# Demonstrating Techniques for Establishing Woody Biomass Plantations on Surface Mine Lands as Feedstocks for Energy Production

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**Joshua S. Brinks, John M. Lhotka, Christopher D. Barton, Richard C. Warner, and Carmen T. Agouridis**

UNIVERSITY OF KENTUCKY

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**Abstract**

Short rotation woody biofuel plantations on reclaimed surface mines in Appalachia can diversify domestic energy supplies and facilitate the reforestation of these disturbed lands. This study examined growth, biomass accumulation and allocation, survival, and nitrogen concentrations following two growing seasons in black locust and American sycamore seedlings receiving irrigation, fertilization, and irrigation + fertilization compared with untreated controls. Fertilization increased basal diameter, height, and stem mass of the American sycamore following two growing seasons. Increased stem production was attributed to accelerated development and not shifts in biomass allocation due to treatment. Irrigation had a general negative effect on sycamore and this is likely attributed to the additive effects of irrigation and poor drainage associated with compacted mine soils. Black locust basal diameter, height and stem mass did not differ among the treatments. Intensive ungulate browse may have confounded the effects of treatments on black locust. Survival was unaffected by treatment in both species, but mean survival was greatest in sycamore (80%) compared to black locust (58%). Total nitrogen uptake was highest in fertilized sycamore (14.5kg $ha^{-1}$) and lowest in irrigated sycamore (1.75kg $ha^{-1}$). The results of our study suggest that granular fertilizer applications can accelerate seedling growth on reclaimed surface mines in the Appalachian region.

## 1. Introduction

Woody biomass is the leading renewable energy supply in the United States and the United States Department of Energy forecasts the potential need for a billion ton annual biomass supply by 2050 (English and Ewing 2002). Increased woody biomass demand will be met through fuel reduction treatments of natural stands, utilization of logging and wood processing residues, and biofuel plantings (Perlack, Wright et al. 2005). Woody biofuel plantations provide an excellent opportunity to diversify and increase domestic energy feedstocks (Johnson, Coleman et al. 2007). Non woody biomass such as switchgrass, miscanthus, and corn provide annual biomass yields, but perennial woody crops can reduce erosion, runoff, and nutrient loss with longer rotations (Nyakatawa, Mays et al. 2006). Woody crops intended for co-firing with coal for electricity generation will prolong coal supplies, reduce $NO_x$ and $SO_x$ emissions, and contribute to national woody feedstock supplies (Boylan 1996).

Niu and Duiker (2006) estimated that 6.5 million ha of marginal lands in the Midwest United States are available for reforestation and other carbon sequestration projects. Implementing short rotation woody plantations on reclaimed surface mines and other marginal lands presents an excellent opportunity to increase wood production without diverting current agricultural or naturally forested lands to plantations (Casselman, Fox et al. 2006). The majority of Appalachian surface mines were previously forested, but many have been reclaimed as grasslands. Therefore, in addition to utilizing disturbed lands for energy feedstocks, woody biofuel plantations facilitate the reforestation of reclaimed surface mines.

Intensive management of forest plantations can greatly increase biomass production (Coyle and Coleman 2005; Cobb, Will et al. 2008; Coyle, Coleman et al. 2008). Fertilization can enhance nutrient availability on highly degraded sites and irrigation can mitigate the impacts of severe

drought (Coleman, M. Jacobson et al. 2004; Casselman, Fox et al. 2006). Mechanical and chemical control of weedy competition can increase productivity of target species (Petersen, Ares et al. 2008). However, early fertilization of tree plantations may exacerbate herbaceous competition and lower biomass production (Ramsey, Jose et al. 2003). Moreover, excessive fertilization can negatively affect site carbon budgets (Crutzen, Mosier et al. 2007) and economic efficiency. A large number of researchers have examined the impacts of fertilization, irrigation, spacing, and site preparation on short rotation woody plantations established on higher quality sites (Coyle and Coleman 2005; Casselman, Fox et al. 2006; Coleman, Tolsted et al. 2006; Liberloo, Calfapietra et al. 2006; Cobb, Will et al. 2008; Coyle, Coleman et al. 2008). Despite an extensive body of research on short rotation woody plantations, the effect of short rotation practices on tree growth has not been well document on high disturbed lands such as reclaimed surface mines. Several research projects have examined various spacing (Wood, Wittwer et al. 1977; Wittwer, King et al. 1978) and site preparation (Casselman, Fox et al. 2006) approaches on surface mines, but very few studies have examined the effects of intensive fertilization and irrigation on growth, survival, and biomass accumulation and allocation on reclaimed surface mines in the Appalachian.

The objectives of this study were to evaluate the effects of irrigation and fertilizer treatments on (1) growth and survival, (2) above ground leafless biomass accumulation and above and below ground biomass allocation, (3) and nitrogen uptake and distribution on a woody biofuel plantation established on a reclaimed surface mine in Appalachia. This paper presents data following two growing seasons.

## 2. Methods

### *2.1 Study Site*

The study is located on a reclaimed surface mine within the Cumberland Plateau physiographic region near Hazard, Kentucky (Knott County). The region is characterized by warm humid summers and cool winters. Yearly rainfall average is 127 cm year$^{-1}$. The average July high temperature is 30° C and the average January low is -5° C. Upland oak-hickory hardwood forests dominated the site prior to being cleared for mining. The soil profile is undeveloped, compacted, derived from shale and sandstone, and large rocks pervade the soil matrix (Conrad 2002).

The study site was previously reclaimed using smooth grading. Intensive machine traffic by large equipment during reclamation used to reclaim mines creates high levels of soil compaction (Sweigard, Burger et al. 2007), which can negatively affect tree growth by inhibiting root growth and reducing soil drainage and aeration (Unger and Cassel 1991). The site was ripped with a D-11 dozer in February of 2008 to alleviate compaction and reduce weedy competition. Twelve 0.209 ha plots, three replicates of each treatment, were established following ripping. In March 2008, half of each plot was planted with American sycamore, while the other half was planted with black locust. 1-0 bare root seedlings were used. Large boulders and rough topography prevented uniform seedling plantings. Mean seedling density across all plots was 1842 stems ha$^{-1}$. Following surface ripping and planting, the vegetation community was populated by early successional and other disturbance dependant plants reported at other reclaimed surface mines in the region (Bell and Ungar 1981; Holl and Cairns 1994; Holl 2002). Dominant genera include: *Rubus*, *Lespedeza*, *Ailanthus*, *Ambrosia*, *Oxalis*, *Cirsium*, *Veronia*, *Leucanthemum*, *Rudbeckia*, *Festuca*, and *Coronilla*.

*2.2 Field Methodology*

For each species, seedling height and basal diameter were tracked in a centrally located 0.019 ha measurement sub plot. All seedlings in the 0.019 ha measurement subplots were tagged to facilitate re-measurement. Each plot half also contained four 0.008 ha destructive sampling sub plots. At least two treated border rows were present around all measurement and destructive harvest sub plots. Control (C), fertilization (F), irrigation (I), and irrigation + fertilizer (IF) treatments were replicated three times and randomly assigned to the experimental plots. All fertilized plots received 36 kg $ha^{-1}$ of granular nitrogen, 30 kg $ha^{-1}$ phosphorous, and 16 kg $ha^{-1}$ of potassium during June of 2008 and 2009. 3.4 million liters of water $ha^{-1}$ were applied to I and IF treatments throughout the 2008 and 2009 growing seasons with a drip irrigation system. 36 kg $ha^{-1}$ of liquid nitrogen, 30 kg $ha^{-1}$ phosphorous, and 16 kg $ha^{-1}$ was applied to IF plots during the 2008 and 2009 growing seasons. To control competing vegetation, glyphosate was administered to all plots at a rate of 0.871 liters $ha^{-1}$ with backpack sprayers during June of 2008 and 2009.

*2.3 Field and Laboratory Measurements*

Initial seedling basal diameters and heights were recorded in measurement sub plots after planting in May 2008. Basal diameter was taken to the nearest tenth of an mm with digital calipers at two locations, 90° apart from one another, and then averaged. Measurements taken following the first growing season were recorded in January of 2009 and following the second growing season in December of 2009. To evaluate the effect of ungulate browse on the responses of the planted seedlings, browse was categorically assessed across four levels using methods adapted from Keigly and Frisina (1998). Where level 1 indicates an uninterrupted growth type, 2 equates a released type, 3 indicates an arrested growth pattern, and 4 represents retrogressed growth architecture.

