THESIS

RESPONSE OF DESIRABLE TREES TO AMINOCYCLOPYRACHLOR IN THE FIELD AND GREENHOUSE

Submitted by

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ABSTRACT

RESPONSE OF DESIRABLE TREES TO AMINOCYCLOPYRACHLOR IN THE FIELD AND GREENHOUSE

Aminocyclopyrachlor is a selective, plant growth regulator herbicide in the pyrimidine carboxylic acid family. Previously, aminocyclopyrachlor was marketed for turfgrass management but was withdrawn by the EPA in light of unexpected non-target injury related to desirable tree species such as white pine (*Pinus strobus*), and Norway spruce (*Picea abies*). Field trials to assess the impact of aminocyclopyrachlor on two tree species green ash (*Fraxinus pennsylvanica*) and honeylocust (*Gleditsia triacanthos*) were conducted to determine the relative sensitivity of green ash and honeylocust to aminocyclopyrachlor, determine a minimum safe spraying distance from green ash and honeylocust trees, and to assess the effect of application timing on tree response. An additional greenhouse dose response trial on tree seedlings was used to compare green ash and honeylocust response to Norway spruce which had demonstrated high sensitivity to aminocyclopyrachlor, and blue spruce which is a common and important native species in Colorado. Field results showed that green ash was not susceptible to herbicide applications while honeylocust showed severe injury, up to 100% for trees closest to applications. Honeylocust trees up to 7 m from the edge of the application displayed moderate to severe injury symptoms, and the fall treatment timings in October and November appeared to be safer in terms of reducing injury on target trees. Soil analysis using LC-MS and HPLC demonstrated that aminocyclopyrachlor dissipation was no different in the soil underneath green ash and honeylocust trees, and that dissipation was likely aided by absorption in tree roots. A

greenhouse dose response showed that honeylocust was a moderately sensitive species, about four times more tolerant to aminocyclopyrachlor than blue spruce or Norway spruce. Green ash was consistently tolerant to aminocyclopyrachlor in the greenhouse, showing only minor response at the higher doses. Taken together these results provide a basic groundwork of research necessary for the writing of better aminocyclopyrachlor herbicide labels, and a better understanding of this herbicide's effect on certain woody vegetation.

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DEDICATION

This work is dedicated to Galen Brunk,
who made family, education, and curiosity his priorities.

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INTRODUCTION

Aminocyclopyrachlor (6-amino-5-chloro-2-cyclopropyl-4-pyrimidinecarboxylic acid; AMCP) is a selective, plant growth regulator herbicide in the pyrimidine carboxylic acid family (Turner, 2009). Its structure resembles the pyridine carboxylic acids, another class of auxinic herbicides, that includes active ingredients such as clopyralid, aminopyralid, and picloram (Bukun et al., 2010). It is characterized by a pyrimidine ring, with reactive groups that include a carboxylic acid group, and an amine group. With a pK_a of 4.65, AMCP is often in its anionic form within a typical soil environment, leading to a higher degree of polarity (Oliveira et al., 2011; Oliveira et al., 2013) with a $\log K_{ow}$ of -1.12 at a pH of 4, and -2.48 at a pH of 7 (Shaner, 2014). Because of this, AMCP has a predicted leaching potential that is relatively high. Some researchers have recovered AMCP at depths up to 70 to 90 cm after one year indicating higher movement potential (Durkin, 2012). Additional work in sandy soil shows higher leaching potential for AMCP than the herbicide quinclorac (Adams and Lym, 2015). Work on Hawaiian soils showed a high leaching potential for AMCP across all tested soils due to its relatively low K_{oc} values (Hall et al., 2015). Its reported half-life ranges from 32.5 days under field conditions (Lindenmayer, 2012) up to 433 days in a sandy loam soil (Shaner, 2014).

The relatively long persistence time for AMCP also allows for longer duration of control for many invasive weed species troublesome in rangeland and turf settings. Trials have shown good control of Canada thistle (*Cirsium arvense)* up to 14 months after application using AMCP, with root-absorbed AMCP having more inhibitory effect than shoot absorption (Lindenmayer, 2012). Field bindweed (*Convolvulus arvensis*), a notoriously difficult weed to control, is controlled for up to 16 months after application due to the ability of AMCP to readily translocate to the root system (Lindenmayer, 2012; Lindenmayer et al., 2013). Long term control of leafy spurge (*Euphorbia esula*) is also possible with AMCP, providing up to 24 months of control greater than 80% with rates of 2 to 3 oz Acre^{-1} (Lym, 2012). Other broadleaf weed species important to rangeland settings that AMCP controls include: spotted knapweed (*Centaurea maculosa*), diffuse knapweed (*Centaurea diffusa*), Russian knapweed (*Acroptilon repens*), Russian thistle (*Salsola* spp.), yellow starthistle (*Centaurea solstitialis*), houndstongue (*Cynoglossum officinale*), Dalmatian toadflax (*Linaria dalmatica*), common sunflower (*Helianthus annuus*), and perennial pepperweed (*Lepidium latifolium*) (Meredith et al., 2013). The long term control of a range of broadleaf weed species due to a high level of root translocation, coupled with selectivity that favors cool-season grasses such as tall fescue (*Festuca arundinacea*) (Parker et al., 2015) makes AMCP an ideal candidate for use in rangeland management.

Besides its use for control of broadleaf weeds, AMCP has also been shown to control troublesome tree species such as Russian olive (*Eleagnus angustifolia* L.) (Getts, 2015). Auxinic herbicides typically cause growth regulator type symptoms that result in epinasty of leaves and petioles, chlorosis, and necrosis (Feucht, 1988). For auxinic herbicides that persist longer in the soil, there is increased risk of uptake via root systems resulting in non-target injury to nearby tree species. This has been seen from the use of aminopyralid near ponderosa pine (*Pinus ponderosa*) (Wallace et al., 2012).

Currently, AMCP holds labels for right-of-way applications with the tradenames Perspective® and Streamline® (Anonymous, 2012; Anonymous, 2014). Previously, AMCP was also marketed as Imprelis® (Anonymous, 2010), but was withdrawn by the EPA in light of non-target injury related to desirable tree species such as white pine (*Pinus strobus*), and Norway spruce (*Picea abies*) (Patton et al., 2013). The Imprelis® label mentioned that extra care should be taken within the dripline of desirable trees; however, it is known that tree roots may reach extensively past the dripline in the absence of restrictive soil or substrate characteristics, and that these extensive roots play a large role in water and nutrient uptake for trees (Stone and Kalisz, 1991). Since tree root growth is influenced by temperature, water availability, and other abiotic factors, maximum radial root growth is different under variable environmental conditions. Because of this, radial root architecture can differ quite widely between different species (Coutts, 1989; Stone and Kalisz, 1991), and understanding radial root growth in terms of risk for non-target herbicide injury can be problematic. More trials, covering important tree species in a range of abiotic conditions is necessary in order to understand how a herbicide will interact with trees in the environment. This information will be necessary to write effective labels for appropriate use of the herbicide since more conservative estimates for caution areas around trees will be required to avoid further issues.

Trials were conducted from 2012 to 2015 to assess the impact of AMCP on two tree species, green ash (*Fraxinus pennsylvanica*), and honeylocust (*Gleditsia triacanthos*). The objectives of these trials were to determine the relative sensitivity between green ash (GA) and honeylocust (HL), determine a minimum safe spraying distance from GA and HL trees at the field site, assess the effect of application timing on tree response to the herbicide in order to determine the safest application timing, and to determine whether AMCP dissipation was similar underneath both GA and HL. An additional dose response trial performed in the greenhouse in 2013 was used to assess GA and HL trees for their relative sensitivity to AMCP, and to compare these species to Norway spruce which has demonstrated high sensitivity to AMCP, and blue spruce which is a common and important native species throughout Colorado.

MATERIALS AND METHODS

Test Site Description

All field tests of GA, and HL trees took place at the Colorado State University Agricultural Research Development and Education Center's (ARDEC) Tree and Turf Research Area. Established in the mid 1990's, the trees at the site included GA and HL separated into nine different blocks which received different water regimes using an irrigation system with pop-up sprinkler heads. All blocks contained sections of GA, HL, and a strip of Kentucky bluegrass. Both species of trees were arranged in columns of three, with nine rows in each block, for a total of 27 GA and 27 HL trees in each block. HL and GA were either placed adjacent to one another, or separated by a strip of Kentucky bluegrass (Figure 1). Each block was able to be independently irrigated, allowing the incorporation of herbicide post-application. Each row was separated from the next by a distance of six m, allowing for controlled experimental conditions to measure distance to herbicide injury. Trees within rows were spaced 3.5 m apart. At the time of the first herbicide applications in 2012, the trees at the site were 16 years old, with heights ranging from 6.5 m on the southern end of the research area to 15 m on the northern end of the area (likely due to previous irrigation studies as well as flooding from the nearby irrigation pond). Tree height was therefore also used as a correlator with visual injury ratings. Soil at the site was that of a Fort Collins loam with its taxonomic class listed as fine-loamy, mixed, superactive, mesic Aridic Haplustalf.

Tree Safety Trials

In order to determine how the season of herbicide application affected injury, different application dates of AMCP corresponding to the spring, fall and summer were used during 2012, 2013, and 2014. Applications in 2012 were made on April 14th, June 22nd, and October 20th. Applications in 2013 were made on July 1st, October 10th, and November 27th (foregoing a spring application to compare two fall timings), and applications in 2014 were made corresponding to two timings on April 21st, and July 28th. Applications were made only once, and observations were made yearly. Plots were arranged by spraying two strips of AMCP at a rate of 210 g ai ha^{-1} halfway between two rows of HL and GA trees, such that the closest trees were one meter away from the edge of the application, the next row at seven m away, and a third row 13 m away (Figure 2).

Applications were set up so that there were six trees one meter from the edge of each application, and at least three trees at seven m and 3 m from the edge of the application. In some cases, there were six trees at seven m from the edge of the application, depending on where the application was made within the block (on the edge, or more toward the middle). Each tree was treated as a biological replication. Between the two strips there were always 12 replications of trees one meter from the edge of the application, at least six replications at seven m and six replications at 13 m. To make sure that all treatments at a given application timing received similar amounts of moisture, the two strips of AMCP were also kept inside of the same block. Strips were separated by at least 4 rows of trees in order to ensure that AMCP from one strip was not affecting trees closest to the other strip. After application, treatments were supplied with 1.25 cm of moisture utilizing the onsite irrigation system to ensure activation of the herbicide.

Trees treated in 2012 were assessed one, two, and three years after treatment (YAT). Trees treated in 2013 were assessed one, and two YAT. Trees treated in 2014 were assessed one YAT. Assessments consisted of measuring from the edge of application to the furthest trees exhibiting injury symptoms. All trees were rated on a scale of 0-100% according to the following criteria: 0% - no response, similar to the check; 1-10% - mild epinasty, and slight stunting or swelling of shoots or leaves; 11-15% - moderate epinasty, swelling and stunting as well as chlorosis but no necrosis; 16-20% - moderate to severe epinasty and stunting, chlorosis, and some necrosis; 21-30% - major epinasty of shoots and leaves, and major chlorosis as well as necrosis; 31-50% - major chlorosis and necrosis such that recovery may not occur; 51-80% - severe response with major necrosis and recovery is unlikely; 81-100% - extreme response and tree death. Heights were collected for all trees within treated areas during the last rating in 2015. Due to limited numbers of trees, individual trees within each distance range were considered as individual replications, and Fisher's LSD at $p < 0.05$ in SAS was used for comparisons of injury.

Height Analysis

Tree heights were correlated with injury symptoms by pooling trees with similar treatment timings into fall, spring and summer for every year of treatments. Spring timings included trees treated in April, summer timings included trees treated in June or July, and fall timings included trees treated in October or November. Each individual tree was plotted for a correlation of height versus its injury rating 1 YAT. The average height of all trees within treated plots for 2012, 2013, and 2014 was determined and plotted. Tests for difference in average height between years was done using Tukey's HSD in R. Correlations between tree height an injury were analyzed by linear regression, with tests of slope using R determining whether a positive or negative slope was statistically different from 0 at $p < 0.05$.

Recovery

Tree recovery was measured by grouping treated trees into pools of one or seven m from the edge of the application. Next, these pools were sorted into groups corresponding to the treatment timing appropriate for a specific application year. Recovery was assessed by taking later ratings for specific trees, and subtracting the rating for a previous year. Groups assessed for trees treated in 2012 were as follows: change in rating between 2013 and 2015, and change in rating between 2014 and 2015. The only group assessed for trees treated in 2013 was the change in rating between 2014 and 2015 because these were the only two ratings available. Since trees treated in 2014 were only rated one year after treatment, no recovery data could be assessed. Individual trees that showed a negative value indicated recovery, while trees that showed positive values indicated a worsening of symptoms. Groups of trees were plotted for each timing, and two sided t-tests were performed using SAS in order to determine whether the mean change in injury rating for each timing was different from zero. Means tested were comprised of varying numbers of trees, depending on available biological replications for each timing and distance. This data is summed in Table 1.

