EXPLORING THE BENEFITS OF COVER CROPS TO AGROFORESTRY TREE PLANTATIONS: AN ANALYSIS OF DIRECT AND INDIRECT NITROGEN TRANSFER IN ALLEY CROPPING SYSTEMS

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ABSTRACT

Exploring the Benefits of Cover Crops to Agroforestry Tree Plantations: An analysis of direct and indirect nitrogen transfer in alley cropping systems

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Incorporating species capable of biological nitrogen fixation into agroforestry systems can improve nutrient cycling and soil health while reducing the need for synthetic nitrogenous fertilizers. Research has confirmed multiple pathways through which biologically fixed nitrogen is transferred to non-nitrogen fixing plants, including indirect transfer through decaying biomass and direct transfer through belowground root exudates and shared fungal networks. I analyzed the benefits of cover crops to the growth and nutrition of important agroforestry tree species through experiments in the field and greenhouse. In the field study, I examined the contributions of *Trifolium pratense* to the health and growth of hybrid poplar (*Populus nigra* L.×*P. maximowiczii* A. Henry ‘NM6’) in an alley cropping system at varying seed rates and tree row spacing. Soil and poplar leaf nutrient analysis revealed higher nitrogen levels in treatments with *T. pratense* at regardless of seed rate at the wider spacing, while narrow spacing treatments showed signs of competition. In the greenhouse study, the transfer of N through root exudates from white clover (*Trifolium repens*) to a hybrid poplar (*Populus nigra* L.×*P. maximowiczii* A. Henry ‘NM6’) and a hybrid chestnut (*Castanea sativa x C. crenata* ‘Colossal’) was quantified via ^{15}N isotope tracing methods. Results revealed that over the course of 9 weeks, white clover supplied 33.3% (±7.4) of chestnut N and 12.1% (±1.1) of poplar N through root eudates. White clover (*T. repens*) was found to exude 0.048 g N m^{-2} day^{-1} and roughly 3.4% of its’ total N over the course of the 63 day experiment. Together these studies reinforce the potential of nitrogen-fixing species to benefit mixed systems through both direct and indirect means.

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1 Introduction

Global demand for food and fiber is expected to increase consistently in the coming decades. Due to limited remaining productive land and a desire to protect remaining natural forests, much of the increased demand will have to be met from existing production systems [1]. However, the growing problem of global land degradation poses a threat to the ability to increase future production to meet future demands [2]. Moreover, altered climatic conditions as a result of human-induced climate change will continue to threaten food and fiber yields and require more resilient agricultural systems to maintain present and future production needs [3–5]. In order to overcome the issues facing food and fiber systems, producers and landowners must embrace agroecological concepts that ensure high yields while sustaining the integrity of the land in use.

Agroforestry practices integrate agriculture and forestry into the same land management scheme in order to protect soil and diversify landowner income while providing multiple ecosystem services [6]. These practices present an important pathway for establishing sustainable production and resilient farming systems that will be critical to meeting future food and fiber demands. A large body of research has highlighted the potential for agroforestry to not only increase yields but also restore soil on degraded lands, improve biological diversity, and protect waterways [2,6,7]. Further, the United Nations has recognized agroforestry as having a critical role in climate change mitigation because of its efficient carbon sequestration and resiliency to changing climatic conditions [8].

One of the many ways that agroforestry practices support sustainable production is through enhanced nutrient cycling. This occurs, in part, because of the ability of trees to capture water and nutrients at deeper layers and subsequently make them available to herbaceous crops with shallow rooting systems [9,10]. However, a perhaps more critical element to nutrient cycling in these practices is the utilization of biologically fixed nitrogen through the incorporation of leguminous species. These species have symbiotic relationships with soil microbes that inhabit their roots and “fix” N_2 gas from the atmosphere into plant-available forms in exchange for energy [11]. Since nitrogen is an extremely important nutrient to plant health and productivity, determining the best methods of utilizing this process for the benefit of food and fiber production has been the subject of much research. Additionally, the high carbon cost of producing synthetic nitrogenous fertilizers and associated risks to ecosystem health during production has made biologically fixed nitrogen an increasingly important form of sustainable fertilizer for production systems.