Destructive harvests were used to develop allometric equations, analyze partition fractions, and test for shifts in allocation due to treatment. For each species, one seedling was excavated from a destructive sampling subplot within each replicate plot. Destructive sampling occurred across 14 days during September and October of 2009 and a total of 24 specimens were excavated (3 per species and treatment combination). Destructive methods were adapted from Coyle and Coleman (2005) who excavated 1-2 trees per replicate plot and Davis and Trettin (2006) who excavated 13 total trees for above and below ground analysis. Harvested trees were defoliated in September prior to excavation. All roots were excavated from a 60 x 60 cm square centered on the tree to a 30 cm depth. This method was derived from similar studies (Albaugh, Allen et al. 1998; King, Albaugh et al. 1999; Pregitzer, DeForest et al. 2002; Coyle and Coleman 2005; Coyle, Coleman et al. 2008) and adapted to accommodate rockiness at our site. Trees were partitioned into roots, stems, and leaves. Diameters and heights were recorded before partitions were oven-dried for 48 hours at 60°C, and then weighed to the nearest hundredth of a gram.

*2.4 Leaf, Soil and Tissue Analysis*

Excavated partitions were sub sampled for tissue nutrient analysis. Subsamples were pulverized with a Spex SamplePrep 800M Electric Mixer/Mill. Total nitrogen concentrations were determined by dry combustion using a LECO CHN 2000 analyzer (Leco Corp. – St. Joseph, MI). The resulting gases were equilibrated in a ballast chamber followed by infra-red detection for $CO_2$ and $H_2O$. $N_2$ was determined by a thermal conductivity detector after reduction of N oxides and removal of $CO_2$ and $H_2O$. Six leaves from each excavated tree were sub sampled from varying cardinal directions and crown depth for leaf area calculations. Leaf area was calculated to the nearest hundredth of a $cm^2$ with a Li-Cor LI-3100 Area Meter. Subsamples were dried at

60 C and weighed to the nearest hundredth of a gram with an Ohaus Adventurer-Pro digital scale to calculate specific leaf area (SLA).

Five soil samples were taken to a 15 cm depth from each replicate plot and aggregated for all species and treatment combinations. Samples were pulverized in an electric mill and processed for chemical analysis. Soil samples were analyzed at the UK College of Agriculture Regulatory Services Laboratory for nitrogen, phosphorus, and potassium.

*2.5 Water Budgets*

In an effort to better understand the impact of irrigation we utilized rainfall and evapotranspiration rates to characterize the site water budget. Mean daily reference evapotranspiration rates were calculated using a modified version of the Penman-Monteith equation with fixed parameters derived from the literature (Allen *et al.*, 2005). Based upon the age of vegetation and observed growth at the site, the estimated reference evapotranspiration was calculated for a 0.5 m tall grass crop. Inputs into the equation were obtained from a weather station (Campbell Scientific ET106 weather/ET station, Logan, UT) located at the study site. Inputs included daily mean solar radiation (MJ $m^{-2}$ $d^{-1}$), air temperature ($^{o}$C), wind speed (m $s^{-1}$), and humidity (%). Latitude, longitude and elevation of the site were utilized to determine constants for the equation. Precipitation quantities were measured using a tipping bucket and recorded on the weather station data logger. Long–term (1972 to 2005) monthly precipitation averages were developed from data collected at a nearby (5.5 km straight line distance) weather station located at the University of Kentucky's Robinson Forest. Irrigation, precipitation, and evapotranspiration data was compiled along with 2008 and 2009 precipitation deviations from 25 year norms for a visual representation of site water budget (Figure 1).

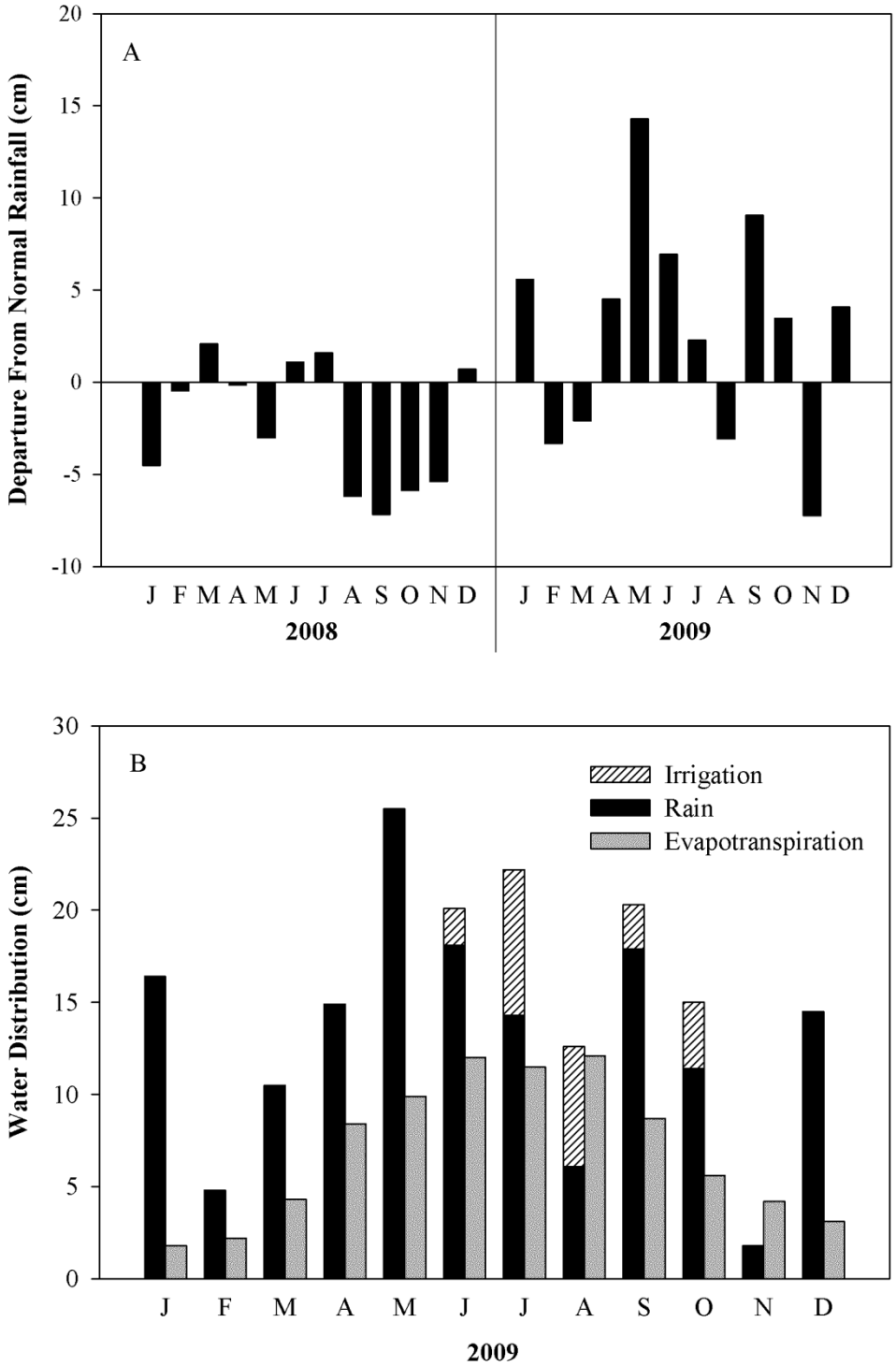


Figure 1. (A) Deviation of monthly precipitation (2008 and 2009) collected at the study site weather station from the long-term (1972 to 2005) monthly average collected at a nearby weather station on the University of Kentucky's Robinson Forest and (B) monthly comparison of water input (precipitation plus average irrigation quantity applied to each plot) to estimated reference evapotranspiration at the study site for 2009.

*2.6 Statistical Analysis*

Destructively sampled trees were used to develop regression equations for predicting root, stem, and foliage biomass. Per stem biomass was predicted using a linearized version of a power function that included species, seedling height, and basal diameter as independent variables. Equations were then used to predict aboveground leafless biomass of each tree located in the 0.019 ha measurement subplots. Analysis of variance (ANOVA) was used to test for differences ($\alpha = 0.05$; n=3) in plot mean height, basal diameter, and per stem biomass among the two species and four treatments following two growing seasons. Pre treatment analysis revealed no significant differences for basal diameter, but American sycamore heights were significantly lower in fertilized plots compared to irrigation and irrigation+fertilizer plots. For analysis of biomass accumulation, we used average per stem biomass by plot as a dependant variable because area based calculations would have differed due to varying plot densities, not disparate growth patterns. Soil samples and tissue samples from destructive harvests (n=3) were used in an ANOVA to detect significant treatment and species differences for nitrogen concentrations, soil chemistry, leaf area, and specific leaf area. A generalized linear model was used for the ANOVA that tested for differences in average seedling survival among treatment and species. The generalized linear model utilized a binomial distribution and a logit link function to account for the binomial nature of survival data (Littell, Stroup et al. 2002).