Soil Analysis

Because differences in evapo-transpiration and soil biota between GA and HL trees could lead to differential organic compound dissipation, the relative AMCP dissipation in the soil underneath both species was of interest. Prior to the July 2013 treatments, eight petri dishes containing filter papers were placed down underneath GA and HL trees using a random pattern in order to accurately record the rate applied at 0 DAT. Next, six soil cores were randomly pulled from the middle of the treated strip underneath both GA and HL at 10, 20, and 60 days after treatment (DAT). All soil cores were pulled using a zero contamination sampling probe system, and plastic core sleeves. Once removed, the soil cores were capped, and placed into a freezer at -20ºC until analysis using an HPLC with a UV detection system. To investigate the extent to which tree root absorption might be contributing to the dissipation of AMCP, a separate study was established in the spring of 2015. Two AMCP treatments of 210 g ai ha^{-1} were placed corresponding to a 90 foot strip underneath rows of GA and HL trees, and a 90 foot strip set away from nearby trees with only ground vegetation within the treated plot. Prior to application, six petri dishes containing filters were placed down using a random pattern within the planned treatment areas to capture 0 DAT data. Next, a total of twelve soil cores were randomly pulled from within the area of both plots at 7 DAT, 30 DAT, and 45 DAT. Groups of three randomly assigned cores were mixed together to get a total of four reps for each time point of the experiment. These mixed samples were stored in the freezer at -20ºC until analysis using an LC-MS system.

Extraction HPLC

Soil core preparation consisted of separating individual cores into depth segments consisting of 0 to 5 cm, 5 to 10 cm, 10 to 20 cm, and 20 to 30 cm (Figure 3). Once depths were separated, samples were crushed, and run through a 2mm sieve. Five g of sieved soil for each sample was weighed, and placed into a 50 mL centrifuge tube. To these samples, 10 mL of deionized water was added. The tubes were then placed on a shaker for 2.5 hours to perform the AMCP extraction. Once extracted, the tubes were placed onto a centrifuge, and spun at 4,000 rpm for 15 minutes to move suspended soil to the bottom of the tube. Further cleaning was necessary, so an aliquot from these tubes was transferred to a 2 mL microcentrifuge tube with a 0.45 µm nylon filter tubes. These tubes were placed into a microcentrifuge, and spun at 15,000 rpm for 15 minutes. The resulting filtered extract was transferred to a 1.5 mL HPLC vial containing a low volume insert, and then analyzed using HPLC.

An additional two gram sample was placed into an aluminum weigh boat and placed into a dryer at 80ºC for 24 hours to perform soil moisture correction on the samples. Average core weights from 10 different soil cores were determined for each depth segment, allowing a translation from extracted AMCP into ng per core segment. Filter papers corresponding to the 0 DAT treatment were cut into 1 cm^2 sections and placed into 50 mL centrifuge tubes. To these tubes 10 mL of deionized water was added, and the tubes were spun at 4,000 rpm for 15 minutes to remove suspended paper from the extraction. Next, 1 mL of supernatant for each sample was diluted 100x to make peaks fall within the standard curve. This diluted solution was filtered through 0.45 µm nylon filter tubes, and placed into low volume inserts of HPLC vials for analysis.

HPLC Analysis

The HPLC system used for analysis consisted of a Hitachi L-7100 pump and an L-7250 autosampler coupled to a an Zorbax Rx C8, 4.6mm x 25cm column. The L-7400 UV detector was set to 250 nm. Samples were injected at a volume of 50 µL with a solvent flow rate of 1.400 mL minute^{-1}. Mobile phase A was composed of methanol at 1% and phosphoric acid at 0.05% mixed with HPLC grade water, and mobile phase B was composed of methanol at 15% and phosphoric acid at 0.05% mixed with HPLC grade water. The solvent program consisted of a start at 100% mobile phase A, with a ramp to 100% B at 10 minutes. Mobile phase B was held for 5 minutes, followed by another 5 minutes at 100% mobile phase A for a total runtime of 20 minutes, and an AMCP retention time of about 10 minutes. Standard samples were made containing AMCP dissolved in mobile phase A at known concentrations of 2, 1, 0.75, 0.5, 0.25, 0.1, and 0.05 µg mL^{-1}, and were analyzed to develop a standard curve for quantification for soil core samples. Additional quality control samples were created by spiking five g of topsoil, collected from the ARDEC Tree and Turf Research Area, with AMCP such that resulting concentrations were equal to 1, 0.5, and 0.1 µg m^{-1}. To compare the recovery numbers to the 0 DAT petri dish samples, average µg AMCP 3.8 cm^{-2} (or the area of a soil core cross section) was determined underneath both GA and HL trees at each time point by adding the total amount of recovered AMCP in each depth zone. Unpaired t-tests with Welch's correction were performed in GraphPad Prism to determine differences between the two species. Additionally, the AMCP concentration in ppb at each depth zone and time point was analyzed using Tukey's HSD and R statistical software to determine the extent to which vertical AMCP movement was occurring in the soil profile.

Extraction LC-MS

Much like the cores used for HPLC analysis, the cores analyzed using LC-MS were also separated into four depths; however, these cores were separated into four 7.5 cm segments. Five g of soil from each sample was weighed and placed into a 15 mL centrifuge tube. To this tube 10 mL of deionized water was added to perform the extraction. Tubes were then placed onto a shaker for 2.5 hours to complete the extraction. Once extracted, tubes were transferred to a centrifuge and spun at 4,000 rpm for 15 minutes in order to settle the suspended solids from the extracted solution. The UPLC-MS/MS required heavier filtering than the HPLC samples as described before, so an extraction aliquot was drawn into a 10 mL plastic syringe, and filtered into a 1.5 mL UPLC vial using an Acrodisc® 13 mm syringe filter with a 0.25 µm nylon membrane (PALL Corporation, Port Washington, NY 11050). These vials were capped and then analyzed using a Shimadzu LCMS 8040 (Shimadzu Scientific Instruments, Columbia, MD 21046).

LC-MS Analysis

The system consisted of a Nexera X2 UPLC with 2 LC-30AD pumps, a SIL-30AC MP autosampler, a DGU-20A5 Prominence degasser, a CTO-30A column oven, and SPD-M30A diode array detector coupled to an 8040 quadrupole mass-spectrometer. Proto levels were detected in positive mode with a MRM of 214.10>68 (Nanita et al., 2009). The MS was set for 100 ms dwell time with a Q1 pre-bias of -22.0V, a collision energy of -25.0V and a Q3 pre-bias of -30V. The samples were chromatographed on a 100x4.6 mm Phenomenex kinetex 2.6 µm biphenyl column maintained at 40°C. Mobile phase A consisted of water with 0.1% formic acid and mobile phase B was methanol with 0.1% formic acid. The gradient started at 5% B and increased linearly to 59% B until 5 min, followed by a linear gradient to 99% until 8 min. The mobile phase remained at 99% B until 10 min, then returned to 5% B at 10.5 min and maintained 5% until the end of the run at 15 min. The flow rate was set at 0.4 mL/min and samples were analyzed as 1 µl injection volumes. An initial extraction performed on 4 soil samples was followed by recurring extractions using 80%, 80%, and 70% acetonitrile solutions respectively. These extractions showed an average recovery of AMCP to be 90.6% from the initial water extraction, so a correction factor of 1.10 was applied to all water extracted samples to compensate for this. Much like the HPLC analysis, average µg AMCP 3.8 cm^{-2} was determined for treated plots at each time point, and each depth zone was also analyzed independently to quantify vertical AMCP movement. Unpaired t-tests with Welch's correction were performed in GraphPad Prism to determine differences between treated plots. Changes at each depth zone over time were also analyzed using Tukey's HSD and R statistical software.

Greenhouse Dose Response Trial

The rates of AMCP used in this study included an untreated control, 1.25, 2.5, 5, 10, 20, 40, and 80 g ai $hectare^{-1}$, or 0.6, 1.3, 2.5, 5.1, 10.1, 20.2, and 40.4 ppb (based on dry soil weight and a soil density of 1.32) respectively. To calculate the g ai ha^{-1} rate based on the concentration we used the following equation:

$$(1) \qquad Rate(gai * ha^{-1}) = Concentration(ppb) * 1.9802$$

The soil used was a custom soil blend ordered from Hummert International that was composed of 32.5% sand, 47.5% silt and 20.0% clay. Initial planting tests with the soil showed poor water penetration, and a medium to coarse textured sand was added during the treatments to alleviate this problem.

Herbicide treatments were made over two days on March 28th and March 29th of 2013. Soil treatments were done by placing 22.5 kg of sand with 77.5 kg of custom blend soil in a 9 cubic foot cement mixer from Sunstate Equipment Co. to achieve the desirable soil texture. As the soil was mixing a 250 mL AMCP solution, with a concentration based on the dry soil weight in the mixer as well as the treatment rate, was slowly applied using a hand-powered spray bottle applicator, and the soil was allowed to mix for 20 minutes after application to distribute the herbicide throughout the soil. The untreated control soil was mixed first, followed by the lowest treatment rate up to the highest treatment rate to avoid cross contamination between the treatments, and the mixer was rinsed thoroughly and dried between treatments. All trees in this study were bare root whips ordered from Lawyer Nursery. Their heights ranged depending on the species of the tree and availability from the nursery. HL trees were between 7.6 and 15.2 cm and were two year old seedlings. GA trees were between 15.2 and 30.4 cm and were two year old seedlings. Blue spruce trees (*Picea pungens glauca* var. Majestic Blue) were between 15.2 and 22.8 cm and were four year old transplants. Norway spruce trees (*Picea abies*) were between 15.2 and 22.8 cm and were two year old seedlings. A total of four replications of each species were planted per dose. Pots were Anderson Plant Bands (AB512) ordered from Stuewe and Sons. The bands were 12.7 cm wide by 30.5 cm tall with a total volume of 4.34 liters. A constant weight of 5 kg of treated soil was used for every pot. All trees were planted into the treated soils by holding the tree at the desired height in the pot, and pouring the treated soil around the roots.

The soil was lightly packed in the pot and then each pot was placed in the greenhouse and sorted into trays randomly by treatment, and then trays were assigned random locations within reps.

Trees were watered once a day as necessary to avoid leaching of the herbicide, and plastic liners in the bottom of the trays were used to ensure any leachate stayed within the tray and would not contaminate other treatments. Visual injury ratings and pictures were observed 60 days after transplanting. The visual injury was rated based on a scale of 0 to 100% as outlined before, and the factor chosen for comparison between the species was EC_{25}. Analysis was performed using the drc package in R, and dose response curves were fitted using a 3-paramater log-logistic equation as described by Knezevic et al. (2007).

RESULTS

Injury Symptoms and Observations

Interestingly, full size GA trees did not show injury symptoms from any treatments applied at the site (Figure 4a), with the exception of a cut stump tree within the row of trees treated in April 2012 (Figure 4b). This particular stump was sending up vegetative growth, and therefore had a large root system feeding only a few individual shoots. On this stump, injury symptoms mostly included cupping of the leaves. There was no visible chlorosis, or necrosis, and the shoots coming off of the stump never defoliated. By the second year after treatment, there were no longer any visible injury symptoms and the shoots from this stump continued to flourish.

Since there was a large difference in response between the GA and HL trees, the data presented for injury ratings will mostly deal with injury symptoms observed on honeylocust trees. Injury symptoms appeared to differ from spring and summer applications to fall applications. Our initial April application in 2012 did not yield any injury symptoms until after the first rainfall in May, indicating that rainfall and irrigation may lead to the activation of herbicide uptake and injury. In cases where moisture was available, injury symptoms appeared relatively quickly; sometimes within 1 week of the AMCP application and appearing as chlorosis on the lowermost leaves of the affected tree. Since AMCP is an auxinic herbicide, the expected injury symptoms included epinasty and malformed growth of new leaves and tissues; however, within two weeks after the May rainfall, the symptoms observed on the honeylocust trees were that of chlorosis and necrosis. This chlorosis started at the base of the petiole, and moved out along the leaf, eventually affecting all of the leaflets (Figure 5a). Next, necrosis started out from the base of the petiole over-taking the leaflets, and eventually the most severe cases of injury results in defoliation of the affected parts of trees. The chlorosis first appeared in the tops of the honeylocust trees, with some exceptions where shoots near the base of the trunk showed the chlorosis first, typically within one to two weeks after the application. The observed injury symptoms from the spring and summer treatments were not localized to the new growth.

The fall treatments were made after the trees had gone into dormancy, and therefore no symptoms were observed until the following spring. In these cases, herbicide injury symptoms differed. Leaf petioles were observed twisting, and leaflets showed pronounced curling (Figure 5b), which was a much more consistent response with plant growth regulator herbicides. Eventually, these epinastic symptoms turned into chlorosis that began at the tip of the leaves, and worked its way down toward the base of the petiole. Severe cases from fall treatments also resulted in defoliation of the tree. In cases where the herbicide did not cause complete defoliation of the tree, injury symptoms tended to be localized to the side of the tree located closest to the strip of herbicide. Symptoms for trees treated in the spring, summer, and fall persisted into the first, and second YAT, with new growth in subsequent years showing epinastic symptoms and termination of new shoots. The most severe cases showed development of trunk growths, as well as vertical splits in trunks of the trees. In these cases, little to no recovery occurred.

2012 Applications

Analysis of visual injury ratings 1 year after treatment (YAT) with respect to timing for 2012 (Figure 6a) applications did not show differences between the April and June application for any trees 1, 7, and 13 m from the edge of the application. Trees 1 meter from the edge of the application showed reduced injury for the October application, relative to the April and June applications. Trees located 7 m from the edge of the application displayed lower injury from the October application as well. Trees located 13 m away from the application did not display injury symptoms from any application timing.

Visual injury ratings taken 2 YAT (Figure 6b) displayed different results. There was no difference in injury between the April and June application timing for trees located 1, 7, and 13 m from the edge of the application. Injury to October treated trees 1 meter away still showed less injury than the April and June treatments; however, this injury was still quite severe at an average rating of 90%. October treated trees 7 m away showed higher injury than the year before. These trees also had a higher injury rating than June treated trees, but had similar injury to the trees in the April treatments. Like the 2012 applications, trees located 13 m away still displayed no injury symptoms for any treatment timing.