Thus far, research has identified three pathways for biologically fixed nitrogen to be transferred from a legume to a non-nitrogen-fixing species: (1) indirect transfer through decaying legume biomass, mineralization of organic N, and subsequent uptake by plants, (2) direct transfer of plant available nitrogen through shared belowground fungal networks, and (3) direct transfer through the exudation of N-rich compounds (primarily NH_4^+ and amino acids) from living roots of legumes to the roots of a recipient plant [12,13]. As it stands, indirect transfer through decaying biomass has been the primary mode of transfer utilized in agriculture systems and is thought to be the most substantial N transfer pathway [12]. However, this process requires the mineralization of organic N into plant-available forms and, as a result, the actual delivery of biologically fixed N to recipient plants can be difficult to control. Direct transfer, on the other hand, supplies N compounds that are readily assimilated by recipient plants. Recent research has highlighted the underestimated importance of direct transfer pathways, particularly through root exudates [14–17]. The slow but steady N transfer through direct pathways could serve as a consistent, low-input, and sustainable form of nitrogenous fertilizer. Regardless of the transfer pathway, utilizing biologically fixed nitrogen for production systems could displace the need for synthetic fertilizers, which are often inefficiently used by cropping systems and result in pollution of soil and waterways[18].

Nitrogen transfer, whether through direct or indirect means, is affected by a number of biotic and abiotic factors. Water stress, low temperatures, and low light availability, for instance, all inhibit both the fixation of nitrogen and the movement of exudate through soil [13]. Transfer can also be influenced by the level of root-to-root contact, plant growth stages, and legume to non-legume density ratios. Roggy et al. (2004) revealed the importance of legume species selection to transfer potential. Species with high soluble N and low lignin content within their leaves, for instance, had higher indirect transfer rates [14]. Exudation potential can differ greatly between species even within the same family, as has been recorded for species of *Trifolium*[17].

Research focused specifically on N transfer within agroforestry dates at least as far back as the 1980's, if not earlier [19,20]. However, it wasn't until after the turn of the century that researchers started identifying and quantifying between the different transfer mechanisms. The development of ^{15}N isotope methods, including natural abundance and tracer application studies, has allowed nitrogen sources to be traced and quantified in such studies [12]. Presently, there is a small but powerful pool of research that has utilized these methods in various species combinations with great success. However, there are some notable research gaps that have yet to be filled. For example, much of this vein of research has focused on the transfer of nitrogen from leguminous trees and shrubs to herbaceous crops and grasses. There are also a number of studies that have focused on transfer from herbaceous legumes to grasses. No research, however, exists on the potential for herbaceous legumes with high nitrogen-fixing capacity and exudation potential, such as clovers, to provide directly transferred N to valuable tree species. A number of studies have illuminated multiple benefits of incorporating species such as clover and alfalfa into tree plantations, including improved nutrient availability to trees and higher foliar N content [19,21,22]. However, few, if any, have actually quantified direct and indirect N transfer in such systems.

As a result of this research gap, I illuminated the potential of two clovers (*Trifolium pratense and Trifolium repens*) to benefit temperate alley cropping systems. These studies will build on existing research regarding the benefits of incorporating legumes into production systems and the transfer of biologically fixed nitrogen. The novelty of these experiments is the focus on trees as recipient of N transfer and herbaceous cover crops as the N donor and comparing transfer between two species combinations. This research consists of two experiments: a two-year field study that focus on the observed benefits of *T. pratense* to a hybrid poplar plantation and a greenhouse study that quantified direct N transfer from *T. repens* to two important agroforestry tree species. The results of these studies will inform land management decisions for agroforestry and tree plantation practitioners regarding soil health and tree nutrition. A preceding section will offer a literature review describing the present issues facing food and fiber systems, and how agroforestry and utilizing the transfer of biologically fixed nitrogen can address them.

2 Literature Review

As the worlds' population continues to increase, global food, fiber, and fuel demands will intensify accordingly. Land conversion and intensified management of existing agricultural lands have been historically used to increase production and meet demands but they may not have a place in an era of sustainability and environmental conscientiousness. Clearing land results in forest losses, substantial greenhouse gas emissions, and damage to a suite of ecosystem services critical to human and ecosystem health. On the other hand, intense land management can result in soil degradation and extensive non-point pollution. In addition, land limitations pose a complex issue that forces tradeoffs between land-use for forestry, agriculture, conservation, and urban development. As a result of these concerns, considerable research has been dedicated to determining how future needs can be met while minimizing the negative effects of land conversion and degradation.