Following the methodology outlined in Coyle and Coleman (2005), shifts in resource allocation were tested by calculating K coefficients (i.e., regression slope) when regressing one tissue partition against total biomass or each other another or total mass using the model:

$$\ln y = a + K \ln x$$

where x and y are tissue partitions or total biomass being compared

Utilizing tissue partitions or total biomass as independent variables adjusts for shifts in allocation due to ontogeny and not nutrient or moisture amendments (Causton and Venus 1981; Hunt 1991; Coyle and Coleman 2005). Analysis of variance was used to test for different slopes (K) among treatments within each species.

## 3. Results

### *3.1 Survival and Growth*

Granular fertilizer treatments increased American sycamore basal diameter and height. Mean sycamore height was 168.5cm in fertilized plots compared to 79.5cm in irrigation plots and 100.4cm in controls (Table 1). Basal diameter in fertilized plots was nearly double (31.6mm) that of other treatments.

Table 1. Mean (± standard error) seedling basal diameter, height, per stem aboveground leafless biomass, and survival by species and treatment following two growing seasons. Treatments are control (C), irrigation (I), fertilizer (F), and irrigation + fertilizer (IF).

| Species | Treatment | Diameter (mm) | Height (cm) | Stem Biomass (g) | Survival (%) |
|---|---|---|---|---|---|
| Sycamore | C | 18.8±2.8a | 100.4±15.5a | 97.95±33.4a | 80.0a |
| | I | 14.1±1.9a | 79.5±9.4a | 48.7±16.3a | 76.5a |
| | F | 31.6±1.4b | 168.5±3.7b | 373.2±37.1b | 80.1a |
| | IF | 16.5±0.9a | 114.8±18.9ab | 85.5±19.6a | 83.3a |
| Black Locust | C | 13.1±0.3a | 83.9±8.1a | 52.4±3.8a | 53.7b |
| | I | 14.5±1.6a | 89.5±7.9a | 65.4±17.2a | 58.5b |
| | F | 17.8±0.1a | 121.7±13.4ab | 113.0±13.0a | 58.5b |
| | IF | 12.8±1.9a | 89.3±13.1a | 56.9±22.8a | 49.9b |

Lower case letters indicate significance ($p<0.05$) across species and treatments.

Average seedling basal diameter and height of black locust did not differ among the treatments following two growing seasons (Table 1). Average basal diameter was between 17.8mm (fertilization) and 12.9mm (irrigation + fertilization). Mean height of black locust was 121.8cm in fertilizer, 89.5cm in irrigation, 89.3cm in irrigation + fertilizer, and 83.9cm in control treatments.

Sycamore survival was unaffected by treatment and ranged from 83.3% in the irrigation + fertilization treatment to 76.5% in plots receiving irrigation. Despite no intra-species differences in survival, sycamore survival was significantly higher than black locust across all treatments, which was less than 60% in all treatments.

Ungulate browsing was higher on black locust compared to American sycamore. Eighty-five percent of all black locusts exhibited browse level of 2 or greater, while only 1 percent of American sycamores displayed a similar browse pattern. Treatment did not appear to influence browse.

*3.2 Allometric Equations*

Per stem root, shoot, and foliage biomass were predicted from seedling diameter and or height using a linearized version of a power function and residual analysis indicated that assumptions of normality and homogeneity of variance were met (Table 2). No significant interactions existed between treatment and metrics of seedlings size in models for stem and foliage biomass. Species was a significant variable for predicting root, stem, and foliage mass. Basal diameter was the only significant independent variable when predicting American sycamore root and foliage mass while height was the only significant variable for black locust foliage biomass. Height and basal diameter were significant for stem mass of both species. A significant interaction between

species and basal diameter was present for root mass. Correction for logarithmic bias of transformed models was completed using the Baskerville (1972) correction factor (Baskerville 1972).

Table 2. Allometric equations used to predict the natural logarithm transformed root, stem, and biomass partitions (g) using the natural logarithm transformed seedling basal diameter (dia; mm) and height (ht; cm).

| Model Type | Black locust | | American sycamore | |
|---|---|---|---|---|
| | Model | $R^2$ | Model | $R^2$ |
| Root biomass | -1.80 + 1.97*dia* | 0.81 | -1.82 + 2.08*dia* | 0.95 |
| Stem biomass | -5.89 + 1.10ht + 1.78*dia* | 0.97 | -6.13 + 1.10ht + 1.78*dia* | 0.97 |
| Foliage biomass | -15.77 + 3.92*ht* | 0.87 | -4.725 + 2.69*dia* | 0.93 |

*3.3 Biomass Accumulation and Allocation*

Per stem leafless aboveground biomass of fertilized American sycamore (373.2 g) was greater than all other treatment and species combinations (Table 1). Sycamore root mass, leaf mass, and total mass were also significantly greater under fertilizer treatments. Irrigation treatments resulted in the lowest sycamore root mass, shoot mass and total mass, but control plots produced the lowest leaf mass. Per stem biomass of black locust did not differ among treatments and ranged from 113.0g in fertilizer treatment and 53.4g in the control. All black locust partition and total masses were lowest in control treatments.

Although fertilizer treatments appeared to reduced root fractions for black locust and American sycamore resource, the effect was not statistically significant and there was no relationship between root fraction and total mass (Figure 2). No significant treatment effects were detected for either shoot or leaf fractions in either species. K coefficient analysis utilized to test for shifts in allocation irrespective of developmental stages did not identify any significant effects of treatment on resource allocation for either species (Table 3).

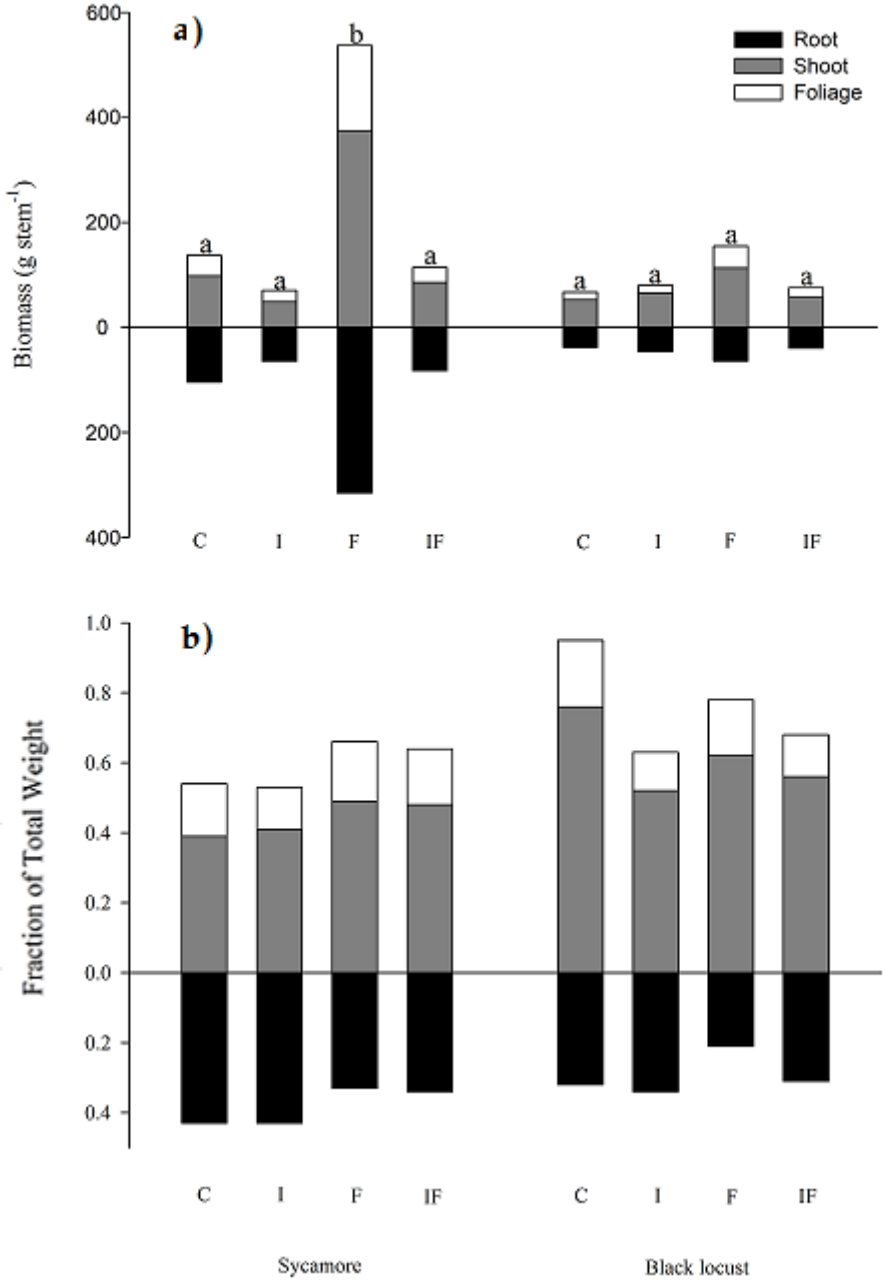


Figure 2. Total (A) predicted per stem biomass by partition and (B) partition fraction expressed as a fraction of the total weight of the seedling. Treatments are control (C), irrigation (I), fertilizer (F), and irrigation + fertilization (IF). Means sharing a letter across species are not significantly different (Tukeys HSD, $\alpha = 0.05$).

