Robinia pseudoacacia* L.)

ABSTRACT

The presence of allelochemicals in the root zone of landscaped areas can have

severe effects on the survival and proliferation of desired species.  These compounds are

exuded by living or decomposing plant tissues as a means of competition for nutrient and

water resources.  When exposed to these compounds, vulnerable species often exhibit

significant root growth inhibition.  Some species used in landscapes of the Intermountain

West exhibit allelopathy.  The allelochemicals produced by some of these plants are

highly specific and effective.  There are very few thorough descriptions of many of the

compounds found in allelopathic species in landscapes.  Native to the American plains

and west, black locust trees have been explored as a source of potent bioactive chemicals.

Allelochemicals from this tree vary in size and structure, but most come from the

flavanoid family of chemicals.  Only a small portion of the described flavanoid chemicals

found in black locust have been shown to be allelopathic.  As grasses and trees are

frequently placed in close proximity in landscapes, additional exploration into the activity

of these compounds is needed to fully understand the potential consequences of including

black locust trees in a landscape setting.  A separation and analysis of field-grown black

locust root extract was performed to determine chemicals present.

Introduction

Over time, plants producing allelopathic compounds have accomplished what chemical companies have been working at for years. Allelochemicals can range from simple to complex and often exhibit significant biological activity (Rice, 1984). This activity can often take the form of root growth inhibition of an invading species. Ji et al. (1996) found that numerous plant flavanoids bind to adenosine receptors in mammals, often with higher affinity for human forms. Adenosine receptors mediate physiological actions of adenosine in the central nervous system (Ji et al., 1996). Some research has suggested that allelochemicals present in phytotoxic plant residues could be used and exploited in numerous agronomic cropping scenarios (Whittaker and Feeny, 1971; Putnam and DeFrank, 1983; Putnam et al., 1983). For these reasons, herbicide developers have focused attention on the mechanisms of allelochemical actions in these phytotoxic plant residues (Weston and Duke, 2003). For many years, allelopathy has been reported across the *Poaceae* family, including Kentucky bluegrass (Sanchez-Moreiras et al., 2004). *Poa* species were among many found to inhibit the germination and establishment of juvenile woody plant species, likely due to allelopathic root exudates (Fales and Wakefield, 1981). Allelopathy has also been reported in fine leaf and tall fescues. Fescues are often used in landscapes as ground covers because they are able to out-compete surrounding plants. Work by Bertin et al. (2007) has shown that *meta*-tyrosine, a potent broad-spectrum phytotoxin, is exuded from roots of *Festuca rubra* (L.) and *Festuca arizonica* (L.) species. This work is merely the second and third found instances of *meta*-tyrosine production in plants, with the first being in *Euphorbia myrsinitis* (L.) (Bertin et al., 2007). Tall fescue has been found to inhibit the growth of

pecan (*Carya illinoensis* (Wangenh.) K. Koch), Sweetgum (*Liquidambar styraciflua* L.) and white ash (*Fraxinus americana* L.) trees (Smith et al., 2001; Walters et al., 1976; Preece et al., 1991). The type and nature of allelopathic chemicals varies greatly. Phenolates, hydroxamates, alkaloids, and quinones have all been found in various genera within the *Poaceae* family, each having distinct effects, such as pollen formation inhibition and hormone production inhibition (Buta et al., 1988). Phytometallophores have also been found in root exudates in the *Poaceae* family (Welch, 1995).