Agroforestry practices address these concerns by integrating agriculture and forestry onto the same land to take advantage of natural ecological feedbacks that restore and maintain soil health, create highly productive systems, and provide a myriad of ecosystem services. This literature review will cover a basic explanation of agroforestry practices as a whole, followed by an elaboration on current issues facing food and fiber systems and how agroforestry can address them. As the focus of this research is on nutrient cycling, particularly nitrogen, most of the focus will be on soil health. However, the systems proposed in this research have multiple beneficial bi-products and, in order to make a thorough case for their utilization, these benefits and will also be detailed.

2.1 Agroforestry

Agroforestry offers a path to sustainable production via an array of practices that integrate of agriculture and forestry onto the same land. The multifunctional properties of agroforestry were described by the Food and Agriculture Organization of the United Nations as:

> ". . . part of the solution to addressing these issues, whether they be environmental, economic or social. Agroforestry systems include both traditional and modern land-use systems where trees are managed together with crops and/or animal production systems in agricultural settings. They are dynamic, ecologically based, natural resource management systems that diversify and sustain production in order to increase social, economic and environmental benefits for land users at all scales." [8]

Agroforestry is common among smallholder agriculture properties in tropic and subtropical regions, where it has been used to diversify income and sustain soil quality for farm-dependent communities. In temperate regions, the domination of agricultural sectors by conventional farming methods has made it difficult for agroforestry to gain popularity. Nevertheless, it has caught on in over five million hectares in the last two decades, showing a slow but global shift towards integrated agriculture [23].

Many of the issues facing agriculture and forest plantations today stem from growing populations and climate change. However, an overly simplistic approach to crop and fiber production may be the primary culprit. Monoculture production of single species can be good for maximizing yields but ultimately require considerable inputs and will likely degrade land quality over time. Different approaches that focus on creating sustainable, productive agroecosystems are needed to effectively address these problems. The synergistic relationships between species and the ecosystem services provided can create systems that require few inputs and produce multiple products.

To sustain future populations, agriculture and fiber systems must be capable of consistent production while maintaining soil and land quality. These management practices must focus on maintaining soil carbon and soil structure, controlling erosion, maintaining or improving soil quality, increasing nutrient cycling efficiency, reducing export of nutrients, and reducing required inputs such as fertilizer and pesticides [24]. Agroforestry integrates agricultural and forestry goals and practices to utilize interspecific benefits that maximize nutrient, water, and light use efficiency [25]. This results in more diverse, productive, and profitable agroecosystems that support sustainable land-use [26]. These systems not only encourage sustainable ecological cycles but also diversify crop production for land owners, making both the land and the owner more resilient to future challenges.

The World Agroforestry Center (ICRAF) called attention to the potential of agroforestry by pointing out several ways that its practices can address the Millennium Development Goals set in 2000. Sustainable agroforestry practices can [27]: .

1. Help eradicate hunger through basic, pro-poor food production systems in disadvantaged areas based on agroforestry methods of soil fertility and land regeneration
2. Lift more rural poor from poverty through market- driven, locally led tree cultivation systems that generate income and build assets
3. Advance the health and nutrition of the rural poor through agroforestry systems;

4. Conserve biodiversity through integrated conservation-development solutions based on agroforestry technologies, innovative institutions, and better policies;
5. Protect watershed services through agroforestry- based solutions that enable the poor to be rewarded for their provision of these services;
6. Assist the rural poor to better adapt to climate change, and to benefit from emerging carbon markets, through tree cultivation
7. Build human and institutional capacity in agro- forestry research and development

The term "sustainable management" has surpassed dealing only in terms of productivity, and now encompasses multiple environmental indicators such as biodiversity, soil health, carbon sequestration, and water protection, among others [28]. Nair (2012) addressed the need for an "evergreen revolution", stating that what is most critical now is a global agricultural revolution that surpasses the production levels of the green revolution of the 1960's while utilizing agricultural and forestry technology that promotes environmental sustainability.