Table 3. Analysis of allometric K coefficient for varying partition and total mass comparisons by treatment and species. Treatments are control (C), irrigation (I), fertilizer (F), and irrigation + fertilizer (IF).

| | Treatment | Sycamore | Black Locust |
|---|---|---|---|
| Root F vs. total mass | C | 0.025 | -0.026 |
| | I | -0.080 | -0.131 |
| | F | 0.055 | 0.004 |
| | IF | -0.163 | -0.214 |
| Shoot F vs. total mass | C | 0.129 | 0.150 |
| | I | 0.067 | 0.088 |
| | F | -0.010 | 0.011 |
| | IF | 0.076 | 0.097 |
| Root F vs. leaf F | C | -0.054 | -0.097 |
| | I | -0.238 | -0.282 |
| | F | -0.136 | -0.180 |
| | IF | -0.156 | -0.200 |
| Shoot F vs. leaf F | C | -0.343 | -0.279 |
| | I | -0.046 | 0.017 |
| | F | -0.211 | -0.148 |
| | IF | -0.208 | -0.144 |
| Shoot F vs. root F | C | -0.075 | -0.174 |
| | I | -0.434 | -0.533 |
| | F | -0.218 | -0.317 |
| | IF | -0.374 | -0.473 |
| Root F vs. shoot F | C | -0.135 | -0.433 |
| | I | -0.731 | -1.030 |
| | F | -0.983 | -1.279 |
| | IF | 0.089 | -0.209 |

Fertilized American sycamore had the highest leaf area at 343.4cm$^2$ (Table 4). This was significantly higher than all other treatments for either species. Irrigation resulted in the lowest sycamore (94.1cm$^2$) and locust (53.4cm$^2$) leaf area. There were no statistical differences among

any locust treatment for leaf area. Specific leaf area varied from 115.48 (locust IF) to 219.12 (locust F), but there were no significant differences.

Table 4. Mean (± standard error) leaf area and specific leaf area (SLA) by treatment and species following two growing seasons. Treatments are control (C), irrigation (I), fertilizer (F), and irrigation + fertilizer (IF).

| Species | Treatment | Leaf Area ($cm^2$) | SLA |
|---|---|---|---|
| Sycamore | C | 170.4±24.8ab | 160.1±1.6a |
| | I | 94.1±10.4abd | 174.8±17.4a |
| | F | 343.4±44.4c | 132.6±8.8a |
| | IF | 181.1±42.3b | 138.3±4.1a |
| Black Locust | C | 63.1±10.7abd | 151.0±20.4a |
| | I | 38.8±8.7ad | 179.8±28.1a |
| | F | 53.5±12.9d | 219.1±57.9a |
| | IF | 57.9±10.1d | 115.5±7.2a |

### *3.4 Nitrogen Concentrations and Uptake*

Black locust in the control treatment had the greatest root nitrogen concentrations at 29.8 g $kg^{-1}$ (Table 5). This was significantly higher than irrigated black locust (20.9 g $kg^{-1}$) and all sycamore treatments. All black locust treatments had significantly higher root nitrogen than sycamore treatments. Stem nitrogen levels revealed similar results; there were no intra species treatment differences, but all locust treatments had significantly higher nitrogen levels compared to every sycamore treatment. Black locust leaves possessed the highest nitrogen concentrations for any tissue or species, but no intra species differences were detected. Total nitrogen uptake of fertilized sycamore (14.5 kg $ha^{-1}$) were significantly higher than all other treatment and species combinations except fertilized black locust (9.1 kg $ha^{-1}$). Total nitrogen uptake by black locust was similar among treatments.

Table 5. Mean root, shoot, foliar, and total nitrogen of sycamore and black locust trees following two growing seasons. Treatments are control (C), irrigation (I), fertilizer (F), and irrigation + fertilizer (IF).

| Species | Treatment | Root N ($g\ kg^{-1}$) | Shoot N ($g\ kg^{-1}$) | Foliar N ($g\ kg^{-1}$) | Total N ($kg\ ha^{-1}$) |
|---|---|---|---|---|---|
| Sycamore | C | 4.7a | 4.9a | 20.2a | 3.4a |
| | I | 5.8a | 4.6a | 17.1a | 1.7a |
| | F | 8.0a | 5.6a | 20.5a | 14.5b |
| | IF | 5.4a | 4.7a | 17.2a | 2.5a |
| Black Locust | C | 29.8b | 16.9b | 34.0b | 4.6ac |
| | I | 20.9c | 13.9b | 30.2b | 4.3ac |
| | F | 24.8bc | 15.8b | 36.4b | 9.1bc |
| | IF | 27.6bc | 16.3b | 33.0b | 4.6ac |

Lower case letters indicate significance ($p<0.05$; $n=3$) across species and treatments.

Granular fertilizer applications resulted in significantly elevated levels of soil phosphorous and potassium, but had no effect on total soil nitrogen. Soil phosphorous and potassium levels were significantly higher in fertilized plots compared to all other treatments. Mean phosphorous was 78.8 kg $ha^{-1}$ in fertilized locust plots and 44.0 kg $ha^{-1}$ in fertilized sycamore more plots. The next highest readings were 10.4 kg $ha^{-1}$ and 10.1 kg $ha^{-1}$ in black locust IF and C treatments, respectively.

## 4. Discussion

### *4.1 Growth Measurements and Survival*

American sycamore height and diameter were significantly greater in granular fertilizer treatments than in the other treatments following two growing seasons. Fertilized sycamore heights were 68% greater compared to controls and more than doubled the heights of irrigation treatments. American sycamore under controlled drainage on a former agricultural field in South

Carolina reported greater height and diameters than at our site (Davis and Trettin 2006). While irrigation has been used to increase growth in short rotation plantations (Cobb, Will et al. 2008; Samuelson, Butnor et al. 2008), this treatment did not increase growth or biomass accumulation for American sycamore or locust. In fact, apparent trends in height and basal diameter data suggest that irrigation may have negatively impacted the growth of sycamore. Other studies reported that American sycamore may be nutrient, not moisture, limited in the relatively humid and wet southeastern United States (Coleman, M. Jacobson et al. 2004; Coyle and Coleman 2005; Davis and Trettin 2006). Although our study site was ripped to alleviate compaction, which has been shown to increase growth (Casselman *et al.*, 2006; Shrestha *et al.*, 2009), bulk density may still be high relative to undisturbed sites (Conrad, 2002). Moreover, mitigating compaction by ripping may be effective in the area where the ripping blade comes in direct contact with the spoil but limited in the area below the ripping and in the space between rips. The 2009 growing season had higher monthly precipitation compared to the 25 year average. Furthermore, there was only one month (August) during the 2009 growing season where precipitation + irrigation was at or below the estimated rate of evapotranspiration (Figure 1). The additive effects of irrigation, high rainfall totals during the 2009 growing season, and poor drainage due high bulk density or insufficient ripping of mine spoil may have produced sustained anaerobic conditions that resulted in reduced height and diameter growth for American sycamore despite its affinity for moist conditions.

Height and basal diameter of black locust was not affected by treatment following two growing seasons. Black locust was outperformed by American sycamore in all growth metrics and treatments. Although nutrient and moisture amendments had no significant impact on survival for either species, American sycamore exhibited significantly higher survival compared to black

locust. These interspecies growth and survival trends may have been influenced by differential ungulate browsing witnessed between black locust and American sycamore. Starfire Mine, since renamed Big Elk Mine, is home to the highest densities of elk in the eastern Kentucky reintroduction zone (Dahl 2008). More than 80% of all black locusts, irrespective of treatment, exhibited browse; subsequently, growth of many trees was completely arrested due to browse of every apical meristem. Numerous specimens were scraped up or completely uprooted. Prior studies of black locust on reclaimed coal mines in Eastern Kentucky (Carpenter and Eigel 1979; Carpenter 1980) reported higher growth metrics than at our site, but it appears that ungulate browsing severely mitigated black locust growth in our study and likely confounded the effects of nutrient and moisture treatments in our study. Dahl (2008) estimated the elk population at approximately 7,000, and without a natural predator in the region populations may continue to rise, densities increase, and ranges will expand. This could have serious implication for forest succession and the success of reforestation plantings throughout the entire Appalachian region as elk migrate out of eastern Kentucky and into neighboring states with surface mine lands that serve as excellent habitat.