Visual injury ratings recorded 3 YAT (Figure 6c) showed severe injury (>90%) for trees 1 meter from the edge of the application for all three application timings. The October treatment resulted in slightly less injury compared to the June timing, but was similar to the injury observed from April application. Injury on trees observed for the June application was also similar to injury on trees observed from the April timing. By 3 YAT, trees 7 m from the edge of the application showed no significant differences in injury for April, June and October treatment timings. Comparison of distance for applications made in 2012 showed that there was a significantly less injury observed on trees 1 meter from the edge of the application than trees 7 m away for 1, 2, and 3 YAT (Figure 7). No injury was observed on trees located 13 m from the edge of the applications.

2013 Applications

Because there was less injury observed on trees treated during the fall vs those treated in the spring and summer for 2012, 2013 applications focused on fall timing applications in order to determine whether October or November treatments were safer for HL trees at our site. In addition a July treatment was also added as a control to compare summer treatments to fall treatments.

One YAT (Figure 8A), average injury for trees treated in July 1 meter from the application was about 100%, with average injury for July and October trees significantly lower at 40, and 44% respectively. Trees 7 m from the application showed a similar trend. July treated trees showed average injury ratings of 73% while October and November trees had lower average injury ratings of 8% and 18% respectively. There were no significant differences between the average injury rating of the fall treatments at either 1 or 7 m from the application, and trees at 13 m did not show injury symptoms.

The 2 YAT (Figure 8B) ratings showed largely similar results. The two fall treatments still showed significantly reduced injury compared to the July treatment for trees both 1 and 7 m from the application, and there were still no significant differences in average injury rating between October and November treatments. Comparison of 1 YAT ratings for trees located 1 meter from the edge of the application showed significantly higher injury ratings than trees located 7 m away from the edge of the application (Figure 9A). There were no observable injury symptoms on trees 13 m from the edge of the application. The 2 YAT ratings (Figure 9B) showed the same result. Average injury rating for July treated trees 7 m from the application were lower than 2012 applications, and October and November treated trees both 1 and 7 m from the application were also lower than 2012 applications.

2014 Applications

Injury ratings 1 YAT for HL trees treated in 2014 showed a higher level of injury on trees 1 meter from the application for the April timing than the July timing (100% and 40% respectively). This same trend was seen for trees 7 m away from the application, with April trees averaging 73% injury while July trees averaged only 8% (Figure 10). Trees 13 m away showed no injury for April treated or July treated trees. With respect to distance (Figure 11), there was a significant drop off in injury between 1 and 7 m from the application, with averages dropping from 100% to 73% for the April timing, and dropping from 40% to 7% for the July timing.

By three weeks after the July application in 2014, the trees located 13 m away from the application appeared to show very slight injury symptoms (<5%); however, by 1 YAT these symptoms could no longer be seen. The fact that these trees were the only trees in the study showing symptoms at 13 m from the edge of the application is most likely due to their larger size (averaging 13.3 m tall) compared to all other trees in the study.

Height Analysis

Trees treated in 2013 and 2014 showed less injury compared to trees treated during 2012. A plot of the average tree height for trees treated in 2012, 2013, and 2014 (Figure 12) shows that trees treated in 2013 were on average the same height as trees treated in 2012. Despite this, the number of trees that were taller than the average tree in 2012 was higher. Trees treated in 2014 were on average taller than both trees treated in 2012 and 2013. It was hypothesized that tree height or size may have affected tree injury observed in 2013 and 2014.

Correlations between tree height and injury rating (Figure 13) showed that summer treated trees located both 1 and 7 m away from the edge of the application showed a trend of lower injury rating as tree height decreased (p-values 0.0004 and 0.0317, respectively). Spring applications showed a similar trend. Trees 1 meter from the edge of the application showed a trend of steep decline (p=0.0027) in injury rating as tree height increased; however, trees located 7 m from the application during spring applications did not show a trend of lower injury rating as tree height increased (p=0.4489). Fall applications for trees located 1 meter from the edge of the application did not display lower injury ratings on taller trees (p=0.3800) but the trees located 7 m from the edge of the application did (p=0.0021). Overall, with the exception of the 7 meter trees for spring applications and the 1 meter trees for fall applications, trees did appear to show a trend of lower injury with respect to increased tree height.

Recovery

In general, trees treated in 2012 did not show signs of recovery in the time between the 2013 rating and the 2015 rating. The data summed up in Table 1 shows trees 7 m away from the application treated in October showed significantly higher injury in 2015 than the rating taken during 2013, showing on average an injury rating of 38.33% higher than the initial rating (p-value=0.0022). Moderate to minor recovery could be seen on trees treated in 2012 during the year between 2014 and 2015 with 7 meter trees treated in both April and October showing significant decreases in injury rating between 2014 and 2015 (p values = 0.0153 and 0.0024 respectively). Trees treated in 2013 also showed signs of recovery between 2014 and 2015. Trees treated in November at both 1 and 7 m from the edge of the application showed significant recovery (p-values 0.0001 and 0.0006), and October trees 7 m from the application also showed significant recovery. In general, trees located 7 m from the application were more likely to show recovery, and fall treated trees were most likely to show recovery following AMCP injury.

HPLC Analysis

To determine whether AMCP dissipated at different rates in the soil beneath GA and HL trees, soil cores were pulled and analyzed using HPLC. The 0 DAT samples (Figure 14) showed no differences between the applied AMCP underneath GA and HL trees (p-value = 0.1099). Looking at the average amount of AMCP found within each core at 10, 20, and 60 DAT (Figure 15), there was no difference in the amount of AMCP underneath GA or HL trees for any of the three time points. Except for a small amount detected in a core pulled from underneath GA trees, almost no AMCP was recovered after just 20 DAT, and no AMCP was recovered 60 DAT. Since literature suggests a relatively long half-life for AMCP (Lindenmayer. 2013; Shaner. 2014) this was a puzzling result.

Looking at the data by depth (Figure 16), we see that all of the recovered AMCP underneath GA trees was in the upper 5 cm of the soil profile 10 DAT. At 20 DAT a small amount was recovered underneath GA trees in the 5-10 cm profile, showing some vertical movement through the profile. Underneath HL trees, the trend was similar. Most of the recovered AMCP was within the first 5 cm; however, unlike GA we also detected AMCP in the

5-10 cm segment as well. There was no detectable AMCP underneath HL trees 20 DAT. No AMCP was detected below 10 cm, for either HL or GA trees at any time point indicating a low amount of vertical movement, contrary to predictions made from chemical properties. It was possible that AMCP was there, but below the accurate detection limit of the HPLC system. The lack of AMCP found after such a short amount of time could also have been due to bioaccumulation in trees from tree root absorption, making the dissipation rate faster. Therefore, another study was designed to address these questions using an LC-MS system, which has a much lower limit for quantification.

LC-MS Analysis

While the HPLC analysis was focused on AMCP dissipation differences between GA and HL trees, the LC-MS analysis was focused on determining the contribution of tree root absorption to overall dissipation of AMCP. Once again 0 DAT samples indicated there was no difference between AMCP applied in the plot located next to GA and HL trees and the plot that was located away from trees, with only Kentucky bluegrass in the plot (Figure 17). By 10 DAT there was a significant difference (p-value = 0.0097) in total recovered AMCP between the 'tree' and 'no tree' treatments, with the plot without trees showing more AMCP at 6.5 µg AMCP 3.8 cm^{-2} versus 5 µg AMCP 3.8 cm^{-2}. Both the 30 and 45 DAT samples showed this same trend (p-values = 0.0040 and 0.0012, respectively), with the plot without trees showing a significantly higher amount of AMCP than the plot with trees present (Figure 18).

The overall detection of AMCP was much better using the LC-MS than with the HPLC. Easily detectable and quantifiable amounts of AMCP were found for all three of the tested time points. By depth (Figure 19), the results from the LC-MS also contrasted widely with the results from the HPLC. AMCP was recovered for every tested depth, all the way down to 30 cm for every time point tested. At the 10 DAT time point, the bulk of recovered AMCP was located within the 0-7.5 and 7.5-15 cm soil segments, with smaller amounts being found at the 15-22.5 and 22.5-30 cm segments. The 30 DAT time point shows that the AMCP had moved slightly, with the most recovered in the 7.5-15 and 15-22.5 cm segments. The 45 DAT time point shows that the AMCP was accumulated mostly in the 15-22.5 cm segment. This demonstrates vertical movement of AMCP through the soil profile, and was true regardless of whether the plot was beneath GA and HL trees or not. The herbicide moved from the 0-7.5 cm segment between the 10 and 35 DAT time points for both the plot with trees as well as the plot without trees. Between the 35 and 40 DAT time points, the level of AMCP stabilized showing no difference. This trend remained true for the 7.5-15 cm segment as well, except that the tree plot showed a reduced level of AMCP between the 30 and 45 day time points. The 15-22.5 cm segment for the plot without trees plots showed an accumulation of AMCP between the 10 and 35 DAT time point, and stabilized between the 35 and 45 DAT time points. The plot with trees showed no difference at this depth to the 10 DAT time point, but did show an accumulation of ACMP between the 30 and 45 DAT time points. Both plots showed an accumulation of AMCP within the 22.5-30 cm segment after 30 DAT.

Greenhouse Dose Response

Visual injury ratings observed 60 days after treatment showed variable results between the spruce trees and the deciduous trees. When looking at the dose response curves (Figure 20), both the Norway spruce and blue spruce trees appeared to have a somewhat similar response ($p = 0.056$) to the herbicide with EC_{25} values of 2.5 and 5.6 ppb respectively. However, GA and HL trees showed a different response, where HL appeared to have a moderately higher tolerance to the AMCP treatments with an EC_{25} of 19.9, which was about four times more tolerant than the spruce species ($p < 0.05$) ppb. Much like the field observations, GA was the most tolerant species, and did not approach a 25% response at the tested doses. Because the upper limit for GA was not adequately predicted by the model, comparison of EC_{25} between GA and HL showed that there was no significant difference between these species. It should be noted that GA did indeed have a very distinct response when compared with HL. An EC_{10} of 107.5 ppb was predicted for GA based on the model; however, even this value had a very high standard error.

DISCUSSION

Injury Symptoms and Observations

The range in response between the two tree species in the trial was quite contrasting, but not necessarily surprising. While information is typically scarce on the topic of AMCP's effect on specific tree species, it has been shown that AMCP applications resulted in only 28% injury in loblolly pine trees one year after treatment (Roten, 2011). In addition, our own greenhouse dose response test indicated a wide variety of potential responses among the tree species tested. Taken together these results show that there could be many different deciduous and non-deciduous trees that do not display a hyper-sensitive response to root absorption of AMCP. It is not known why GA is tolerant to AMCP, although several different factors can affect overall sensitivity to a given herbicide. Differences in absorption or translocation of the herbicide (Adu Yeboah et al., 2014; Goggin et al., 2016), enhanced metabolism (Han et al., 2013), and changes to how the herbicide interacts with the target site (Powles and Yu, 2010) can all influence whether or not a herbicide is effective in altering plant growth or function. Future studies investigating this in GA could help in the development of quick markers, to help determine whether given trees will be tolerant to AMCP applications.

One interesting note about the AMCP injury symptoms was that they manifested differently depending on the timing of the application. The fall and spring applications (assuming symptoms appeared right away) resulted in twisting and epinasty of affected leaves; however, when treated during summer months after the foliage had already reached full size, the symptoms included chlorosis, necrosis, and defoliation. This is likely because in the summer months there is less actively growing foliage tissue, whereas in the spring the treatments are made right after bud burst so leaves are still growing. Fall applications occur after dormancy, so any herbicide injury witnessed would not be until the following spring during active tissue growth. The fact that herbicide injury tended to exist on the side of the tree closest to the application is consistent with a mechanism of root uptake, as only certain portions of the root system would have been exposed to the herbicide (Feucht, 1988). Generally, trees that were very severely injured in the first year did not recover in subsequent years. After the initial treatment of AMCP trees in April of 2012, it was also noted that no injury symptoms were observed until a week after the first major rainfall of the spring, which was 1.2 cm of rain. Because of this, later treatments utilized the irrigation system at the site to make sure at least 1.25 cm of moisture was available to ensure activation of the herbicide in a timely fashion.

2012 Applications

The 1 YAT ratings for trees treated in 2012 were indicative that the October treatment timing was a safer time to apply the herbicide in the case of HL trees. The high level of injury for trees treated in April and June 1 YAT was quite predictive for how those trees would look in subsequent years, as trees that were highly injured after the first year typically did not make recoveries. Oddly, although the injury ratings were relatively low for HL trees during the October treatment timing in 2012, the 2 YAT ratings showed an increase in the injury rating from the previous year, making these trees look much more similar to the April and June treated trees during the same year. One possible explanation for this could be due to application of AMCP next to nearby pine trees for a separate trial. While applications were kept far apart to avoid carry over between different treatments, it is possible that the roots of the HL trees in the October treatment reached into the zone of application for another treatment. The trees located 7 m from the edge of the application of the October treatment showed high levels of injury compared to the April and June treated trees at the same distance, which might indicate that these trees have a more extensive root system. Despite this, the trees located 1 meter from the edge of the application still showed slightly lower injury compared the 1 meter trees treated in April and June. Even though this is true, the level of injury on these trees was still high with an average injury rating of close to 90%. The farthest distance to injury noted from the 2012 applications was for trees located 7 m from the edge of the application. Interestingly, 7 m was fairly close to the average tree height for trees treated during this same time, and well outside the dripline of the affected trees.