Some work into allelopathy in the black locust tree (*Robinia pseudoacacia* L.) has already proved enlightening. Lin et al. (1973) screened numerous species for allelopathic and antimicrobial compounds, and black locust tested positive in bioassays. Allelopathy has been reported in both roots and leaves of the black locust tree. Arborists and landscape managers have been warned of allelochemical production in black locust bark and roots (Coder, 1999). The lumber industry has given attention to black locust, noting that flavanoids isolated from harvested heartwood contribute to wood longevity (Smith et al., 1989). These same compounds are thought to act as a signal to initiate *Rhizobium* sp. symbiosis with black locust roots (Scheidemann et al., 1997). Extracts from black locust leaves proved significantly phytotoxic in bioassays with three types of herbaceous plants (Nasir et al., 2005). This work isolated robinetin, myrcetin and quercetin (Figure 1). Aqueous solutions of these compounds in further bioassays confirmed the phytotoxic role of these flavanoid allelochemicals (Nasir et al., 2005). Highly phytotoxic cyanamide (NH2-CN) was found present in black locust in January of 2008 (Kamo et al., 2008). This is only the second time the compound has been found in any plant.
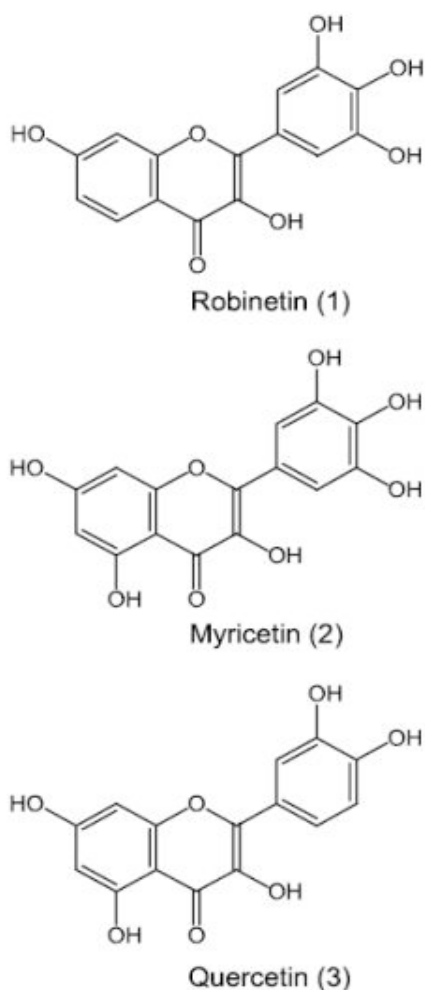
Robinetin (1)

Myricetin (2)

Quercetin (3)

Figure B-1. Structures of isolated allelopathic flavanoid monomers from black locust leaf litter.

Source: reprinted from Journal of Chemical Ecology, Vol. 31, No. 9, p. 2183, Sept. 2005, with permission of Dr. Yoshiharu Fujii.

Advances in detection and screening of volatile allelochemicals have elucidated 2-hexenal and *cis*-2-hexenol in black locust (Fujii et al., 2005). Flavanoids and associated oligoflavanoid chemical species have a broad range of biological sources and

activities.  White and green teas have been found to contain (+/-) catechin, epicatechin, kaempferol and other related compounds (Frei et al, 2006; Ververidis et al., 2007).  Green tea (*Camelia sinensis* L. (Kuntze)) has been shown to contain a diverse set of flavanoid monomers and oligomers (Frei et al, 2006).  One such flavanoid in green tea, EGCG has been shown to bind a T-cell receptor with high HIV virus affinity.  (Williamson et al., 2006; Yamaguchi et al., 2002).  While these compounds have been described to have antioxidant and anti-viral properties, other flavanoids like resveratrol have been found to reduce tumor growth rates in humans (Joe et al., 2002).  Other described sources of bioactive flavanoids include chocolate, citrus fruits, and the *Ginkgo biloba* (L.) tree (Frei et al., 2006).  A family of flavanoids, the oligomeric proanthocyanidins (OPC) are potent pharmacologically active compounds.  These are dimers, trimers, or oligomers of monomer flavanoid compounds such as quercetin or catechin.  OPCs can be found in cranberry, bilberry, sea buckthorn, red grapes, and grape seed oil (Frei et al., 2006; Kennedy et al., 2002; Rösch et al., 2004).  OPCs are powerful antioxidants and have been shown to inhibit expression of a protein that constricts blood vessels (Schramm and German, 1998).  In black locust, these compounds have been found to exist in a family named prorobinetidins (Coetzee et al., 1995).
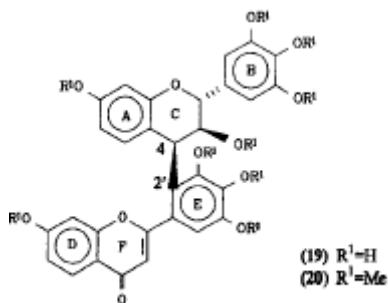
(19) R¹=H
(20) R¹=Me

Figure B-2. Structure of prorobinetidin 20 from black locust.

Source: from Tetrahedron. 51(8):2339-2352.  Reprinted with permission of J.P. Steynberg.