*4.2 Biomass Accumulation and Allocation*

Granular fertilizer applications led to large gains in total mass and aboveground leafless biomass in American sycamore. Fertilizer treatments increased total mass by 287 % over irrigation + fertilization treatments and 608 % compared to control plots. Per stem aboveground leafless biomass nearly quadrupled in fertilizer treatments (373.18g) compared to controls (97.95g) and nearly eight times greater than irrigation treatments (48.7g). Stocking densities were not consistent across all plots, but using a mean stocking rate of 1849 stems $ha^{-1}$ mean annual biomass production was 0.36 Mt $yr^{-1}$ after two growing seasons. This is significantly lower than

other studies. Fertilized direct seeded black locusts on a reclaimed mine in Eastern Kentucky produced 3.3 Mt $yr^{-1}$ following two growing seasons (Carpenter and Eigel 1979), but their study had much higher stocking rates. Even greater gains were witnessed for American sycamore in Western Kentucky (Wood, Wittwer et al. 1977), the Savanna River Site in South Carolina (Coyle and Coleman 2005), and on former agricultural land in South Carolina (Davis and Trettin 2006). Irrigation appeared to negatively affect sycamore aboveground leafless biomass and total mass, but had a positive effect on total leaf mass. Although negative effects of irrigation on a variety of growth metrics are not widely documented, an increase in leaf mass following irrigation is in congruence with other studies (Coleman, M. Jacobson et al. 2004; Coyle and Coleman 2005).

Fertilized sycamore had nearly double the leaf area of the next highest treatment (sycamore IF). The positive effects of fertilization on leaf area were also witnessed for hybrid poplars on a Minnesota plantation (Coleman, Tolsted et al. 2006). However, it was not of the same order of magnitude. Fertilization increased mean leaf area by 20% in at their site whereas a 102% increase was witnessed for sycamores and 46% increase in locusts at our site compared to controls. American sycamore leaf area was more than 80% lower in the irrigation treatment when compared to the other treatments. This pattern further suggests that irrigation had a negative effect on American sycamore at our site and supports similar trends present in American sycamore seedling height, diameter, aboveground leafless biomass, and stem and foliage nitrogen. We attribute the negative effects of irrigation to the additive impacts of poor drainage of the mine spoil and irrigation treatments creating sustained anaerobic conditions that limited nutrient uptake by lowering chemical and electrical gradients across root surfaces. Irrigation treatments at highly compacted sites may create sustained anaerobic conditions that are detrimental to growth; even for species with an affinity for wet sites.

Even though we detected significant effects of treatments on leaf area, differences in specific leaf area were insignificant between species and treatments despite a lower mean SLA in sycamore in F and IF plots. SLA is a measure of leaf thickness and is an indirect measure of photosynthetic capacity (Evans and Poorter 2001). Our results suggest that fertilizer and nutrient amendments did not significantly increase photosynthetic capacity per unit leaf area; however, total woody biomass production was positively correlated with leaf area.

Analysis of K coefficients indicated no effect of treatments on biomass allocation for either American sycamore or black locust. Coyle and Coleman (2005) reported similar results for sycamore at the Savannah River Site in South Carolina. They did attribute slight shifts in fine root allocation to intensive management treatments. The pervasiveness of large boulders throughout the soil matrix at our study prevented an accurate analysis of fine vs. coarse root production, thus we were unable to test for allocation shifts of fine roots. However, trends of declining root fraction with fertilizer applications for both species at our site are aligned with allocation patterns presented by Coyle and Coleman (Coyle and Coleman 2005). It is possible that characteristics of the soil matrix (cast mining overburden) affected tree response to resource amendments at our site. Coleman and Coyle (2005) analysis was also done following three growing seasons; it is possible that shifts in allocation are only possible once trees reach a particular developmental stage that has not happened at our site due lower site quality and fewer growing seasons.

### *4.3 Nitrogen Concentrations and Soil Uptake*

Nutrient and moisture amendments treatments applied in this study had little effect on intraspecies nitrogen concentrations. However, black locust exhibited significantly greater levels

of nitrogen across all treatments and partitions compared to American sycamore. Generally, black locust nitrogen concentrations were two to four times greater for roots and more than double in stems and foliage. Higher nitrogen concentrations in black locust is not surprising given that the species is known for its ability to form symbiotic relationships with soil bacteria that fix nitrogen (Rice, Westerman et al. 2004). Elevated stem nitrogen concentrations have been correlated with higher ungulate browse frequencies (Tripler, Tripler et al. 2002). Thus, the difference in nitrogen concentrations between black locust and American sycamore could provide a plausible explanation of why browse was observed on 86% of black locust seedlings and only 1% of American sycamore seedlings. American sycamore nitrogen concentrations at our site were in congruence with levels established at a plantation in Georgia under varying irrigation and fertilizer regimes (Cobb, Will et al. 2008). They reported foliage sycamore nitrogen concentrations of 15.3 g $kg^{-1}$ under control treatments and 18.5 g $kg^{-1}$ to 20.3 g $kg^{-1}$ in fertilized plots. Despite similar nitrogen concentrations as in our study, Cobb et al. (2008) detected significantly elevated levels of foliar nitrogen concentrations under treatments of irrigation with nitrogen fertilizer. Although foliar nitrogen concentrations were similar to our study, mean stem nitrogen concentrations were greater at our site (4.6 g $kg^{-1}$ to 5.6 g $kg^{-1}$) compared with 3.5 g $kg^{-1}$ to 1.6 g $kg^{-1}$ in sycamore under a control and fertilizer treatments in their study following six growing seasons. Increased nitrogen concentrations in response to fertilizer treatments were also reported for hybrid poplars at a Minnesota plantation (Coleman, Tolsted et al. 2006). Conversely, no significant effects of fertilizer applications on foliar nitrogen concentrations were indentified at a green ash (*Fraxinus pennsylvanica*) plantation established on a ripped mine in Ohio (Kost, Vimmerstedt et al. 1998). However, they reported similar foliar nitrogen concentrations (15.0 g $kg^{-1}$). Although nitrogen levels at our site were

similar to other studies, we lacked a significant effect of fertilizer and irrigation on concentrations.

Nitrogen concentrations were higher in black locust tissue, but due to much greater biomass accumulation total nitrogen uptake was significantly greater in fertilized sycamore plots compared to all other treatments except fertilized locusts. Thirty-seven kg of elemental nitrogen $ha^{-1}$ were applied annually to fertilized plots. Mean nitrogen uptake in fertilized sycamores plots was 14.53 kg $ha^{-1}$. Conversely, total nitrogen in fertilized black locust plots was only 9.11 kg $ha^{-1}$, but due to black locust's ability to form symbiotic relationships with nitrogen fixing bacteria, it is unclear what proportion of that was a result of fertilizer applications and what was a function of nitrogen fixing. Moreover, despite a difference of 59.47 kg $ha^{-1}$ between applied and sequestered nitrogen following two growing seasons, there was no significant residual nitrogen loading in fertilized soils.

Total nitrogen uptake and percent uptake of applied nitrogen at our site was lower than similar studies. Total nitrogen uptake at an upland sycamore plantation in Tennessee was approximately 140 kg $ha^{-1}$ following three growing seasons (van Miegroet, Norby et al. 1994). The amount of total nitrogen applied in fertilizer treatments sequestered by the target crop in their study was 31% compared to 24% at our site. Furthermore, American sycamore at an upland Western Kentucky plantation sequestered 86.2 kg $ha^{-1}$ and 58.5 kg $ha^{-1}$ of nitrogen following three growing seasons and applications of 167 kg N $ha^{-1}$ and 112 kg N $ha^{-1}$, respectively (Wood, Wittwer et al. 1977). This represented 52% of total nitrogen applied in both treatments. Greater comparative total nitrogen uptake and percent uptake of applied nitrogen at these sites compared to our study may be a function of inherent differences in chemical and physical soil properties, extended study durations, and reduced weedy competition

Despite annual herbicide applications across all treatments, weedy competition remained present; the most aggressive genera were *cirsium*, *ambrosia*, and *lespedeza*. Therefore, it is plausible that intense competition accounted for a considerable proportion of the remaining 23.47 kg $ha^{-1}$ of nitrogen in fertilized sycamore plots. Nitrogen leaching or runoff may also explain the unaccounted nitrogen. Although soil nitrogen was unaffected by fertilizer applications, significantly elevated levels of phosphorous and potassium were present in fertilized soils. Nitrogen and phosphorus runoff are a primary concern for intensively managed plantations (Nyakatawa, Mays et al. 2006).