2013 Applications

Because the trees in the 2012 treatments appeared to show slightly less injury on trees treated in October, it was hypothesized that different application timings during the fall may provide even safer alternatives. The results of the 2013 comparisons were much starker than from the previous year, providing a clear contrast between trees treated during the summer, and trees treated during the fall. While trees treated in July of 2013 showed a high level of injury for trees both 1 and 7 m from the edge of the application, October and November treated trees showed much less injury both 1 and 2 YAT. It is unclear why these trees responded so differently from the October treatment of 2012, though tree size may play a part. Analysis of furthest distance to injury yielded the same results as trees treated in 2012, with only trees 1 and

7 m from the edge of the application showing injury. The trees 7 m from the edge of the application showed very little injury for fall treated trees in 2013, which is highly contrasted with similar trees from the 2012 treatments.

2014 Applications

Trees targeted for application in 2014 were made on some of the tallest trees at the site, averaging just over 12 m tall. Because we were nearing the end of time we had allotted for the site, applications could only be made for two timings in April and July, corresponding to two more repetitions for spring and summer treatments. Like the April treated trees in 2012, trees treated in 2014 showed quite a high level of injury 1 YAT, with trees 1 meter from the edge of the application showing nearly complete death, while trees 7 m away averaging close to 70% injury. In contrast, the July treated trees showed relatively low injury of 40% for trees 1 meter from the application and 8% for trees 7 m from the application, which is heavily contrasted with the response of trees treated in the summer months for in 2012 and 2013. It is possible that tree size or some factor related to better vigor could be responsible, as the average height for April treated trees in 2014 was 10.4 m, while the July treated trees averaged 13.3 m. These data suggest that the spring application timing may result in the highest injury, and therefore is not the safest time to apply AMCP to avoid tree injury. Although no official rating was recorded, injury on July treated trees in 2014 occurred rather quickly, within two weeks of the application date. It was noted that the very tips of trees located 13 m from the edge of the application showed signs of herbicide injury no greater than 5%; however, these symptoms did not persist to the official 1 YAT rating.

Height Analysis

Due to lower injury for specific timings in 2013 and 2014, which were not expected based on higher levels of injury in 2012, it was hypothesized that tree size may help predict the potential level of injury that HL could experience. Average tree height for trees treated in 2012, 2013, and 2014 suggested an increasing trend of larger trees in 2013 and 2014, so a correlation of tree height vs. injury rating was performed. These were inclusive correlations that included tree ratings 1 YAT and at both 1 and 7 m for trees treated in 2012, 2013, and 2014. In general there was a negative trend showing a drop in injury rating as tree height increased, with the exception of fall applications with trees 1 meter away, and spring applications with trees 7 m away. It is unclear why these two exceptions do not show the same trend, but in general the data suggests larger trees are less likely to be heavily injured after non-target AMCP injury. While this may help explain why less injury was observed on trees treated in 2013 and 2014, other factors such as changes in climate, rainfall, soil moisture, average temperatures and other abiotic factors probably also contributed to this result. Future studies looking at non-target AMCP impact on trees should take these other factors into consideration.

Recovery

One goal of this experiment was to determine whether or not injured trees made a recovery over a long-term period. In general, trees treated in 2012 did not show any recovery or got worse between 2013 and 2015. However, between 2014 and 2015, October treated trees 7 m from the edge of the application showed recovery, which suggests that the trees got worse in the initial year after application, and then started to recover in the second year. Overall, results of the change in rating for individual trees year to year suggested that fall months and being farther from the application were more likely to result in tree recovery. This coupled with lower injury ratings compared to spring and summer application timings indicates that applications during the dormant season of the tree in the fall may be the best time to apply AMCP. This was also suggested for the use of aminopyralid in ponderosa pine and for the establishment of pine forests as a method of plant growth regulator herbicide selectivity to avoid pine injury (Paley and Radosevich, 1984; Wallace et al., 2012).

HPLC Analysis

One hypothesis to explain why GA trees were not susceptible to the applications of AMCP was that GA trees were absorbing AMCP at a lower rate than HL trees; however, the initial HPLC soil analysis determined that the level of AMCP beneath GA and HL trees was similar at all tested time points, indicating that dissipation was similar underneath both species. This result does not suggest lower absorption rate as a reason that GA was tolerating the AMCP application, since a lower absorption rate would have resulted in higher amounts of residual AMCP underneath GA trees compared to HL. There was more vertical movement under HL 10 DAT than compared to GA, in which no detectable AMCP was found at the 5-10 cm depth. Vertical movement was very limited, and no detectable AMCP was found below the 10 cm depth for any time point or tree species. This result contrasts with predicted leaching potential for AMCP based on its chemical properties, but is consistent with a hypothesis by Oliveira et al. (2011) that AMCP leaching potential may be overestimated due to hysteretic desorption. The lack of recovered AMCP just 20 DAT was a very surprising result. Given the long half-life of AMCP reported in literature, it was expected that AMCP would be recovered many days after treatment. It was possible that the background noise was simply too high using the UV detection method, and that we weren't getting good enough resolution to detect small quantities of AMCP within the soil. It was also possible that AMCP dissipation was being sped up by the absorption of the herbicide into the nearby tree and grass vegetation, and therefore wasn't showing up in the soil samples.

LC-MS Analysis

In order to determine whether or not AMCP was being readily absorbed by tree roots, and therefore dissipated from the soil profile, comparisons between a treated plot next to GA and HL trees had to be compared to a treated plot that did not have trees nearby. To make sure that there was an adequate quantification limit, an LC-MS was used instead of the HPLC. The results of this trial indicated that there was a significant difference in recovered AMCP beneath the plot without trees and the plot with trees at 10, 30, and 45 days after treatment, with the plot next to trees showing significantly less recovered AMCP. Since there was no significant difference between the amounts of AMCP recovered in both plots at 0 DAT, this result shows that tree root absorption was indeed contributing to a faster dissipation rate of AMCP. Analysis of the AMCP by depth in both plots showed much better recovery of the herbicide compared to the HPLC analysis. AMCP was found well below 10 cm, all the way down to the lowest sampling depth of 30 cm at every time point tested. As time went on, it was also apparent that AMCP was slowly moving through the vertical profile, accumulating more in the 15-22.5 and 22.5-30 cm depths, while simultaneously vacating the 0-7.5 and 7.5 to 15 cm depths. Also interestingly, average detection for AMCP at each depth was on average less than 50 ppb, which was below comfortable quantification limits using the HPLC. This means that AMCP was likely in the soil below 10 cm in the soil cores analyzed using the HPLC, but we simply could not detect it. This, in conjunction with tree root absorption, was the most likely reason that we did not find AMCP much after 20 DAT using the HPLC analysis method. Future tree trials should use LC-MS analysis to avoid this issue, since labeled use rates of AMCP will result in lower amounts of AMCP in the soil, and high resolution is required to see it.

Greenhouse Dose Response

Results of this experiment in conjunction with the field data indicate strong evidence of a high tolerance to AMCP by GA, with the GA trees in this study showing only minor response at the higher treatment rates. This result indicates that this species may be relatively safe when using labeled use rates of AMCP nearby. HL appeared to show a moderate tolerance to the herbicide relative to the higher tolerance of GA, and the much more sensitive Norway spruce and blue spruce. Norway spruce, which has been shown to have a much higher sensitivity to the herbicide served as a good indicator for sensitivity between the species. Norway spruce was the most sensitive tree species followed closely by the blue spruce, which showed similar symptoms and sensitivity. This indicates that blue spruce is another species in which close attention should be paid when AMCP applications must be made nearby. In the future, additional dose response trials could be performed for other key desirable tree species. While this study had only a 60 DAT observation time due to time and space constraints, other studies could extend the time of observation, allowing for long term study of tree response and recovery on tree seedlings within the greenhouse. Further study on the response of GA and HL would be helpful to determine more rate response information at higher rates of AMCP, so that accurate predictions can be made about highly tolerant tree species.

TABLES AND FIGURES

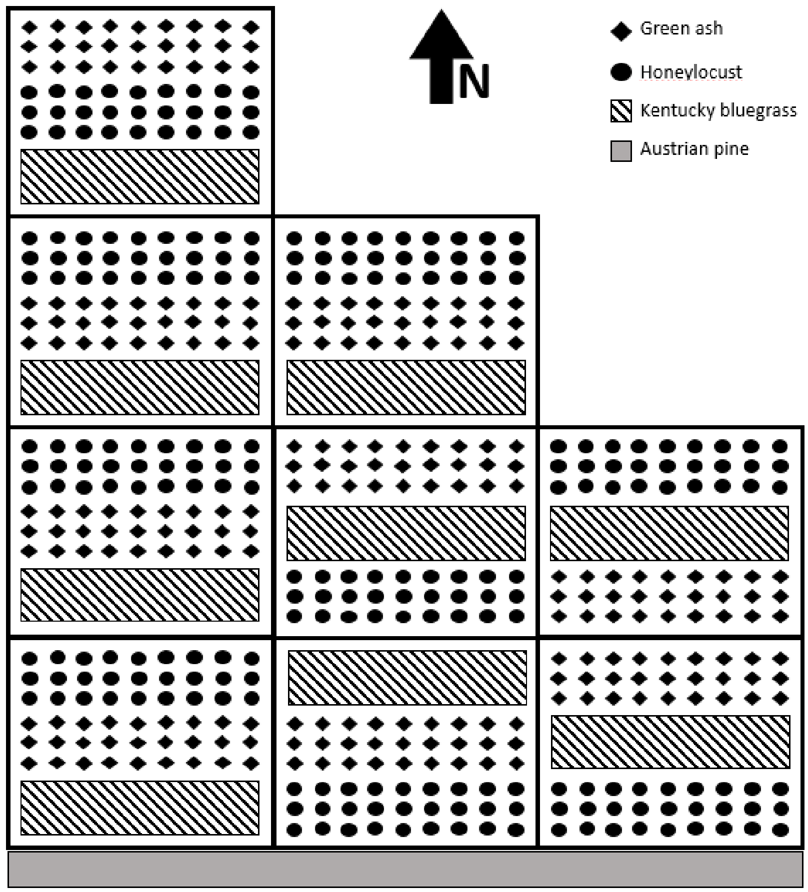

Figure 1. Site map of the ARDEC Turf and Tree Research area. Each of nine blocks was randomly populated with honeylocust, green ash, and Kentucky bluegrass.

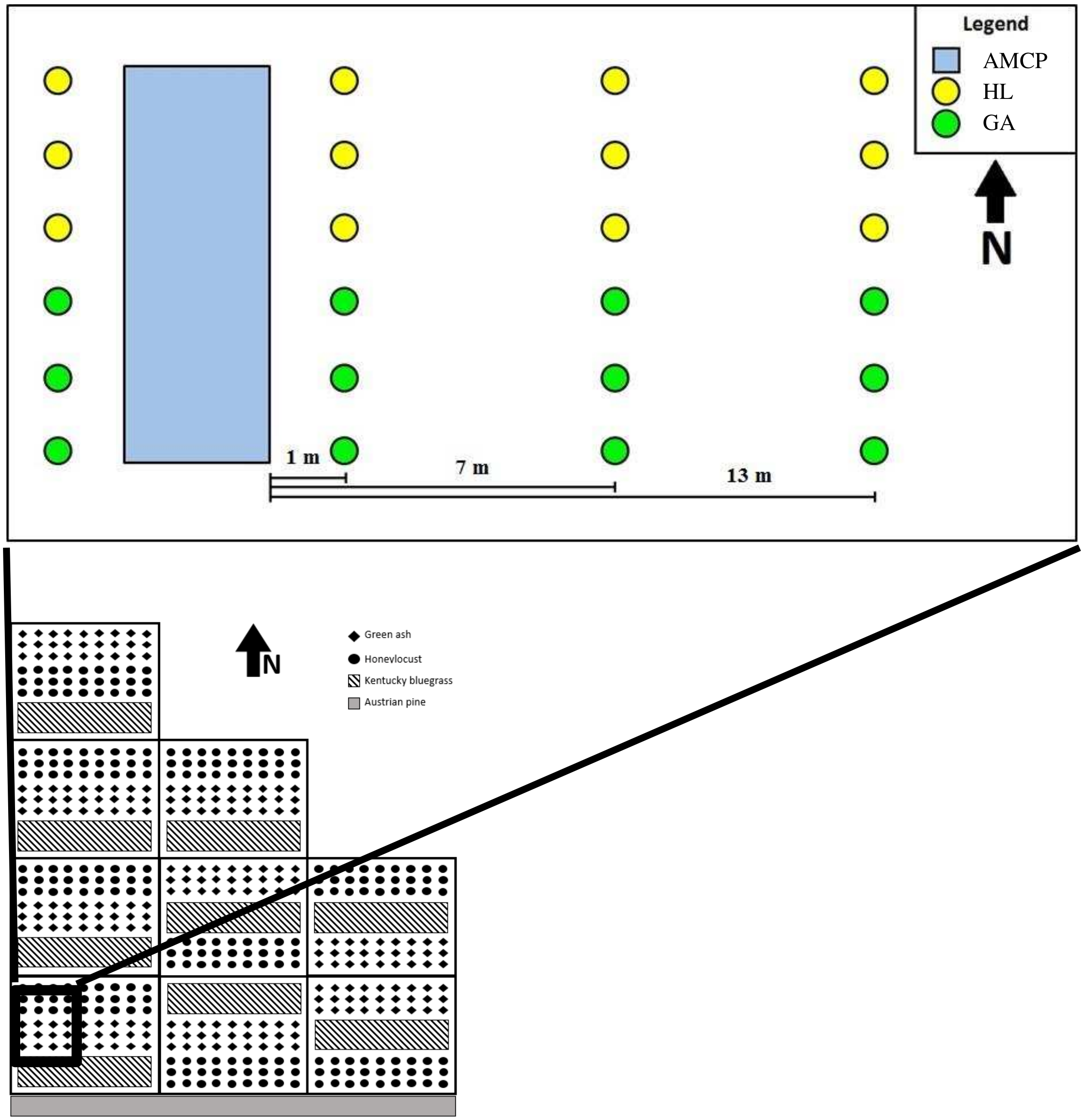

Figure 2. Application layout map for the tree safety trials. Honeylocust = HL and green ash = GA. Two treatment strips were applied for each timing within the same block, allowing for similar watering for herbicide incorporation. Strips were placed far enough away from each other to avoid overlap of injury symptoms on trees between the treated strips.