They consist of flavanoid dimer and trimers.  While flavanoid monomer bioactivity and allelopathy has been demonstrated (robinetin, myrcetin and quercetin), the allelopathic potential of the prorobinetidins remains unexplored.  (Coetzee et al., 1995; Fujii et al., 2005)

While exploration into the black locust tree has isolated three flavanoid monomers and oligomers, the most recent research in *Robinia pseudoacacia* (L.) has brought attention to the species' allelochemical vulnerability.  Black locust has been found susceptible to allelopathic inhibition by other plants (Furubayashi et al., 2007).  The herbaceous species *Solidago altissima* (L.), *Andropogon virginicus* (L.), *Coronilla varia* (L.), *Daucus carota* (L.), *Festuca arundinacea* (L.) and *Phleum pretense* (L.) have all been found to inhibit growth of black locust trees (Larson et al., 1980).  My study determined the presence of allelochemical exudation in a tree species with use in the Intermountain West.  Extraction, isolation and description of allelopathic compounds was performed.

Materials and Methods

Experimental Design

A representative sample of fine roots from 20 field-grown black locust trees was obtained and placed into storage at -80 degrees C.  All roots obtained had a measured diameter no larger than 5 mm.  Following cold storage, the roots were chopped into 1-cm pieces, then macerated using a mortar and pestle.  The sample was freeze-dried for 24 h using a Virtis Sentry Freezemobile freezedryer (SP Industries, Warminster, PA). After dehydration, the sample's mass was determined to be 321.21 g.  The sample was placed in a 500-mL flask filled with 400 mL of 80 % aqueous methanol.  The sample was left in the methanol (chemical formula $CH_3OH$) for 72 hours then vacuum filtered and concentrated.  Using an R-151 Rotovaporator (Buchi, Inc.  Postfach, Switzerland.), methanol was removed from the resulting extract.  The remainder was placed on the freeze drier for removal of water in the extract, producing a yellow-brown amorphous gum.  To this gum, 150 mL of hexane (chemical formula $C_6H_{14}$) was then added.   The solution was placed into a 500-mL separation flask.  To this, 150 mL of dichloromethane (DCM) was added (chemical formula $CH_2Cl_2$).  The separation flask was capped and shaken vigorously for 30 s.  Upon waiting 5 minutes for solvent separation and subsequent extract partitioning, the lower layer (DCM) was poured into a 250-mL flask. The upper layer (hexane) was then poured into another 250-mL flask.  The DCM fraction was poured back into the separation flask and allowed to settle.  To this, 150 mL of 80 % aqueous methanol was added.  The solvent mixture was shaken vigorously for 30 s in the separation flask.  An additional 5 min was allowed for solvent separation and extract partitioning.  The lower layer (methanol) was poured into a 250-mL flask.  The upper

layer (DCM) was poured into another 250-mL flask.  The three resulting fractions were again vacuum-filtered.

The most polar methanolic fraction of this separation was then subjected to NMR analyses to confirm presence of desired polar metabolites.  Seeking the highly polar oligoflavanoid chemicals cited in previous literature, the methanol fraction was then subjected to further fractionation by elution through a 1000-mL column filled with Sephadex LH-20 ionized beads and excess of methanol.  A Spectra/Chrom CF-1 (Spectrum Molecular Separations, Houston, TX) fraction collector was used to collect the column eluant at 5-min intervals.