More effective fertilizer regimes can increase profit margins, mitigate the negative effects of runoff, and improve site carbon budgets (Canadell and Raupach 2008). Our results suggest that annual nitrogen additions of 15 kg $ha^{-1}$ would be sufficient for early growth of American sycamore and black locust on reclaimed surface mines with similar overburden and an equivalent level of weed control. Soil analysis indicates that a fertilizer mix with reduced phosphorus and potassium may be appropriate, but several studies suggest that tree growth can also be phosphorous limited (Misra, Turnbull et al. 1998; Miller, Zutter et al. 2003). Maintaining an appropriate nutrient balance will be necessary as to avoid mitigating the positive effects of nitrogen applications if phosphorus is lowered below a critical mass. Further research is needed to determine optimal fertilizer rates and mixtures for American sycamore and black locust grown on reclaimed surface mines in Appalachia.

## 5. **Conclusion**

This study evaluated the effect of irrigation and fertilization on growth, biomass accumulation and allocation, survival, and nitrogen concentrations of black locust and American sycamore

following two growing seasons. Annual application of granular fertilizer increased growth and biomass accumulation of American sycamore. No treatment evaluated was effective at enhancing the response of black locust. However, the effect of the treatments on black locust was likely confounded by intensive ungulate browse. Treatments had little effect on biomass allocation patterns and nitrogen concentrations of either species.

Further examination is needed, but two-year results suggest that manual broadcast of granular fertilizer may be a preferred over irrigation and irrigation plus fertilizer (i.e., fertigation) for increasing biomass production of woody biofuel plantations on reclaimed surface mines in Appalachia especially given the installation and maintenance costs of a drip irrigation system. Our study also indicates that early fertilizer application on reclaimed surface mines also accelerate young stand development by increasing height growth. Accelerated stand development has important economic and ecological implications in ongoing reforestation efforts within the Appalachian coal region. By promoting more rapid canopy closure, fertilization may help mining companies receive bond release from regulatory agencies more quickly and may hasten the beneficial impact of ecosystem services provide by forests on severely disturbed mine sites.

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## APPENDIX: PROJECT TIMELINE AND QUARTERLY REPORTS

**Demonstrating Techniques for Establishing Woody Biomass Plantations on Surface Mine Lands as Feedstocks for Energy Production**

GOEP-Barton PO2 8850700011339

**March 2008**

***Work Accomplished:***

- Trinity Coal agreed to partner with the University in their effort to undertake this project. They agreed to provide a suitable reclamation site for establishing the plantation on their Big Elk mine (formerly Starfire) in Perry County, Kentucky. Trinity Coal also agreed to provide a cost share in the form of services rendered that the investigators will need to undertake the project. Specifically, they agreed to prepare the site for planting through mechanical ripping of the proposed project area (approximately 20 acres).

- The investigators visited the Big Elk mine on December 5, 2007 and located a suitable site that is in close proximity to a large pond that could be utilized for our irrigation needs and near a source of electric power that will be needed for pumping (Figure 1). The site is also accessible to water from a shallow aquifer that has been characterized by the Kentucky Geologic Survey as having a quality suitable for irrigation purposes. The boundary of the area was surveyed by GPS and maps of the study site are currently being created. The center of the study area is Lat: 37.414936, Long: -83.120423.

Figure 1. Study location at Big Elk (Star Fire) mine in Perry County.

- The study area was ripped by Trinity Coal operators using a D-10 dozer equipped with a single shank from March 1 - 3, 2008.

- On March 6, 2008 the investigators identified sixteen plots for the irrigation, fertigation, fertilized only and control treatments (n = 4) at the site and marked all plot corners with metal posts (Figure 2). In all, eight plots were identified for the planting of American sycamore seedlings and eight for black locust seedlings. Thus each treatment will be replicated twice (n = 2) for each species. Four additional plots (mixed species) were established that will be planted with both sycamore and block locust.

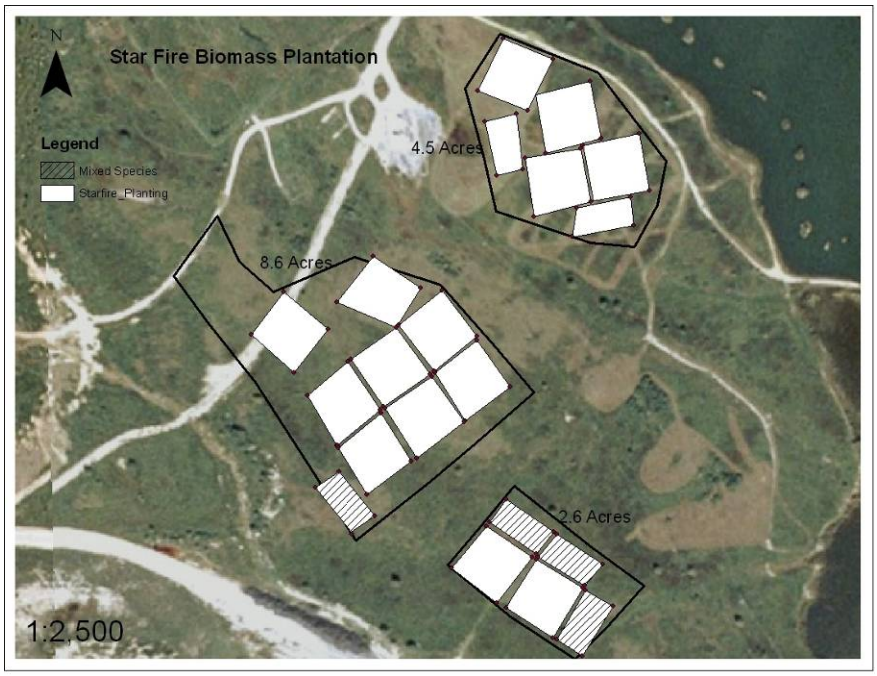


Figure 2. Plot layout for biomass plantation and treatment establishment.

- Williams Forestry and Associates (Calhoun, GA) planted 10,800 American sycamore and 10,800 black locust seedlings at the plantation site on March 9, 2008 (Figure 3). Seedlings were planted in both the marked plots and in areas between plots (all areas identified in Figure 1) to minimize edge effects.

Figure 3. Plantation establishment in ripped spoil.

- A graduate student to work on the project was identified and his employment will begin in April 2008.

- Monthly water samples were collected and analyzed for pH, EC, DO, Ca, Mg, Na, K, Fe, Mn, sulfate, nitrate, ammonium, chloride and alkalinity.

- On-site weather station was activated.

- Irrigation system design and ordering of materials needed for construction was initiated.

- Description of irrigation and fertigation system:

    1. Each plot has two lines 150ft long of ½" PE tubing fitted with 1gph emitters

    2. Each plot has twenty lines 150ft long of 15mil 12" spacing irrigation T-tape (approximately .33gpm/100ft)

    3. Each plot is equipped with a valve box containing an electric solenoid valve, a flow meter and three pressure reducers. 25psi for the ½" PE tube lines and two 10psi each feeding a ¾" PE header line.

    4. Each ¾"PE line feeds ten T-tape lines.

    5. Each valve box is connected to a common 1 ½" PVC supply line running from the pump to the furthest plot approximately 1200ft.

    6. Pump is a 2hp booster pump drawing water from a pond through a strainer and foot valve suspended approximately two feet below the water surface. The suction line is a 2" line and 100ft long.

    7. Plumbing attached to the pump output includes pressure indicators, Fertigation injector bypass system, check valve, 150micron filter, filter flush valve and Fertigation tank filler. A 100g plastic storage tank is used to mix soluble fertilizer and supply this via the fertigation injector.

    8. Irrigation control is done by Rainbird ESP Irrigation controller.

9. Generator to supply power for this remote system is a 7Kw belt driven unit powered by an 18Hp Propane fueled engine. Propane as supplied from a 500gal gas storage tank placed on site. Control electronics for the generator include an "Intelligent" inverter. This inverter would automatically start and stop the generator whenever the Irrigation controller would start irrigating. After a short warm-up period the pump would start. The inverter also monitors the batteries that supply power to the electronics (when the generator is not running) and will automatically start the generator to charge the batteries should they run low. Three 75Ahr deep cycle batteries are used.