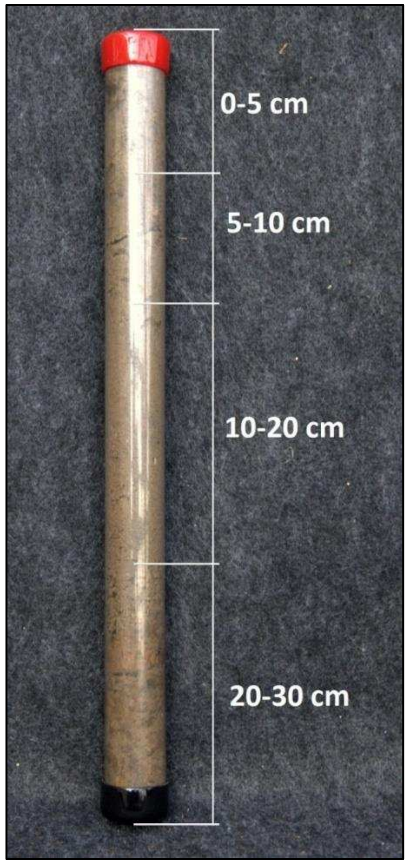

Figure 3. Example soil core in zero contamination sleeve. Cores were 30 cm deep, and were separated into four depth zones for analysis of AMCP vertical movement.

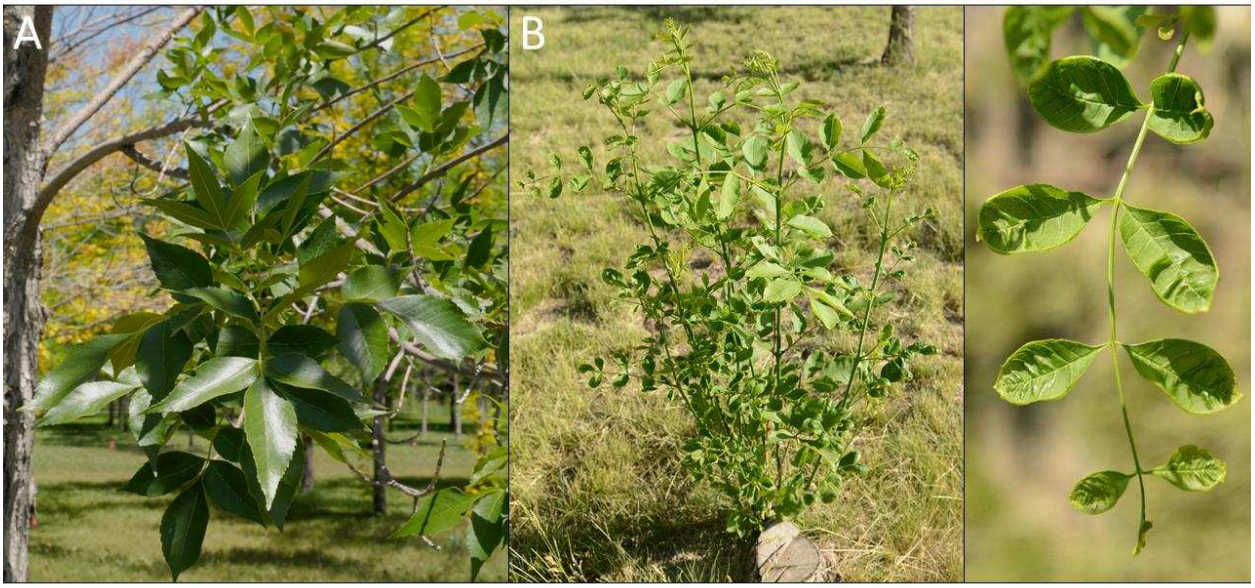

Figure 4. Injury symptoms associated with the green ash trees after a nearby soil application of aminocyclopyrachlor. (A) All full-sized green ash trees at the research site did not show injury symptoms for any treatment timing, or year applied. (B) Only a small cut-stump tree that was sending up shoots showed injury symptoms, which included mild epinasty, and leaf cupping. By 2 YAT, these injury symptoms no longer persisted.

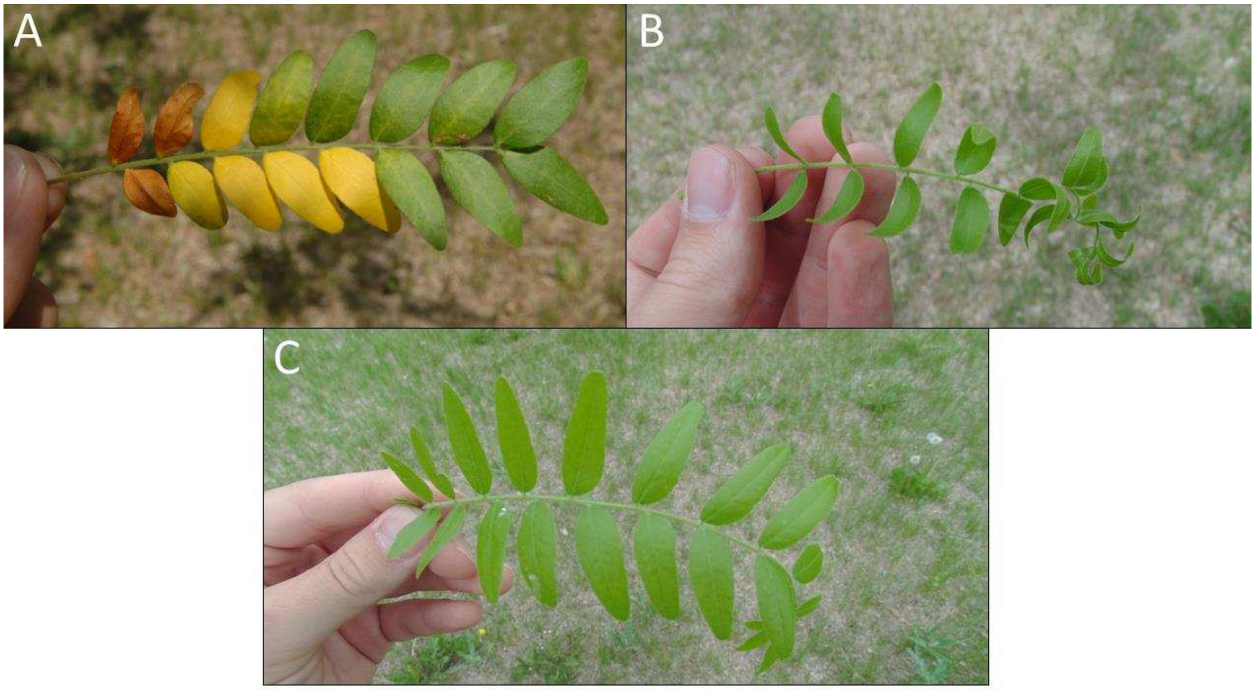

Figure 5. Injury symptoms associated with honey locust trees after nearby soil treatments with aminocyclopyrachlor. (A) A leaf showing injury symptoms that were typical of applications made in the spring and summer, showing chlorosis and necrosis, but no epinastic symptoms. (B) A leaf showing injury symptoms associated with applications made in the fall, showing epinastic symptoms typical for plant growth regulator herbicides. (C) A healthy honey locust leaf.

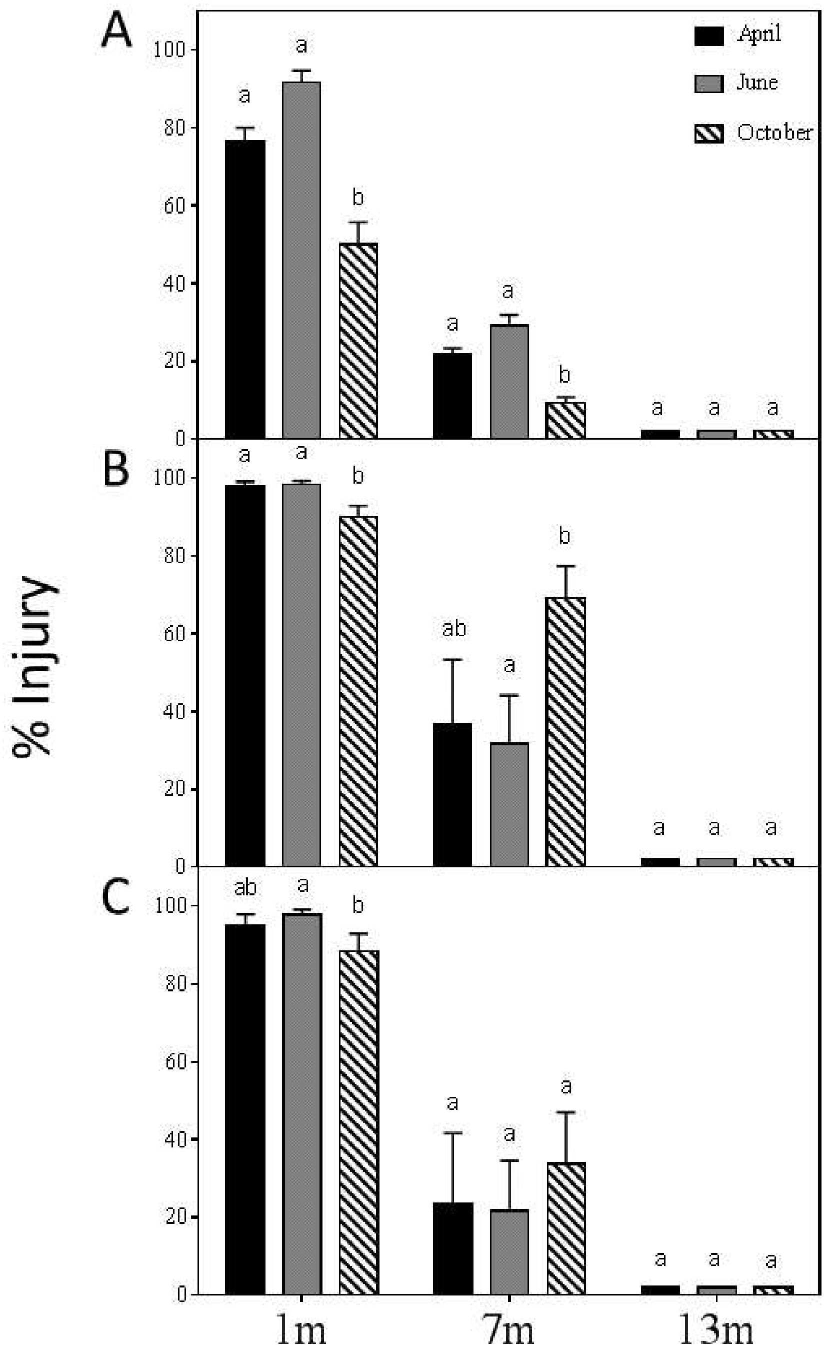

Figure 6. Percent injury on honeylocust trees at different soil applied aminocyclopyrachlor application timings made in 2012. The x-axis represents the distance from the edge of the application to the trunk of the tree. All comparisons were made within distance on mean injury rating ($n \geq 3$). Letters indicate differences in means at $p < 0.05$, and error bars represent standard error. (A) The recorded injury ratings for all honey locust trees 1 YAT. (B) The recorded injury ratings for all honey locust trees 2 YAT. (C) The recorded injury ratings for all honey locust trees 3 YAT.