All fractions were subjected to preparative thin-layer chromatography (TLC) using aluminum-backed, silica-gel TLC sheets (Sorbent Technologies, Atlanta, GA) to identify fractions containing desirable compounds (Figure 2, 3).  Approximately 100 μL of each fraction was spotted onto the base of a 6 x 10 cm TLC sheet.  Seven sheets were used to accommodate all fraction subsamples.  The fractions were spotted 4-5 mm away from one another on the sheet.  The fraction solvent portions were then allowed to evaporate off the sheet, leaving only dry compound on the silica.
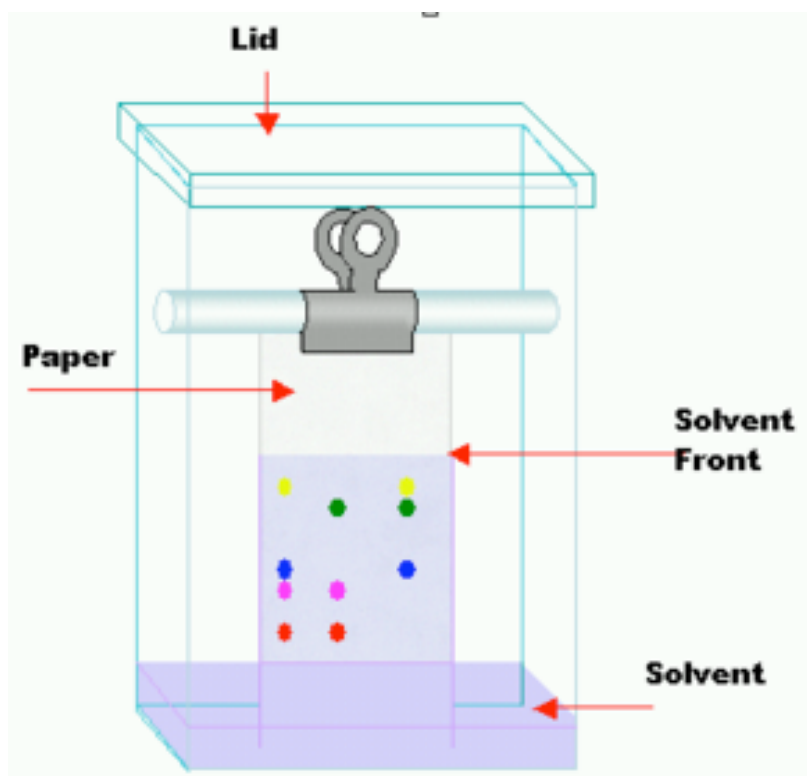
Figure B-3. Diagram of a TLC procedure. The aluminum-backed silica-gel paper is allowed to uniformly absorb the well solvent. Compounds are separated as they are carried up with the solvent front.

Source: http://en.wikipedia.org/wiki/Image:Cromatography_tank.png
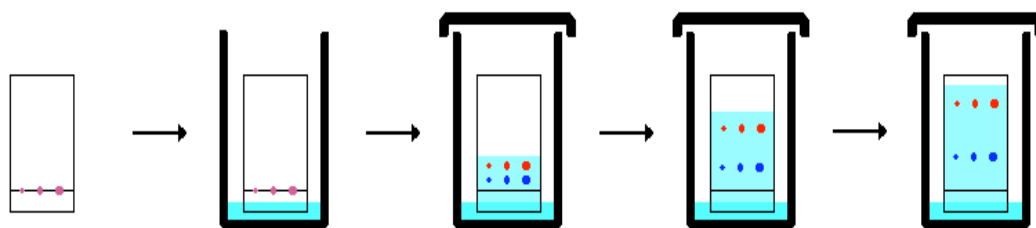


Figure B-4. Illustration showing the sequence of events in thin-layer chromatography. The solvent is absorbed and carried up the silica along with the compounds of interest.

Source: http://en.wikipedia.org/wiki/Image:Tlc_sequence.png

The TLC sheets were placed in 5-mm-deep wells containing solvent of increasing polarity.  The first seven sheets were placed in a pure DCM solvent well.  The second seven sheets were placed in a well of 3 parts DCM: 1 part methanol mixture.  The third group of seven sheets was placed in a well of 1 part DCM: 1 part methanol mixture.  The fourth group was placed in a well of 3 parts methanol: 1 part DCM mixture.  The final group of seven sheets was placed in pure methanol.  Well solvent was allowed to ascend the TLC sheets via capillary action and compounds separated by chemical polarity.  When the solvent ascended to the top of the sheets, they were removed from the well and allowed to air-dry.  Each sheet was then placed under a UV-B light to visualize compounds that had separated from the mixture contained in each fraction.  Compounds appearing dark under the UV-B light were those containing double or triple-bonds and were absorbing the incident radiation.  TLC plates of interest were then stained in a phosphomolybdic acid (chemical formula: $H_3[P(Mo_3O_{10})_4]$) solution to allow visualization of all compounds under the visible light spectrum. After staining, the sheets were placed on a hotplate for 5 s to fix the stain onto the silica. Fractions of interest were pooled, and concentrated by removal of methanol via Rotovaporator.   The dried extract was dissolved in fully deuterated (radio-labeled) methanol ($CD_3OD$) and subjected to additional NMR analysis.