2.1.1 Global Agroforestry Trends

Agroforestry practices are arguably millennia old, as similar concepts utilizing the benefits of trees in agriculture have been found in indigenous agricultural systems. Today, roughly 1.2 billion people depend on agroforestry farming systems in some form around the world [29]. It is practiced primarily by smallholder farmers in developing tropical countries that cannot afford the expensive input-dependent agricultural methods of the industrial temperate countries.

True measures of land area dedicated to agroforestry are difficult to determine. One method is measuring the amount of agricultural land that has partial tree cover and assuming these trees were intentionally integrated into production. According to one study, roughly one billion hectares of global agricultural land has more than 10% tree cover [6]. In Southeast Asia and Central America, much of the agricultural land has tree cover exceeding 30% [6]. In the tropics, the true agroforestry land area is disputed. However, the FAO summed up the agriculture and forestry trends in the tropics very well when it said, "the number of trees in forests is declining, but the number on farms is increasing"[8]. One of the major reasons for the growing popularity of agroforestry in the tropics is a dwindling wood supply due to deforestation [6]. This has encouraged farmers to meet their wood demands by integrating trees into their agricultural systems.

Agroforestry is less popular in temperate climates, partly because industrial agricultural practices are more achievable by the developed countries that make up these areas. Tree cover on agricultural lands is described as "moderate" in West and Central Europe, but elsewhere in temperate climates tree cover is typically less than 10% [29]. However, agroforestry does have a small but significant foothold in many temperate regions. In the United States, for instance, agroforestry traces its roots back to the Great Depression of the 1930's, when tree windbreaks were established to combat the rampant soil erosion of the Dust Bowl [30]. Today, agroforestry has become more popular in the U.S. for managing rangelands and protecting water from the harmful agricultural runoff with riparian buffers[31].

2.1.2 Common Systems and Characteristics

By its nature, agroforestry is susceptible to a varied set of definitions that may not accurately portray its purpose. It is more than just planting trees on farmland. P.K.R. Nair[32] stressed two characteristics to define agroforestry. First is the deliberate growing of woody perennials on the same unit of land as agricultural crops and/or animals either simultaneously or sequentially [32]. Second, agroforestry requires a significant interaction between woody and non-woody species within a system [32]. Furthermore, agroforestry systems typically produce more than one marketable product. Trees themselves can offer a number of economically viable products including firewood, lumber, fruits, and nuts.

These requirements are not difficult to meet, and thus there is a range of methods practiced around the world that could be defined as agroforestry. Indeed part of its attractiveness comes from its room for creativity and ingenuity for landowners to utilize such interactions. As a result, classification of agroforestry systems is an ongoing and relatively informal subject. However, some systems have been well established and are considered important pillars to agroforestry as an agricultural philosophy. These common systems are described in the table below.

Table 1 . Summary and description of common global agroforestry systems[32,33].

Practice	Description
Alley Cropping	Single or multiple tree rows grown at a predetermined spacing with agricultural or horticultural crops planted between to grow simultaneously
Forest Farming (or multi-strata farming)	Culltivation of non-timber forest products under the protection of forest canopy that is altered to allow correct shade
Riparian Buffers	Strips of vegetation (including trees, grasses, and shrubs) that are grown alongside waterways to capture sediment, nutrients, and other agricultural runoff with their roots and prevent them from polluting water systems
Silvopasture	Combining trees, livestock, and forage plants together to provide better microclimatic condtitions for livestock while producing timber
Windbreaks	Linear plantings of trees designed to benefit soil and water conservation while enhancing crop production
Shifting Cultivation (swidden agriculture)	Agricultural systems whre land is cleared of natural vegetation, cropped for a few years, and then left untended for several years to regenerate natural vegetation and soil quality
Taungya	Growing agricultural crops between rows in forest plantations during early years of growth. Usually done as an agreement between a government agency that owns the plantation and local communities who farm between tree rows.
Homegardens	An assemblage of plants including trees, shrubs, vines, and herbaceous species that are grown adjacently and managed for household consumption

Many landowners, particularly in tropical regions, have been practicing the integration of trees into agriculture for years without having a name for it [6]. These smallholder farmers have long recognized the beneficial interactions between different species. More research is needed, however, to establish and understand the mechanisms behind such benefits.