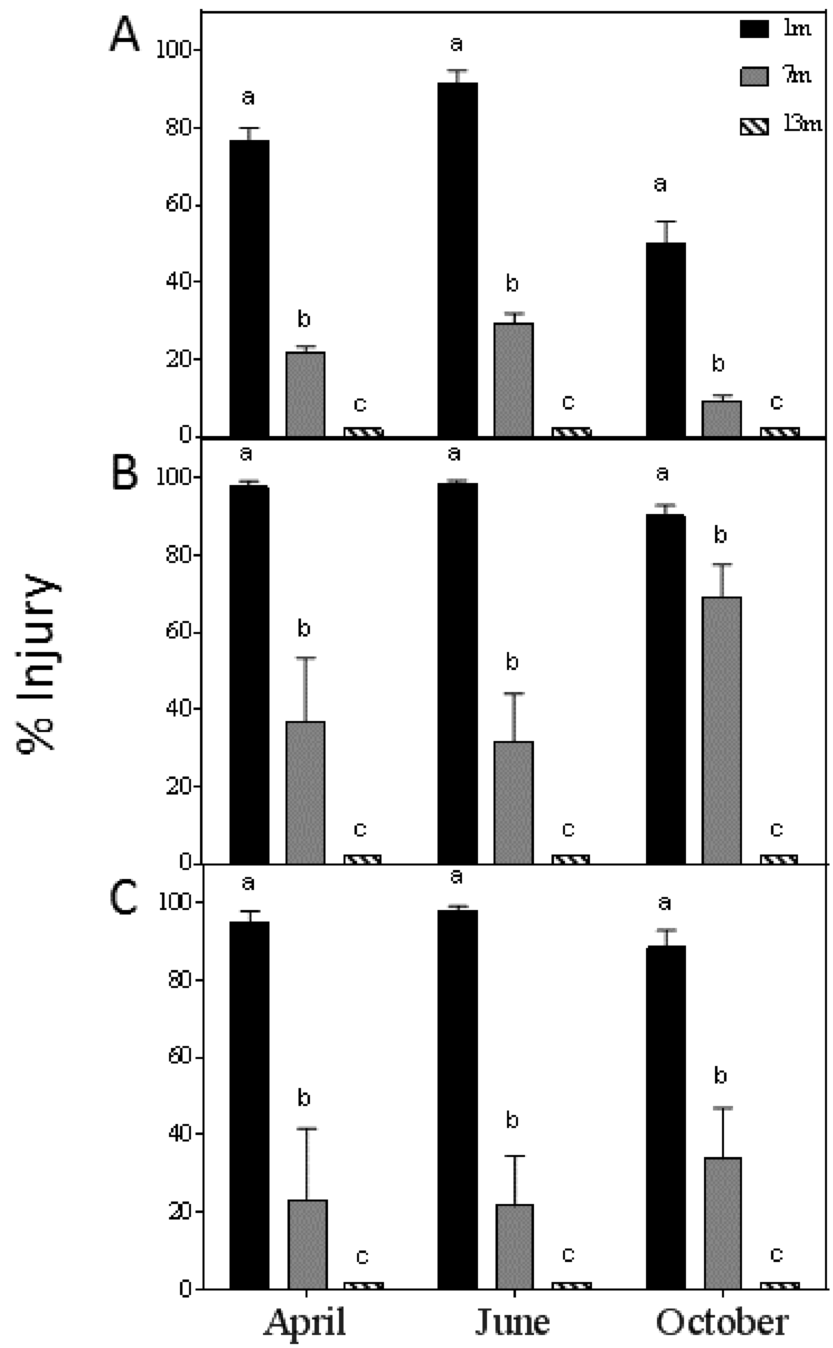

Figure 7. Percent injury on honeylocust trees for different distances from the edge of a soil applied aminocyclopyrachlor applications made in 2012. The x-axis represents different herbicide application timings. All comparisons of distance from application were made within application timing on mean injury rating ($n \geq 3$). Letters indicate differences in means at $p < 0.05$ and error bars represent standard error. (A) The recorded injury ratings for all honey locust trees 1 YAT. (B) The recorded injury ratings for all honey locust trees 2 YAT. (C) The recorded injury ratings for all honey locust trees 3 YAT.

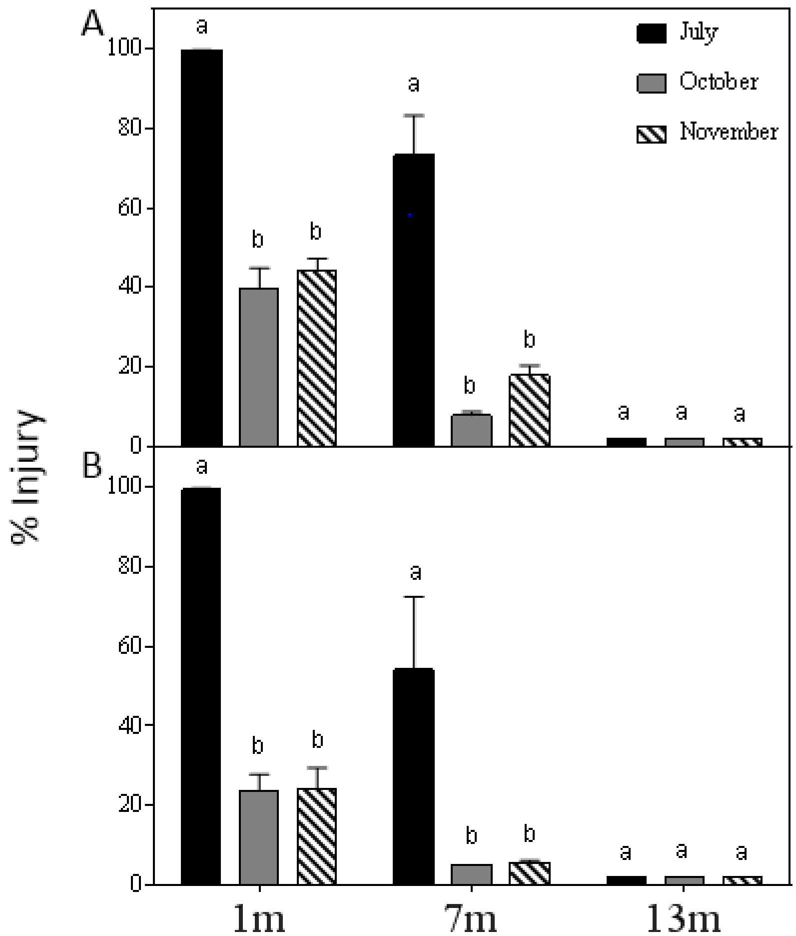

Figure 8. Percent injury on honeylocust trees at different soil applied aminocyclopyrachlor application timings made in 2013. The x-axis represents the distance from the edge of the application to the trunk of the tree. All comparisons of application timing were made within distance on mean injury rating ($n \geq 3$). Letters indicate differences in means at $p < 0.05$ and error bars represent standard error. (A) The recorded injury ratings for all honey locust trees 1 YAT. (B) The recorded injury ratings for all honey locust trees 2 YAT.

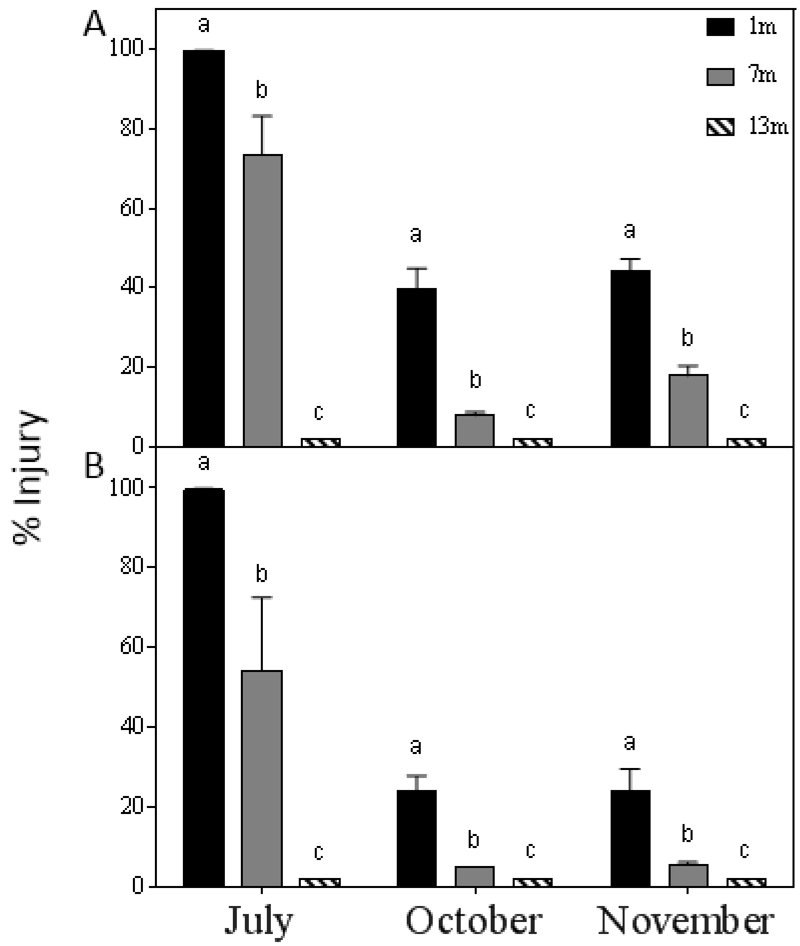

Figure 9. Percent injury on honeylocust trees for different distances from the edge of a soil applied aminocyclopyrachlor applications made in 2013. The x-axis represents different herbicide application timings. All comparisons of distance from the edge of the application were made within application timing on mean injury rating ($n \geq 3$). Letters indicate differences in means at $p < 0.05$ and error bars represent standard error. (A) The recorded injury ratings for all honey locust trees 1 YAT. (B) The recorded injury ratings for all honey locust trees 2 YAT.

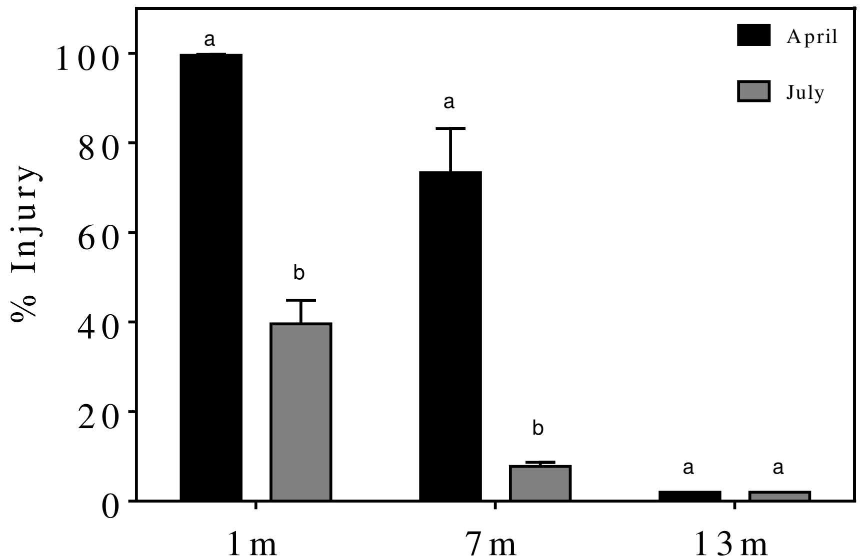

Figure 10. Percent injury on honeylocust trees at different soil applied aminocyclopyrachlor application timings made in 2014. The x-axis represents the distance from the edge of the application to the trunk of the tree. All comparisons of application timing were made within distance on mean injury ratings ($n \geq 3$) taken one year after treatment. Letters indicate differences in means at $p < 0.05$ and error bars represent standard error.

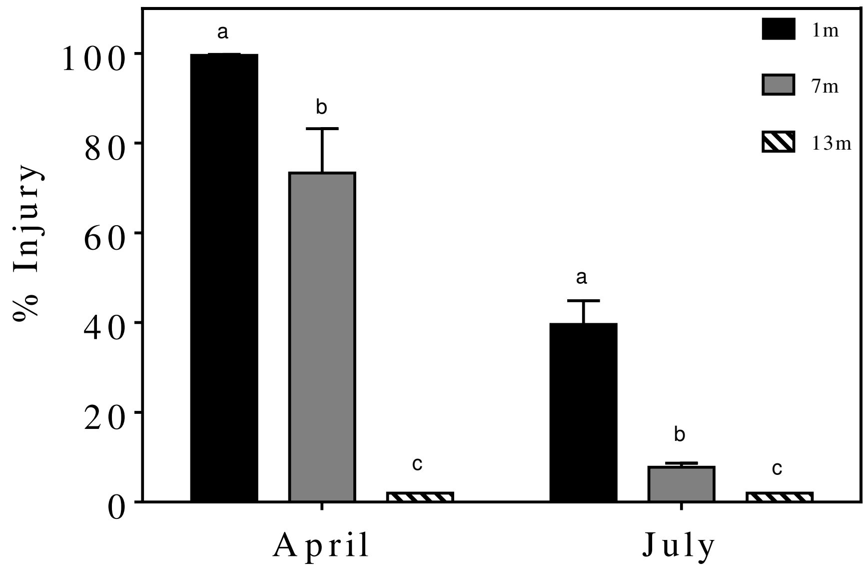

Figure 11. Percent injury on honeylocust trees for different distances from the edge of a soil applied aminocyclopyrachlor applications made in 2013. The x-axis represents different herbicide application timings. All comparisons of distance from the edge of the application were made within application timing on mean injury rating ($n \geq 3$) taken one year after treatment. Letters indicate differences in means at $p < 0.05$ and error bars represent standard error.