***Problems Encountered:***

None this period.

***Projected Activities Next Quarter:***

- Spoil/soil samples will be collected from each treatment plot and analyzed.
- Construction of irrigation system will begin.
- Irrigation/fertigation on selected plots shall begin in June 2008. Irrigation/fertigation schedules will be established.
- Fertilization of "fertilizer only" treatment will be initiated.
- Soil water samplers (lysimeters) will be installed in all plots.
- Soil water solution and pond water will be sampled and analyzed each month.

**May 2009:**

***Work Accomplished:***

- April 29 - May 20, 2008: The graduate student assigned to the project established sub plots within each of larger plots. All trees in sub plots were individually tagged, and height and diameter measurements will be monitored across the two-year period.

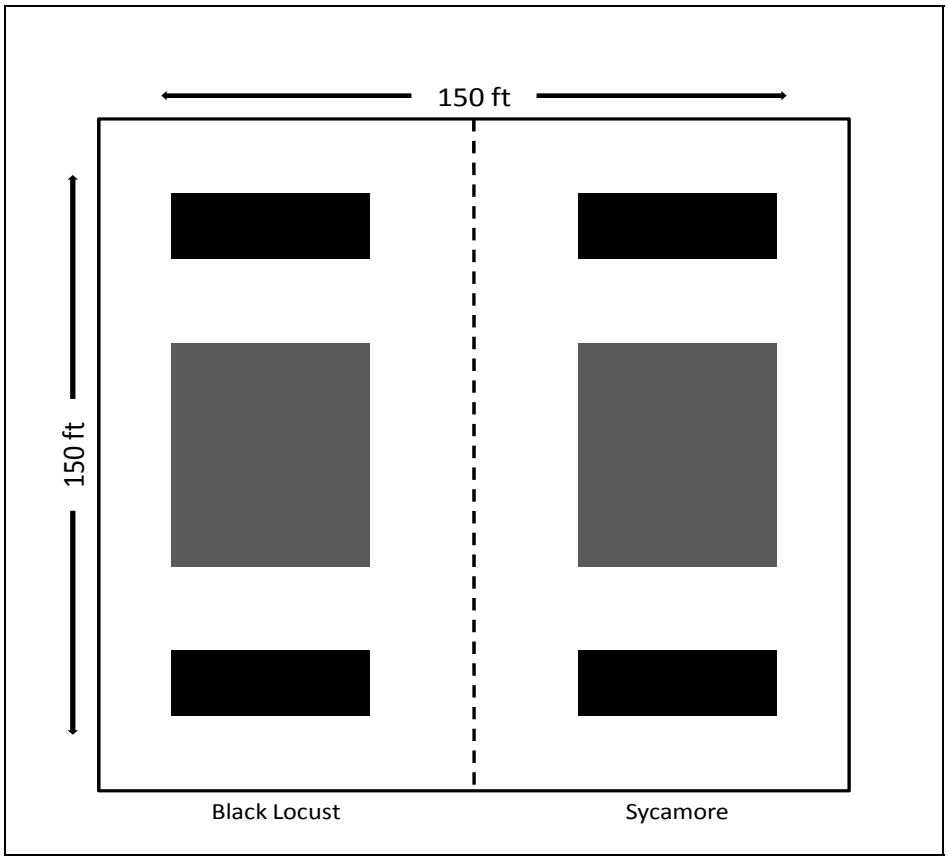


Figure 4: Plot layout showing measureable (grey) and destructive (black) subplots.

- June 3, 2008: Treatments for each plot were established and 500 lbs. of 20-20-20 (N-P-K) fertilizer was applied to each plot with fertilizer treatment.

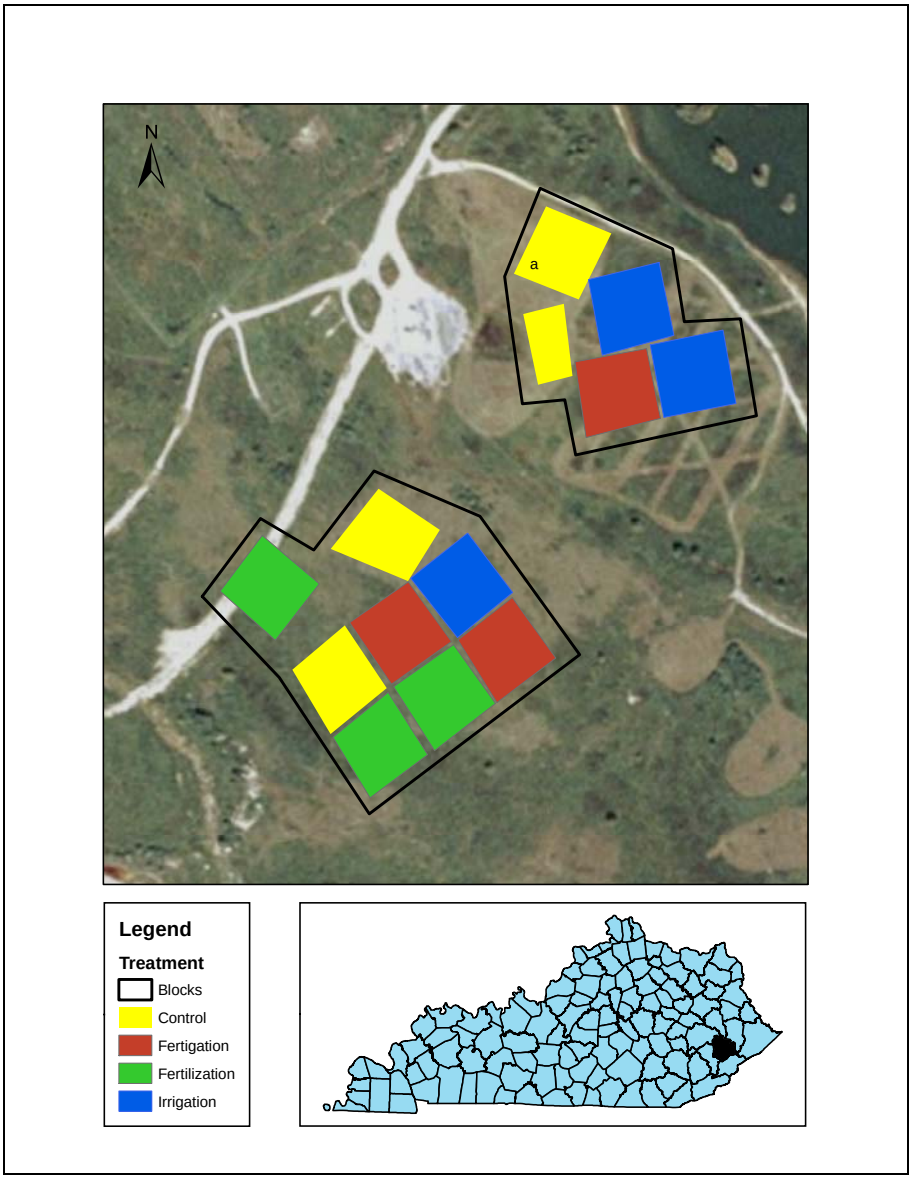


Figure 5: Finalized treatment layout

- July 11-18, 2008: Herbicide (3% glyphosate with surfactant) applied to all plots with back pack sprayers.

- September - October 2008: Irrigation system installed and operating by University of Kentucky Department of Biosystems and Agricultural Engineering. First seasons irrigation and fertigation treatments applied.