The set of practices associated with agroforestry have potential to create sustainable nutrient cycles, restore soil health, and establish a suite of other ecosystem services while remaining economically viable. The remainder of this section will cover the many issues facing global food and fiber systems, particularly in terms of soil health, followed by an argument on how agroforestry can effectively address them.

2.2 Current Obstacles Facing Food and Fiber Systems

Despite the advancements of the modern era, agriculture is still an important source of income for many around the world and often the only source of food for those in developing nations. Although considerable strides have been made towards ending world hunger in recent years, one in eight people still suffer from undernourishment, primarily in developing countries [34]. Moreover, roughly 1.4 billion people are still living off of less than US $1.25 per day, of which 70% live in the rural countryside and depend on farming for at least part, if not all, of their livelihoods [35]. Growing populations, land degradation, environmental pollution from fertilizer use, and climate change are all threatening the ability of present food systems to meet global demands. Additionally, planted forests are currently and will continue to play a large part in sustaining the global wood supply for future generations, which is why it is so important to address the issues facing them today. Plantations are largely made up of exotic species that are intensively mono-cropped and have a range of effects on the land and people involved in them. These forests are usually spaced regularly, at an even age, and lack most of the principal characteristics of natural forests [36]. As a result, they generally do not have very high of resiliency to environmental, or even social, challenges and are often unsustainable to the land they occupy. The next few sections will detail a few of the more salient concerns facing agriculture and plantation forestry in relation to sustainable production.

2.2.1 Population Pressures, Poverty, and Land Limitations

The global population is expected to increase to 9.6 billion people by 2050 with much of the growth occurring in developing nations [37]. As a result, agricultural production must increase by 70-100% over the next few decades to meet the food demands of future populations [38]. The FAO estimates that there is approximately 4.4 billion hectares on Earth that are theoretically suitable for cropping, of which only 1.6 billion are currently cultivated [1]. While cultivated land area grew by 12 percent over the last fifty years, much of it was at the expense of forests and other environmentally important ecosystems [1]. Furthermore, the practicality of converting further land for agricultural uses poses an issue. Most of the land has low food potential due to low fertility and ecological fragility, as well as limited profitability due to a lack of existing infrastructure. Moreover, the cost of damaging or completely removing the ecosystem services provided by the existing ecology in these areas is incalculable in the context of advanced climate change.

Forest plantations also present a growing source of land competition. Global wood demand has been positively correlated to the population and will continue to do so in the future. Although the range of actual forest planation area is disputable (between 3-7% of global forest area), it undisputedly accounted for 35% of global wood supply in 2000 [39]. This number is projected to grow consistently into the first half of the 21st century, to 46% by 2044 [39]. Some experts believe that with the optimal environment for growth, plantations could contribute two-thirds of the worlds roundwood supply in the near future [36]. This growth, when combined with the expected agricultural and urban growth will require new innovations for integrating multi-faceted land use practices into global food and fiber systems [40].

The pressures of growing food and fiber demands and limited land availability ultimately mean that much of the increased production will have to come from currently cultivated land. It is estimated that roughly 80% of the future food demand will have to be met through intensified management of existing global crop systems [1]. Historically, the need for increased production from limited land has been met with intensive, high-input management methods, including extensive fertilizer and pesticide use. While this form of management is capable of improving yields, it can also bring about a litany of negative side effects, including increased non-point source pollution and alteration of soil chemistry, which can lead to degraded soil and limited production in the long-term [2]. Moreover, in developing countries where agriculture is often the major source of income, access to pesticides and fertilizers can be very limited. In Africa, for instance, commercial fertilizers can be as much as 2-6 times the cost compared to Europe or Asia and there affordability has had drastic effects on food production[27]. In Zambia, studies have shown that up 90% of maize-dependent families experience hunger for at least three months of the year, getting much worse during periods of drought [23]. In areas like this, increasing yields through higher inputs is simply not an option.