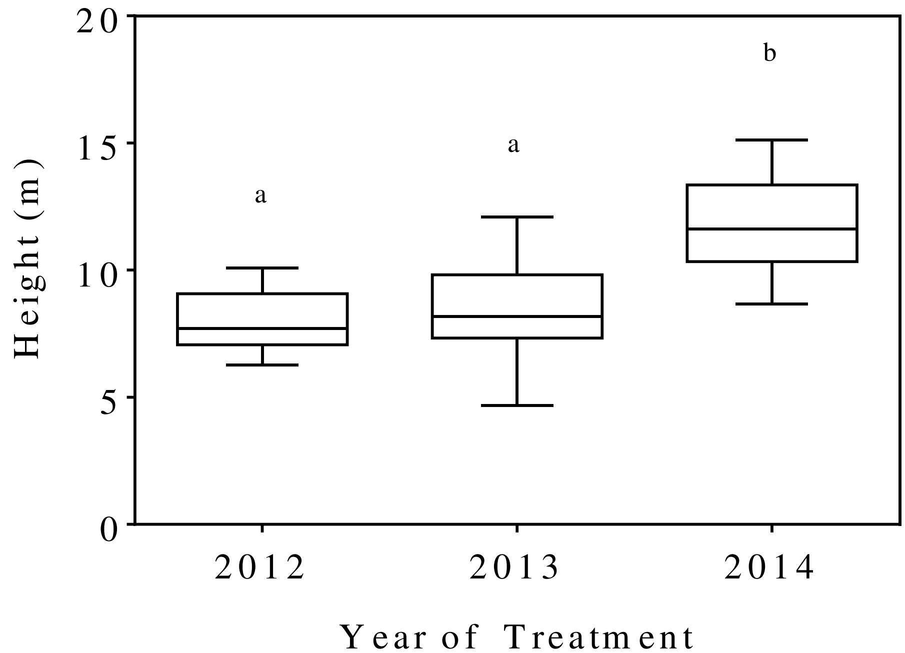

Figure 12. Box and whisker plots showing the mean and distribution of height for honeylocust trees treated in 2012 (n = 36), 2013 (n = 60), and 2014 (n = 45). Letters above each box and whisker plot indicates significant differences in mean tree height for each year.

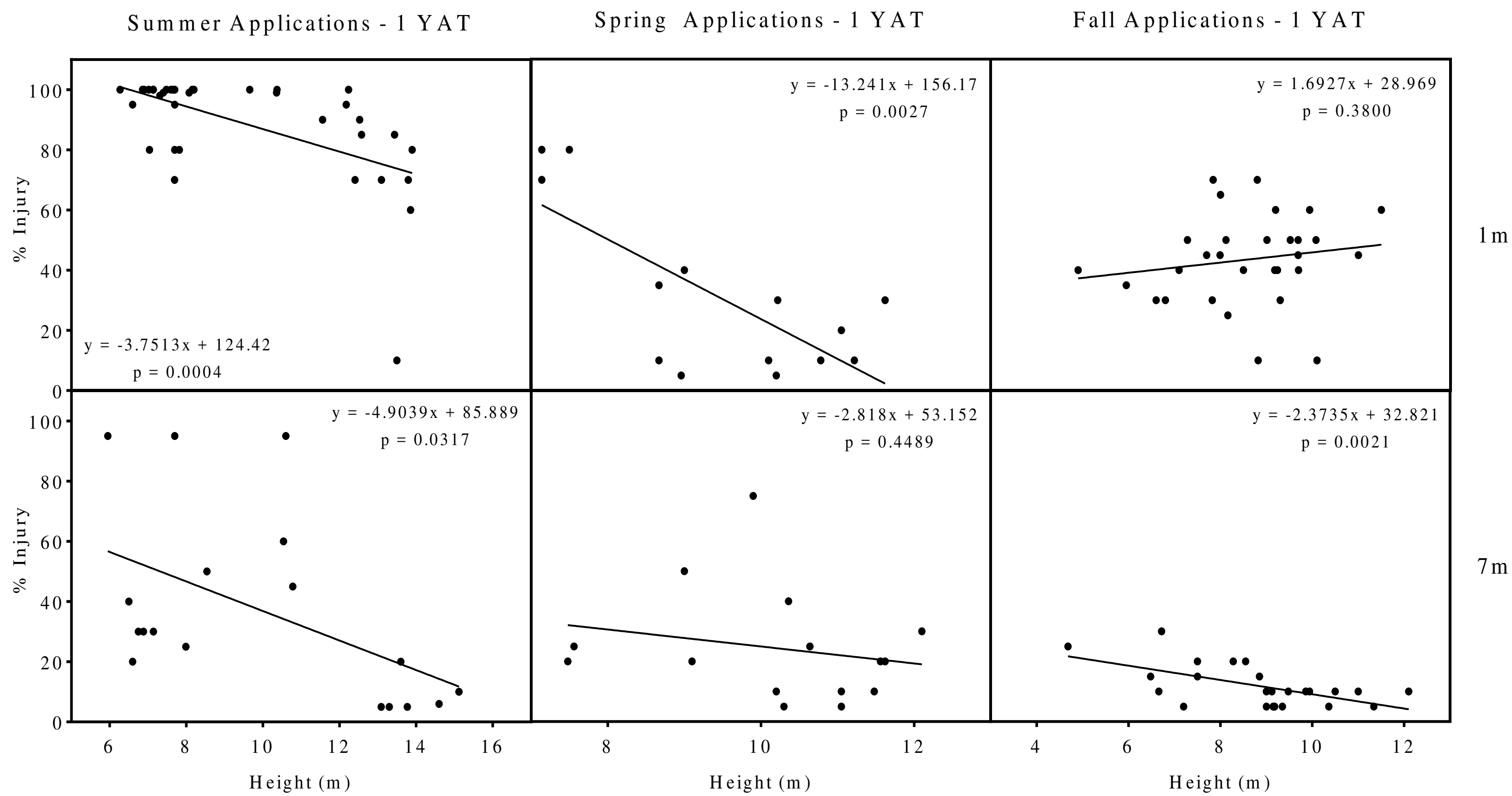

Figure 13. Correlations showing tree height of individual honeylocust trees plotted against the injury rating recorded one year after soil applied aminocyclopyrachlor treatments. Plots include trees treated in 2012, 2013, and 2014. Spring applications (middle column) include trees treated during April and May, summer applications (left column) include trees treated in June and July, and fall applications (right column) include trees treated in October and November. The top row corresponds to all trees 1 meter from the edge of the application and the bottom row corresponds to trees that were 7 m from the edge of the application. P-values < 0.05 indicate that the slope of the trend lines are significantly different from zero.

Table 1. Change in honeylocust tree damage rating between 2013 and 2015. Columns labeled for change in rating can be either positive or negative. Negative values indicate recovery, and are colored in green. Positive values indicate an average higher injury rating, and are colored in red. Asterisks in the p-value column indicate values that were found to be significant, with the level of significance listed below this table. The SE column refers to the standard error of the mean change in rating, and the n indicates the sample size of the mean.

Year of Treatment	Timing	Distance to Application	Δ Rating 2015 - 2013	SE	p-value	n	Δ Rating 2015 - 2014	SE	p-value	n
2012	April	1 meter	18.33	4.41	0.0533	3	-2.67	1.45	0.2079	3
2012	April	7 m	1.67	16.67	0.9295	3	-13.33	1.67	0.0153**	3
2012	June	1 meter	6.17	3.82	0.1351	12	-0.50	0.42	0.2562	12
2012	June	7 m	-7.50	13.17	0.5937	6	-10.00	4.46	0.0752*	6
2012	October	1 meter	38.33	6.67	0.0022***	6	-1.67	2.11	0.4650	6
2012	October	7 m	24.67	13.06	0.1175	6	-35.17	6.20	0.0024**	6
2013	July	1 meter	----	----	----	----	-0.17	0.30	0.5863	12
2013	July	7 m	----	----	----	----	-19.17	8.60	0.0764*	6
2013	October	1 meter	----	----	----	----	-15.83	8.25	0.0814*	12
2013	October	7 m	----	----	----	----	-2.78	0.88	0.0133**	9
2013	November	1 meter	----	----	----	----	-20.00	3.48	0.0001***	12
2013	November	7 m	----	----	----	----	-12.22	2.22	0.0006***	9

* $p < 0.10$

** $p < 0.05$

*** $p < 0.01$

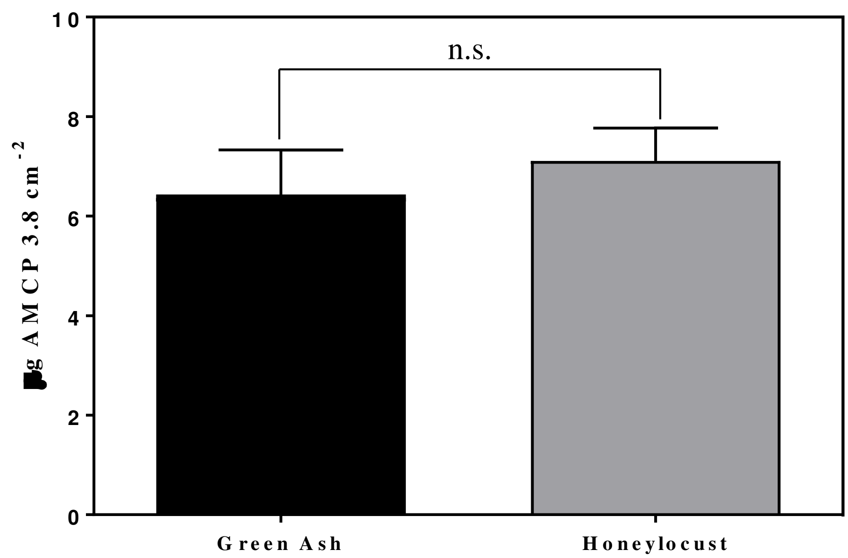

Figure 14. . High-Performance Liquid Chromatography results depicting the rate of aminocyclopyrachlor applied the day of treatment (or zero days after treatment) relative to the area of a cross section of a soil core. Aminocyclopyrachlor was recovered using petri dishes (n = 8) placed on the ground adjacent to both green ash and honeylocust trees with filter papers placed inside of them during the application. There was no significant difference (n.s.) between levels underneath GA and HL trees. Error bars represent the standard error. The application took place on July 1st, 2013.

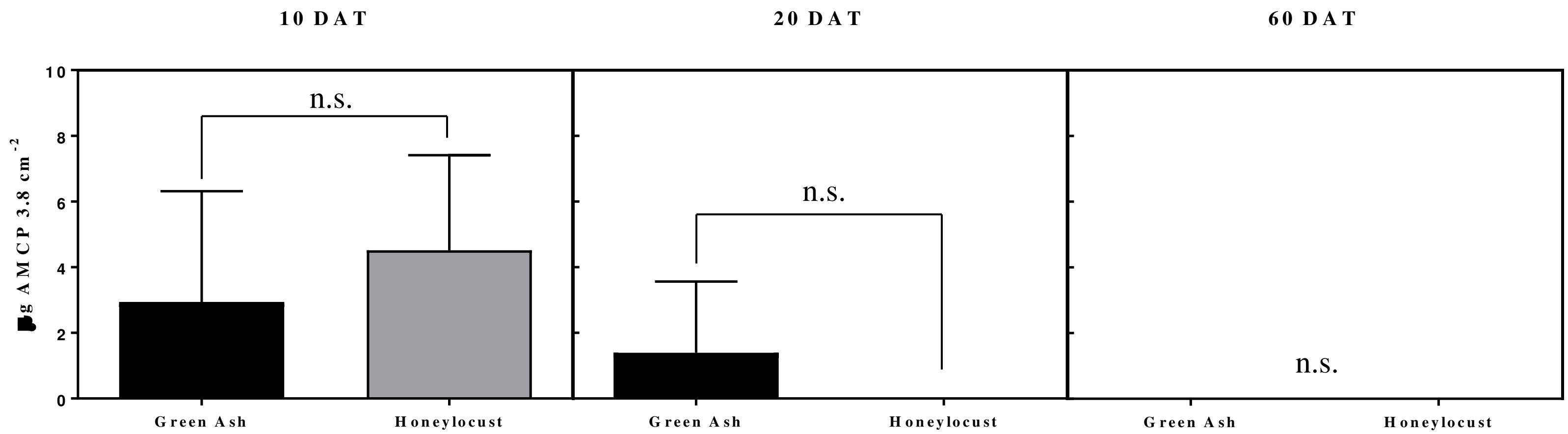

Figure 15. High-Performance Liquid Chromatography results depicting the total amount of aminocyclopyrachlor recovered from soil cores (n = 6), pulled from the treated areas adjacent to green ash and honeylocust trees, relative to the area of a cross section of a soil core. The left graph shows results from soil cores pulled 10 days after treatment, the middle graph shows results 20 days after treatment and the right graph shows results 60 days after treatment. There was no significant difference (n.s.) between detected aminocyclopyrachlor underneath green ash and honeylocust trees at any time point measured. By 60 days after treatment, no aminocyclopyrachlor could be detected. Error bars represent the standard error. The application took place on July 1st, 2013.