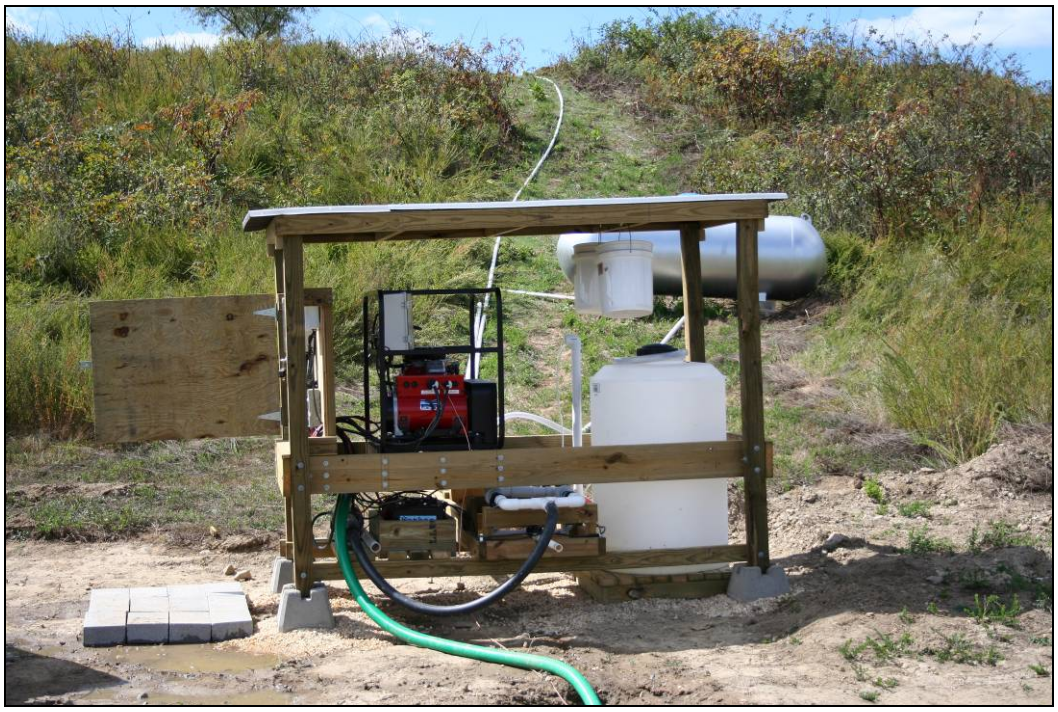

Figure 6: Irrigation pump (above) and piping throughout plots (below).

- December 2008 - January 2009: First growing season measurements (diameter and height; Figure 7) collected from tagged seedlings in measureable sub plots (Figure 4).

Figure 7: Impact of fertilizer already evident.

| Treatment | N | Height and Diameter Differential | Mean | Std Deviation | Minimum | Maximum |
|---|---|---|---|---|---|---|
| Control | 192 | Basal Diameter (mm) | 3.41 | 2.79 | -1.25 | 14.35 |
| | | Height (cm) | 13.11 | 12.96 | -18.70 | 77.00 |
| Fertilizer | 202 | Basal Diameter (mm) | 7.24 | 3.52 | 0.60 | 18.60 |
| | | Height (cm) | 25.90 | 20.43 | -34.80 | 87.40 |
| Irrigation | 226 | Basal Diameter (mm) | 3.50 | 2.41 | -0.05 | 11.73 |
| | | Height (cm) | 11.23 | 12.82 | -18.40 | 75.60 |
| Fertigation | 217 | Basal Diameter (mm) | 3.65 | 3.92 | -0.50 | 10.30 |
| | | Height (cm) | 12.68 | 15.79 | -19.60 | 97.50 |

Figure 8: Preliminary growth data from first season. Mean height and diameter for fertilizer treatment is more than double that of other treatments.

- February 2009: Destructive samples taken for first season biomass analysis.

- May 19 - 29, 2009: Herbicide (3% glyphosate with surfactant) applied to all plots with back pack sprayers. Weeds presenting moderate to high competition (Figure 8).

Figure 8: Weedy competition is moderate to high in areas. Photo taken May 2009

***Problems Encountered:***

None this period.

**September 2009:**

***Work Accomplished***

- June 3, 2009: 550 lbs. of 19-19-19 (N-P-K) granular fertilizer was applied to each plot with fertilizer treatment.

- June 24, 2009: Irrigation and fertigation treatments begin

Figure 9: Sycamore in the background exhibiting positive effects of granular fertilizer

- July 2009: First growing season data analysis:
-
    - Regression equations developed to predict biomass for both species

**Black Locust:** log(Weight) = -4.67 + 0.80*log(Height) + 1.97*log(Diameter)

**Sycamore:** log(Weight) = -4.50 + 0.80*log(Height) + 1.74*log(Diameter)

- Per stem biomass calculations:

Table 1: Black locust mean, minimum and maximum biomass per stem by treatment.

| TREAT | Obs | N | Mean (g) | Std Dev | Minimum | Maximum |
|---|---|---|---|---|---|---|
| C | 84 | 84 | 11.43 | 12.65 | 0.55 | 68.65 |
| F | 96 | 96 | 30.77 | 23.49 | 1.88 | 109.22 |
| I | 119 | 119 | 15.32 | 15.95 | 1.40 | 91.73 |
| I+F | 100 | 100 | 16.48 | 16.95 | 0.73 | 96.50 |

Table 2: American sycamore mean, minimum and maximum biomass per stem by treatment.

| TREAT | Obs | N | Mean (g) | Std Dev | Minimum | Maximum |
|---|---|---|---|---|---|---|
| C | 108 | 108 | 14.59 | 13.22 | 1.27 | 77.20 |
| F | 106 | 106 | 31.01 | 27.60 | 0.96 | 109.49 |
| I | 107 | 107 | 11.23 | 9.20 | 0.70 | 60.45 |
| I+F | 115 | 115 | 9.95 | 7.75 | 0.62 | 35.61 |

- Per acre biomass calculations:

Table 3: Black locust (BL) and American sycamore (AS) per acre biomass assuming 5'x'5 (1742 stems acre$^{-1)}$ spacing and 100%.

| SPC | TREAT | MEAN G stem$^{-1}$ year $^{-1}$ | SPACING | STEMS acre$^{-1}$ | G acre$^{-1}$ year $^{-1}$ | KG acre$^{-1}$ year $^{-1}$ | LBS acre$^{-1}$ year $^{-1}$ |
|---|---|---|---|---|---|---|---|
| BL | C | 11.44 | 5'x5' | 1742 | 19928.48 | 19.93 | 43.84 |
| BL | F | 30.78 | 5'x5' | 1742 | 53618.76 | 53.62 | 117.96 |
| BL | I | 15.33 | 5'x5' | 1742 | 26704.86 | 26.70 | 58.75 |
| BL | I+F | 16.49 | 5'x5' | 1742 | 28725.58 | 28.73 | 63.20 |
| SY | C | 14.59 | 5'x5' | 1742 | 25415.78 | 25.42 | 55.91 |
| SY | F | 31.01 | 5'x5' | 1742 | 54019.42 | 54.02 | 118.84 |
| SY | I | 11.23 | 5'x5' | 1742 | 19562.66 | 19.56 | 43.04 |
| SY | I+F | 9.96 | 5'x5' | 1742 | 17350.32 | 17.35 | 38.17 |

***Projected Activities Next Quarter:***

- Collect second growing season measurements
- Collect second growing season destructive harvests
- Perform biomass and leaf area analysis
- Prepare manuscripts for publication

**July 2010**

***Work Accomplished***

- June 2009 – September 2009: Irrigation and fertigation treatments completed.

- September 2009 – October 2009: Excavated twenty-four destructive harvests for tissue chemistry and biomass partitioning analysis.

- December 2009: Height and diameter measurements recorded for the second growing season:

Table 4

Predicted mean per stem mass, basal diamter, height, survival, and browse following two growing seasons.

| | | Stem Mass (g) | Diameter (mm) | Height (cm) | Survival % | Browse % |
|---|---|---|---|---|---|---|
| Sycamore | C | 97.95±33.4a | 18.8±2.8a | 100.4±15.5a | 80.0a | 1.0 |
| | I | 48.7±16.3a | 14.1±1.9a | 79.5±9.4a | 76.5a | 0.0 |
| | F | 373.2±37.1b | 31.6±1.4b | 168.5±3.7b | 80.1a | 3.3 |
| | IF | 85.5±19.6a | 16.5±0.9a | 114.8±18.9ab | 83.3a | 0.0 |
| Black Locust | C | 52.4±3.8a | 13.1±0.3a | 83.9±8.1a | 53.7b | 78.4 |
| | I | 65.4±17.2a | 14.5±1.6a | 89.5±7.9a | 58.5b | 88.3 |
| | F | 113.0±13.0a | 17.8±0.1a | 121.7±13.4ab | 58.5b | 93.1 |
| | IF | 56.9±22.8a | 12.8±1.9a | 89.3±13.1a | 49.9b | 84.7 |

Lower case letters indicate significance ($p<0.05$; $n=3$) across species and treatments. Control [C], irrigation [I], fertilizer [F], and irrigation + fertilizer [IF]

- January 2010 – February 2010: Lab samples processed and statistical analysis performed.

Figure 10: Elk are present at the site in high densities and browse Black locust in large quantities

- April 2010: Results of first growing season presented at the 17th Central Hardwoods Conference, Lexington KY.
- May 2010 - July 2010: Preparation of full manuscript for publication.
- June 2010: Applied granular fertilizer for third growing season.

***Problems This Quarter:***

- None

***Projected Activities Next Quarter:***

- None, project completed.
- Duration: 28 months
- Data presented at conference and two year results prepared into manuscript (attached) for publication.