Regardless of location, not having the forethought to engage in sustainable and environmentally friendly agricultural and forestry practices and instead intensifying global inputs to production systems would likely trade one problem for another. In order to truly address the problems of land competition and growing demands, system management must focus on creating healthy soils with sustainable nutrient cycling systems that do not require large amounts of synthetic inputs. Taking advantage of biological nitrogen fixation (BNF), for instance, can provide access to what is an essentially unlimited source of atmospheric nitrogen for plant use. This can be done with mixed systems approaches that incorporate leguminous species. Practices such as these are common to agroforestry systems, and utilize ecological concepts to both increase and diversify production from limited land. For example, a study on African farmers found that improved yields from incorporating nitrogen-fixing trees reduced household hunger periods by 2-3 months [41]. Furthermore, a meta-analysis of studies done on African maize systems found that the presence of nitrogen-fixing trees systems doubled productivity when compared to unfertilized fields [42].

Agroforestry as a whole offers a low-cost method of improving yields from limited land and providing food security, particularly for Africa and other developing areas. In a survey, roughly 43% of farmers who had engaged in agroforestry in developing African countries witnessed a notable improvement in productivity [43]. A study from Western Kenya proved that the presence of trees on farms provided a safe and stable source of fuel wood for both energy and income [43]. This was particularly beneficial to Kenyan women, who are often responsible for obtaining fuelwood. Furthermore, in Rwanda it was found that one hectare of agroforestry farmland was capable of producing 140% of the fuelwood required by a six-person family [43]. The excess fuelwood could then serve as source of additional income [44]. A study in Mozambique found that the transition to agroforestry systems helped boost overall household income, largely due to decreased labor requirements [45]. Clearly, agroforestry has enormous potential for catalyzing sustainable development in rural areas, improve economies in the long-term, alleviate poverty, and ensure resiliency to climate change despite growing populations and land limitations [46,47]. An increase of production by 1% corresponds to a 1% drop in the number of people living on a less than $1(US) a day [23]. Increased income and food security have cascading effects that improve the livelihoods of the rural poor. One such effect is improved health conditions. Agroforestry has been linked either directly or indirectly to health benefits in a number of ways. The ecosystem services provided by such systems improve the quality of air, soil, and water, all of which can directly influence the health of individuals who depend on them [6]. Agroforestry has even been linked to improvements in the fight against HIV/AIDS [27]. These practices can generate additional income, improve nutrition, reduced labor demands, and stabilize communities [48]. The foundation of these benefits to human livelihoods lies in the ability of agroforestry practices to create production systems that are sustainable on multiple fronts. In the developing areas discussed above, perhaps the most important front to production is enhancing nutrient cycles. This research will stress the importance of biological nitrogen fixation as source of nutrients for important food and fiber crops, the results of which can have cascading social benefits.

2.2.2 Effects of Agricultural Fertilizer Use

A common reaction to higher food and fiber demand from limited land is using more synthetic fertilizers. However, the use of fertilizers comes at a significant cost to the surrounding ecosystems, particularly riparian systems, and they often do not effectively address soil nutrition deficiencies. Global fertilizer consumption (measured in N, P_2O_5, and K_2O) reached nearly 187 million metric tons in 2014 and is expected to increased up to over 200 million by 2018 [49]. Prior to the mid-twentieth century, the primary source of fertilizer was livestock manure. The discovery of the Haber-Bosch process for converting nitrous gas into ammonium and improved methods of extracting rock phosphorous brought chemical fertilizers onto the forefront of agricultural technologies. They were a major part of the green revolution's advancements in improving yields and have been critical to diminishing global hunger. However, the improvements were not without their drawbacks, such as widespread soil chemical alteration and degraded water quality [18]. Extensive research has shown that the use of agrochemicals can be extremely harmful to the environment both on-site and off-site [50]. A major negative effect of fertilizer use at the site of application is a growing nutrient imbalance in the soil. For instance, the heavy application of nitrogenous fertilizers paired with minimal potassium applications has thrown soil nutrient balance off in many areas [51]. Plant nutrient uptake and health requires not only a sufficient amount of individual nutrients but also an appropriate balance between nutrients. Thus, fertilizer practices have ironically resulted in extremely low use-efficiencies of the nutrients added to the system [51].