The dissolved sample was loaded onto a 250-mL flash chromatography column (60 x 4 cm).  The column was filled with 5 cm of silica gel, as directed in the procedure of Still et al.(1978) (Figure 4).  After sample loading, the column was eluted into fractions using solvent of increasing polarity.  Ten fractions were collected using 100 mL of 1 part DCM: 1 part methanol solvent.  Following this, ten fractions were collected

using 100 mL of a 3 part methanol: 1 part DCM solvent. Ten more fractions were

collected using 100 mL of pure methanol. Finally, ten fractions were obtained by eluting

the column with 100 mL of a 3 part methanol to 1 part distilled, deionzied water solution.
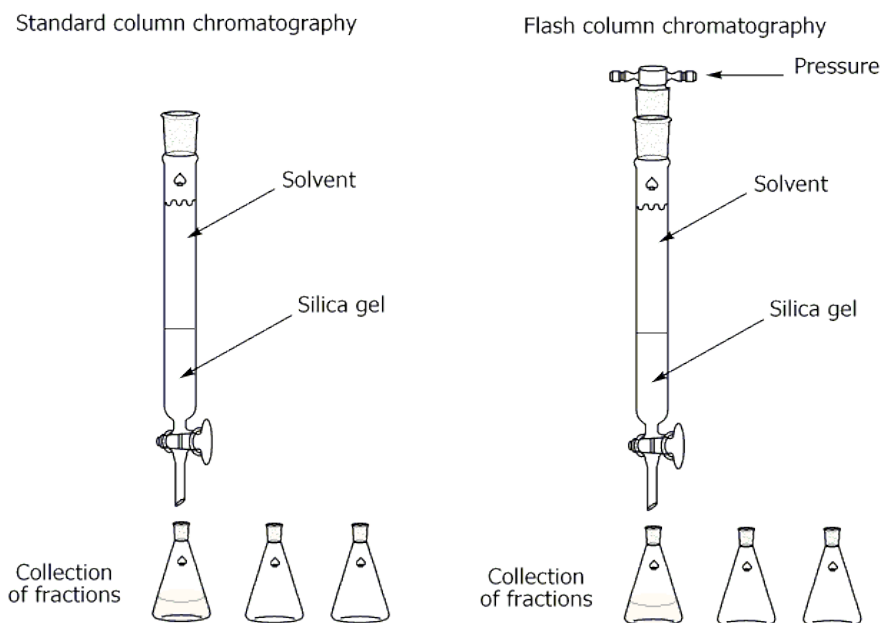
All fractions were collected at 30 s intervals.



Figure B-5. A diagram illustration of column chromatography techniques and fraction

collection.

Source: http://en.wikipedia.org/wiki/Image:Columnchromatography.gif

Following elution, all 40 fractions were analyzed for chemical content using TLC

separation, as described above. Upon analysis and staining of TLC sheets, fractions of

interest were concentrated by removal of solvent using Rotovaporator and water using a

freeze-dryer. Resulting dry material was weighed, then dissolved in deuterated methanol

and subjected to a final NMR analysis for presence of desirable compounds.

*NMR–*

NMR (Nuclear Magnetic Resonance) spectroscopy (at 300 MHz, 7.058 tesla) using a JEOL ECX-300 (JEOL, Ltd., Tokyo, Japan) with standard JEOL software (Alpha Data Systems, Tallahassee, FL) with labeled chloroform (chemical formula $CHCl_3$), was performed on the methanolic fraction of the crude extract to screen for presence of novel compounds by ^{1}H spectra.  Further NMR spectroscopy was performed using the same frequency and pulse strength on fractions from the Sephadex column, and those from the silica gel column.  Desired flavanoid monomers and oligomers' proton spectra were screened by referring to NMR spectra in previously cited literature.