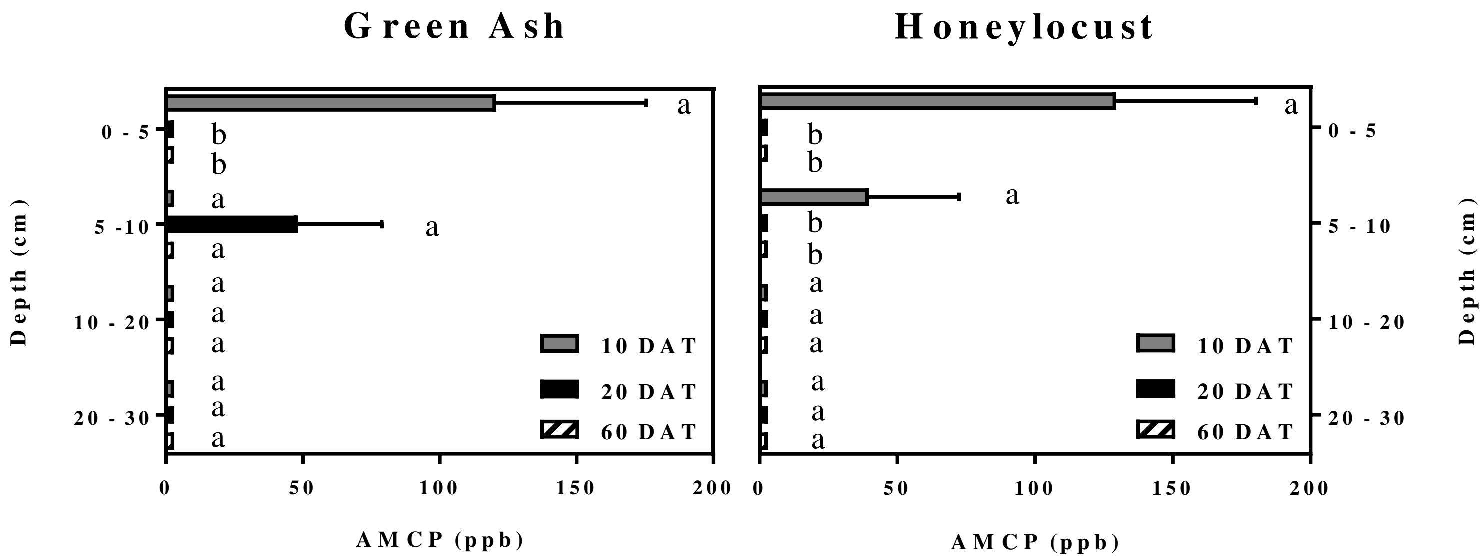

Figure 16. Aminocyclopyrachlor concentration in the soil as a function of depth and days after treatment for both green ash (left) and honeylocust (right) determined by high-performance liquid chromatography. Tukey's HSD comparisons of time point made were made within depth zones to determine how aminocyclopyrachlor moved over time. Different letters indicate significant differences at $p < 0.05$. The application took place on July 1st, 2013.

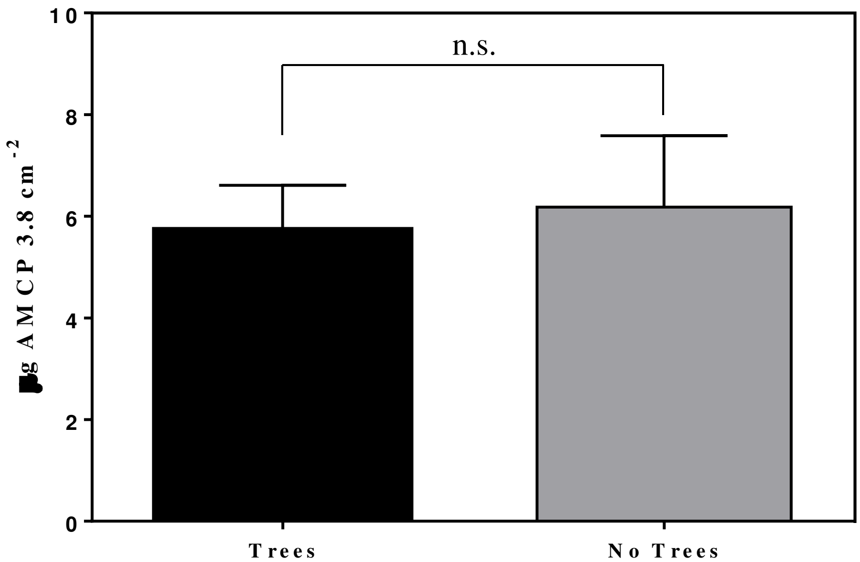

Figure 17. Liquid Chromatography-Mass Spectrometry results depicting the rate of aminocyclopyrachlor applied the day of treatment (or zero days after treatment) relative to the area of a cross section of a soil core. Aminocyclopyrachlor was recovered using petri dishes (n = 6) placed on the ground adjacent to both green ash and honeylocust trees as well as a separate treated strip where no trees were present. There was no significant difference (n.s.) between the rate applied for the treated plot adjacent to the trees, and the treated plot that was not adjacent to the trees. Error bars represent the standard error. The treatments occurred in the spring of 2015.

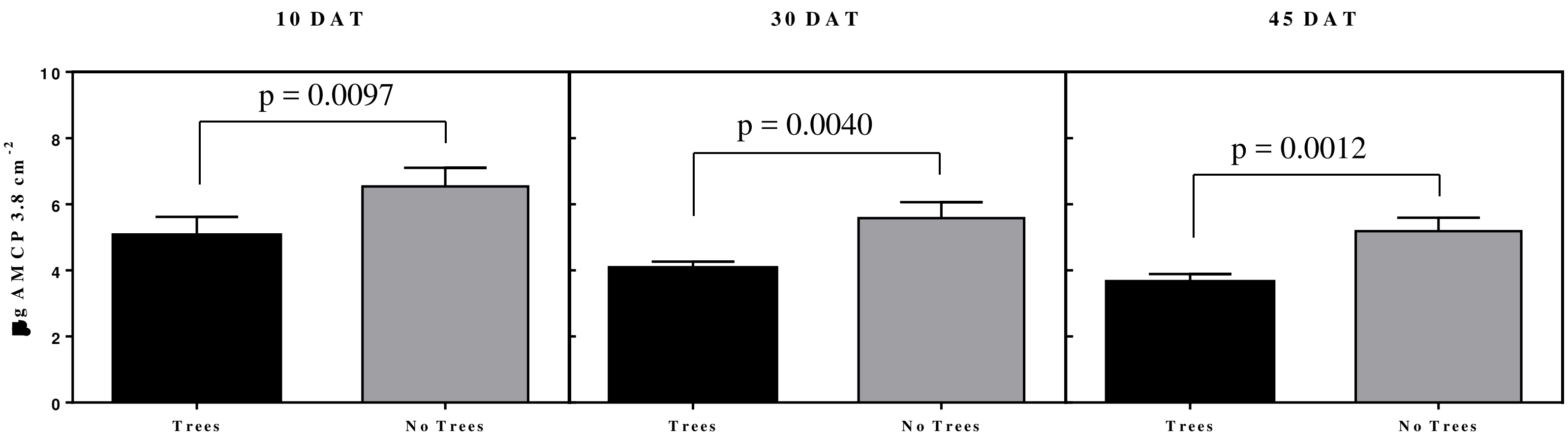

Figure 18. Liquid chromatography- mass spectrometry results depicting the total amount of aminocyclopyrachlor recovered from pooled soil cores (n = 4), pulled from the treated areas adjacent to green ash and honeylocust trees and another treated plot without trees. The y-axis represents the total amount of aminocyclopyrachlor recovered within sample, normalized to the area of a cross-section of one soil core. The left graph shows results from soil cores pulled 10 days after treatment, the middle graph shows results 30 days after treatment and the right graph shows results 45 days after treatment. Error bars represent the standard error. The applications took place in the spring of 2015.

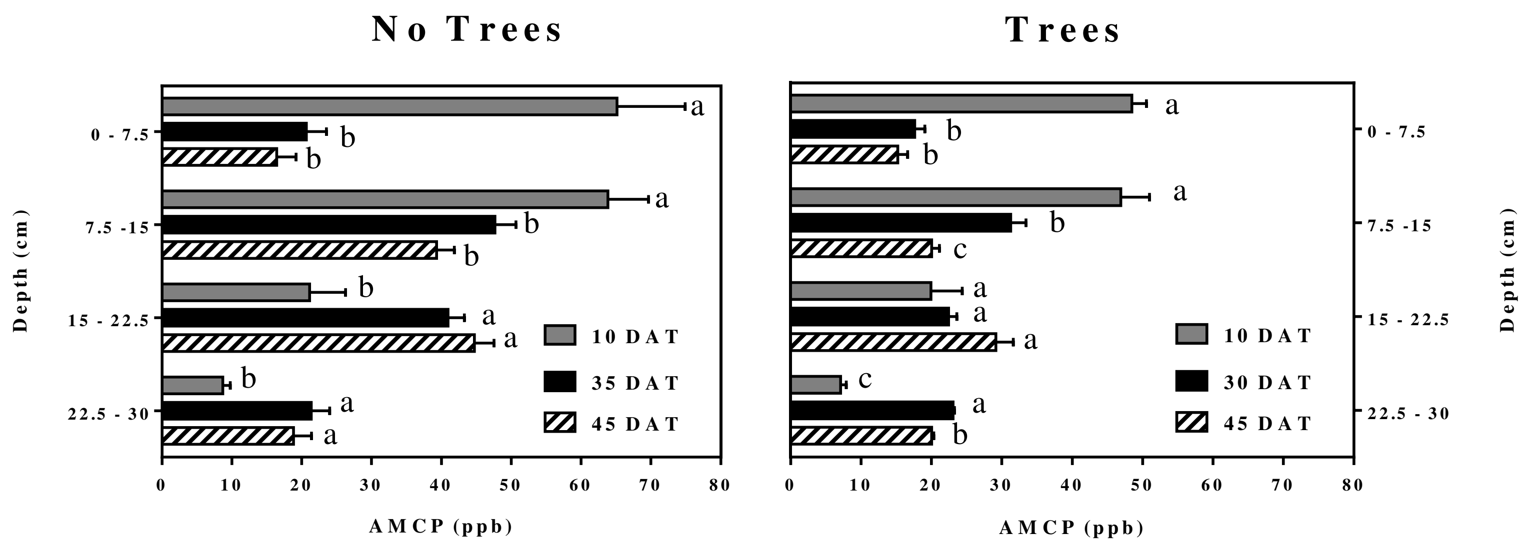

Figure 19. Aminocyclopyrachlor concentration in the soil as a function of depth and days after treatment for both the plot without trees (left) and the tree plot (right) determined by liquid chromatography-mass spectrometry. Tukey's HSD comparisons of time point were made within depth zones to determine how aminocyclopyrachlor moved over time. Different letters indicate significant differences at $p < 0.05$. The application took place in the spring of 2015.

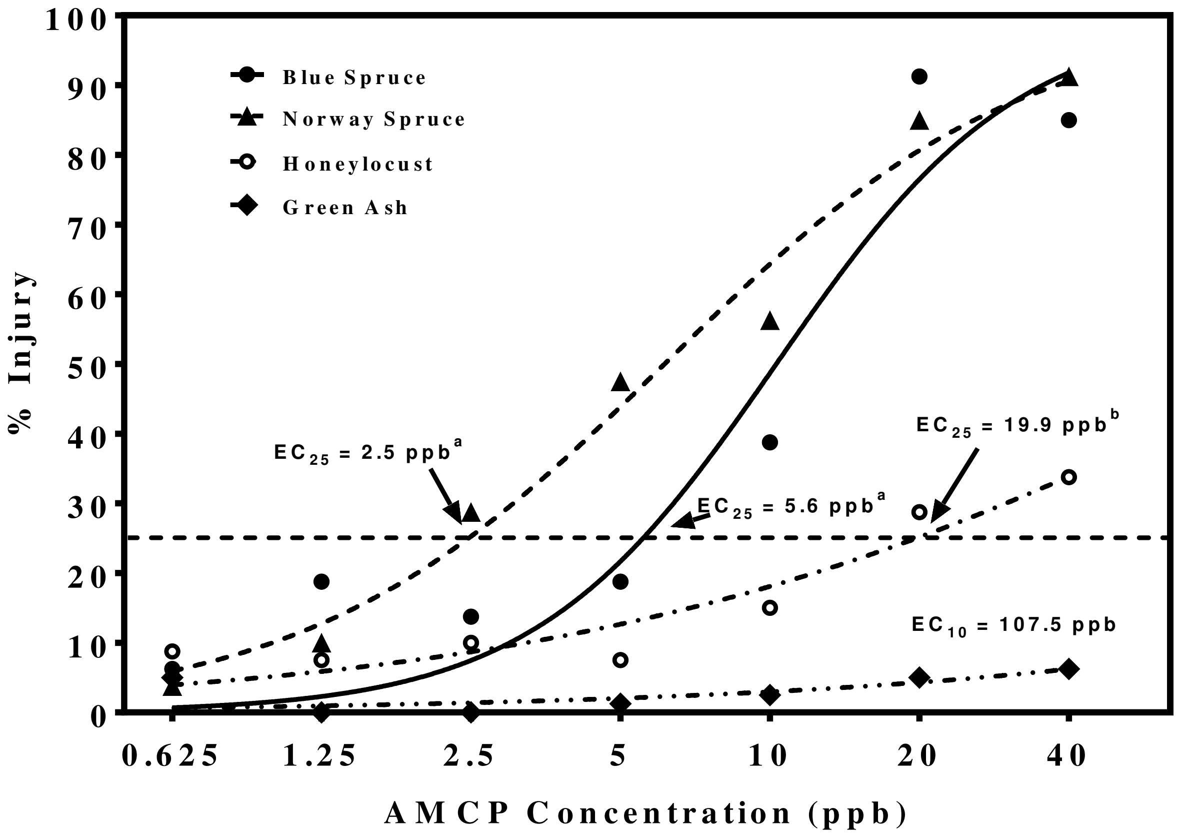

Figure 20. Dose response curves of green ash, honeylocust, Norway spruce and blue spruce trees from greenhouse dose response trial conducted in 2013. Norway spruce and blue spruce showed similar EC_{25} values, while honeylocust was about four times more tolerant. Green ash did not reach a 25% response, and was the most tolerant species in the dose response. Each point is the mean injury of four trees (n=4). EC_{25} comparisons between tree species was performed using the drc package in R. EC_{10} is reported for green ash, since standard error for EC_{25} was too high, due to poor upper limit prediction. Letters indicate differences in predicted EC_{25} at $p < 0.05$.

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