The on-site nutrient imbalance created by fertilizer use has disastrous potential for future crop yields and is a major concern for sustainable agricultural production. However, some of the worst effects of fertilizer use occur outside of the fields on which they are applied. Synthetic nitrogen and rock phosphates have become increasingly less efficient for fertilizing crops and more environmentally harmful [52,53]. It is estimated that only between 25-50% of nitrogen applied to croplands is subsequently taken up by crops [51,53]. Only 20% of phosphorous fertilizer is estimated to be utilized by plants within the year that it is applied [53]. Since there is no process for creating synthetic phosphorous, this level of inefficiency is particularly cost-ineffective when considering global prices are rising and supplies are diminishing. Additionally, the inefficient use of the applied nitrogenous and phosphate fertilizer increases the movement of nutrients into nearby bodies of water and water tables. The abundance of nutrients in the water leads to the production of algae blooms, the decomposition of which reduces oxygen levels in the water, making them toxic to living creatures. This process is called eutrophication, and currently affects over 60% of the US coastal rivers and bays, and is responsible for the “dead zone” where the Mississippi River feeds into the Gulf of Mexico [54]. Agricultural fertilizers serve as a source of non-point pollution that is extremely difficult to prevent from reaching bodies of water [55]. This form of pollution is a widespread problem in much of East and Southeast Asia, Europe, North America, and Central Asia [1]. Nitrogenous fertilizers are also responsible for emitting nitrous oxide, a lesser known greenhouse gas that has a higher global warming potential than carbon dioxide [56]. A study conducted on the carbon footprint of the Tropicana orange juice company found that as much as 58% of their emissions were a direct result of the application of nitrogen fertilizers [50]. The Intergovernmental Panel on Climate Change (IPCC) reported that a substantial amount of the global nitrous oxide production is tied to fertilizer use, representing another negative externality from intensive agriculture [57].

Agroforestry is capable of alleviating fertilizer requirements as well as their pollution pressures on water sources. Multiple studies have shown the potential for leguminous species to be utilized as a synthetic nitrogen replacement [20–22], which when combined with other agroforestry practices can enhance nutrient cycling on multiple fronts. This will be discussed in greater detail in sections 2.3 and 2.4. Moreover, riparian buffers (a common agroforestry practice) present a prime opportunity to protect waterways and wetlands from agrochemical runoff. Due to the widespread runoff issues in the modern agricultural systems, primarily in temperate systems, riparian buffers have grown in popularity in both the United States and in Europe. The deep roots of trees and shrubs in these agroforestry systems are capable of capturing nutrient and soil runoff before it enters rivers, streams, and other bodies of water during rain events. In Poland, for instance, a series of shelter belts and buffers has significantly reduced water pollution from nitrate runoffs and improved water cycling in the region [26]. This has negated considerable potential damage from continued fertilizer use in the area. The ability of riparian buffers to reduce water pollution is well documented. However, the studies presented in Chapters 3 and 4 emphasize the ability of mixed systems to not only protect marginal lands (such as riparian borders), but also to improve their soil health and even make them productive.

2.2.3 Biodiversity, Pests, and Disease

It is a generally held belief that monocultures are more susceptible to damage from pests and disease when compared with natural ecosystems. This is particularly true for plantation forests, which often do not receive the same pesticide inputs as agricultural systems [58]. This susceptibility is largely thought to be due to the extreme lack of diversity in most plantations, which typically only grow one, often non-native, species. In diverse systems, the variety of plants and animals that occupy an ecosystem result in a series of checks that can prevent any one species from becoming dominant, including pests [59]. An inordinately large number of instances regarding devastating pest-related losses have been recorded monocultures when compared to more diverse systems, indicating that biodiversity can reduce pest-related damage [59]. Although this concept is largely favored, it is difficult to research. However, studies have isolated a number of factors that explain the prevalence of pest and disease in plantations [36,39,58,60]:

1. Monoculture stands offer a large, uninhibited food source to any pest that specifically preys on the species. This allows for extreme population bursts.
2. The proximity of trees in plantations allows for contact of branches and roots allow rapid spread of infections from both insects and disease
3. Lack of genetic variation within single species plantations, particularly when planted by cuttings or in coppicing rotations, reduces the potential for genetic resistance to pests
4. Single species growing on the same site over long periods of time allows for pests and disease populations to build up.
5. Use of exotic species in areas means they do not have the populations of natural controlling agents that help reduce pest populations
6. Poor site selection can lead to tree stress, leaving them susceptible to attack

Some studies have particularly stressed the need for genetic diversity within single species plantations and suggested it is critical to stand health [36]. This notion does not bode well for many short-rotation tree plantations, which are typically planted from cuttings and coppiced for several rotations, resulting in extremely low genetic diversity. This form of plantation establishment is typical to short-rotation woody crops (SRWC's), which are already very common in Asia and growing in popularity elsewhere for producing biofuels. Moreover, warming temperatures will likely provide more suitable conditions for pest population growth, especially in areas where pests are usually suppressed by seasonally cold temperatures [36].