Results

*Thin-Layer Chromatography*

Upon TLC screening of the 127 Sephadex column fractions, numbers 19 and 20 (corresponding to eluant times of 95 and 100 min, respectively) when separated in a pure methanol solvent well yielded the most distinct visualization of compounds.  Three dark bands were seen on the TLC sheet.  The middle band appeared smeared and was not as distinct as the lower and top bands, indicative of acid / base equilibrium reactions on the sheet.  The smeared appearance also may have been indicative of the presence of more than one compound in the band.  Due to this, further column chromatography was performed.  Of the three, the middle and lower bands (corresponding to the most polar of the three) absorbed UV-B radiation.  The top band was visualized upon staining.  Upon TLC separation from the flash chromatography (silica gel) column, fractions containing compounds that were previously visualized in TLC sheets were isolated.  TLC sheets

were prepared to reproduce the three bands seen in TLC sheets from the Sephadex

column. Fractions 14 and 15 produced the top band, which did not absorb UV-B

radiation. These fractions were obtained using the less polar 3 parts methanol: 1 part

DCM solvent. The corresponding TLC was performed using a pure methanol solvent

well. Fractions 22 and 23 produced a very narrow and distinct middle band. This band

did absorb UV-B radiation like the middle band from the Sephadex column. The

corresponding TLC was performed using a 5 parts methanol: 1 part deionized, distilled

water solvent solution. These fractions were thought to contain the highly polar

flavanoid and oligoflavanoid compounds of interest. Like the sheet from the Sephadex

column, this band absorbed UV-B radiation. The TLC was performed with a 3 parts

methanol: 1 part water well solution.


*Column Chromatography*

From the Sephadex LH-20 column separation of the methanol fraction of the root

extract, a total of 127 fractions were collected over a span of 635 minutes. All sample

chemical components exhibiting color appeared to be eluted from the column within 300

min.

From the silica gel column (flash chromatography column), most sample

chemical components appeared to be eluted using highly polar solvent mixtures.

Fractions 33 through 37 contained a highly polar, UV-B absorbing component, rising 2

cm ($R_f$ of .185) on a TLC sheet with a well solvent of 3 parts methanol to 1 part water.

Two additional compounds were isolated in earlier fractions eluted with less polar

solvents. Fractions 33-37 contained 1.27 mg dry compound. After solvent removal and

drying, fraction dry compound masses were determined.  Fractions 22-23 contained

0.78 mg dry compound.  Fractions 14-15 contained 0.84 mg dry compound.


*NMR*

Initial NMR $^1$H spectra of the crude root methanolic extract methanol fraction

revealed a rich and diverse pool of chemical species.  This warranted further separation

as screening for flavanoid monomer and oligomer presence was inconclusive.  The $^1$H

spectra of the crude methanolic extract and subsequent methanol fraction (in $CDCl_3$ and

$CD_3OD$, respectively) of the desired compound were identical to those of robinetin and

prorobinetidin.  Abundance of these compounds in the NMR sample was at 0.1 or less,

demonstrating the very low concentration of compound present in the starting material.
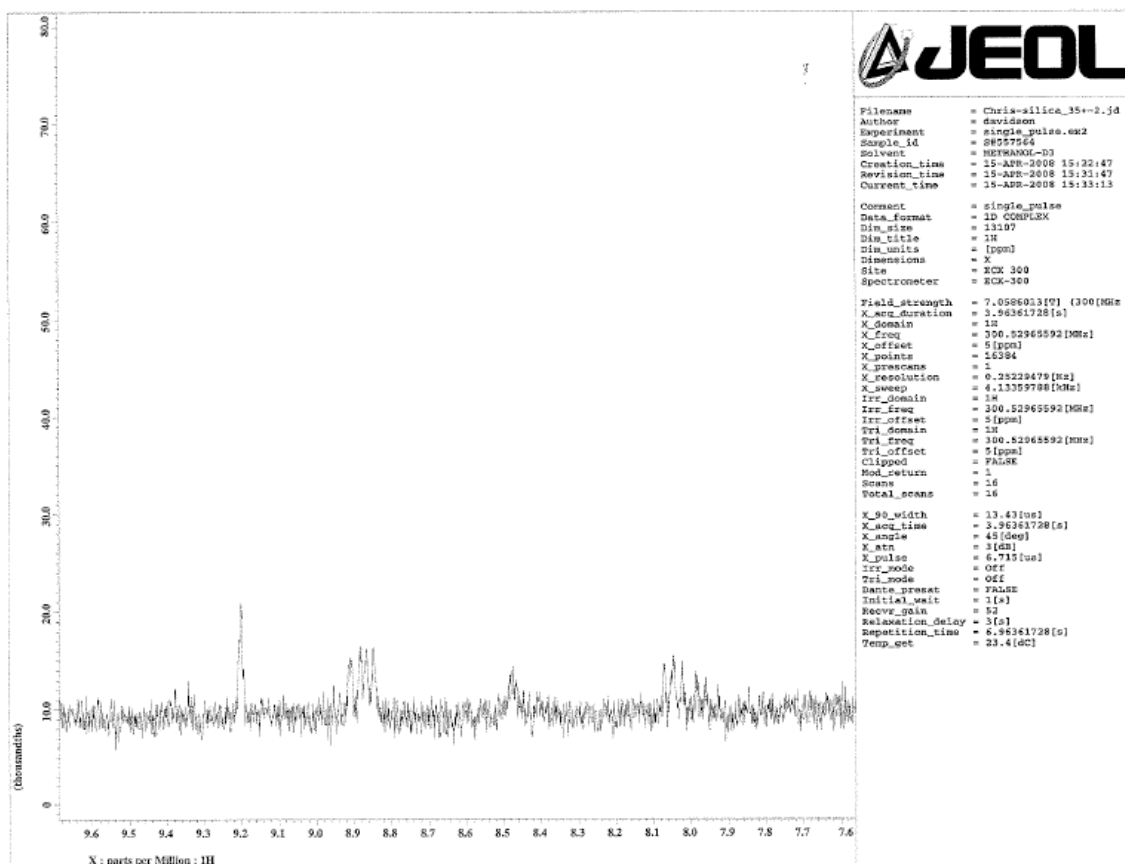
(Figure 5)

Figure B-6. NMR spectrograph of pooled silica column fractions 33-37.

Discussion

While at least one of the flavanoids and prorobinetidin forms was positively identified in the black locust methanolic extract, upon drying of the final purified fractions, a very low amount of the active material was recovered. As a consequence, bioassays to test the recovered compounds' activity and allelopathic potential were not possible.

With the large amount of literature describing the rich biochemical diversity contained in black locust, further work in the tree's flavanoid pool is needed. Flavanoid-

based allelopathy has been cited in many plants native to habitats near that of black

locust (Stermitz et al., 2003). There is a high probability that the prorobinetidins remain

a family of black locust metabolites that have significant allelopathic potential. Given the

extensive amount of literature citing the invasive nature of black locust, there is evidence

to suggest that it is able to gain a competitive advantage from an endogenous mechanism.

Considering the phytotoxic nature of described flavanoid monomers including

cyanamide, allelopathy is likely the mechanism allowing black locust to spread over such

a broad geographic area. Identification of chemotypes in black locust may also be

possible. Such chemical profiling may offer insight into the species response to varying

soil and macroclimates.

To properly screen for bioactivity and allelopathic potential of the

prorobinetidins, a much larger sample of root tissue will be required. While the 376-g

sample did yield NMR spectra confirming flavanoid and prorobinetidin presence, it did

not provide nearly enough purified dry matter to conduct bioassays. Ideally, bioassays

with logarithmically diluted sample concentration would be performed, to demonstrate a

dose-dependent root growth inhibition. This was demonstrated in a study by Nasir et al.

(2005) with black locust flavanoid monomers. A minimum of 3 kg of root material

should be harvested and subjected to methanolic extraction to ensure enough purified

compound to test for allelopathy and dose-dependent growth inhibition.

Future work concerning allelopathy in black locust may also consider the source

of all isolated active chemicals. All but one article in the literature to date regarding

allelopathy in black locust has not sought a genetic or *in vivo* source of these chemicals.

While work by Nasir et al. (2005) demonstrated allelopathic flavanoid monomer

precipitation from decomposing leaf litter, there is likely a genetic source for at least

one of the compounds found in the literature. Proteomic investigations into low-copy

enzymes responsible for synthesis of additional flavanoids, Prorobinetidins, cyanamides

or additional novel chemicals could yield insight into how these compounds are produced

in the cells of black locust. Once isolated, such low-copy proteins could be sequenced in

the search for responsible gene(s). Transgene cultivars of numerous woody species could

then be produced, utilizing the genetic mechanisms that may be producing these

compounds *in vivo*. This would result in plants with an internal capacity to defend

against competing root systems. Such plants would host an increased ability to secure the

resources needed for growth and maintenance in a competitive situation.

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