In summation, monoculture forest plantations and agriculture systems lack the natural constraints that keep pest and pathogen populations in check and provide suitable conditions for quick and devastating spread of infection [36]. Frequent monitoring and applications of pesticides are the most common tools against pests but may not represent a long-term solution. Some have turned to integrated pest management (IPM) methods that seek to take advantage of the natural constraints on pests that are missing in plantations [59]. Agroforestry practices, however, offer a higher level of plant diversity that can lead to a greater number of predators and reduced plant stress and susceptibility. By its nature, agroforestry combines multiple species of crops, trees, and animals to operate in niche complementarity [6]. Agroforestry realizes that interspecific competition can often be less harmful than the intraspecific competition for resources found in monocultures. Perhaps the most exciting benefit of biodiversity in agroforestry is its potential to offer a low-cost form of pest control. As more species are integrated into a production system, habitat complexity increases and provides the conditions necessary to increase the presence of natural predators, which in turn reduce pest[61]. As a result, the use of pesticides is likely to be far more infrequent when compared to traditional agriculture. This idea has been solidified by multiple studies that have shown natural enemy populations to be higher and damage from pests to be lower in agroforestry systems [62]. In a study on coffee plantations, for instance, it was found that coffee agroforestry bolstered pest-control services [63]. In fact, these effects were seen even in the presence of an adjacent forest, showing the importance of perennial species to natural enemy habitat. Perennial biodiversity has also been shown to reduce the presence of weeds by increasing shade and improving soil quality, which allows crops to out-compete advantageous weed species [62].

The systems proposed in these studies are inherently biodiverse and can result in higher system resiliency. In fact, a similar study on pecan-cover crop alley cropping system in the United States found that the presence of the cover crop improved predacious species populations, such as lady beetles, which provided pest control functions for the trees [64]. The economic benefits linked to biodiversity have been estimated at trillions of dollars each year [65]. It has direct economic, cultural, and aesthetic benefits as well as a number of indirect benefits towards other ecosystem services. Since biodiversity is intrinsic to agroforestry, the benefits of the practices are bolstered.

2.2.4 Future Effects of Climate Change and Possible Feedbacks

The struggle to feed future populations on existing lands suffering from degradation and intensive management is further complicated by global climate change. By now there is little disagreement among experts that it has become a serious threat to future generations. When it comes to agriculture, a changing climate can have an array of effects on yields and sustainable production depending largely upon the location of the system at both a global and local scale. This makes predicting future conditions and subsequent impacts on agriculture extremely difficult.

Two key components of climate change are increased atmospheric CO_2 concentrations and warmer global climates, both of which to some degree may benefit terrestrial plant ecosystems, including agricultural crops. In fact, it has been suggested that a moderate warming of up to 3°C could have overall positive effects on agricultural yields in the first half of the 21st century, provided adequate moisture levels [5]. It is likely, however, that any positive effects of global warming on agriculture in the first half of the century will be vastly outweighed by the second half as temperatures would continue to warm above 3°C [5]. The other predicted effects of climate change also have potential to negate any positive effects, such as changes in precipitation patterns and increases in extreme weather events.

What is perhaps more important is that the effects of climate change will not be homogenous across all nations. While warmer temperatures may extend growing seasons and benefit crops in temperate regions like the United States, Canada, Scandinavia, and Russia, they will likely have disastrous effects for the tropics [1,5]. The disproportionate negative effects of climate change on tropical developing nations is troubling, as these countries have high existing poverty rates and dependence on agriculture. The aggregation of all climate effects is likely to increase the number of undernourished people by 10-150 million, most of which are expected to come from these areas [1]. Yields of maize, for instance, are expected to decrease 10% by 2055 in Africa and Latin America on smallholder farms as a result of climate change [66]. Many farm-dependent families in these countries already struggle to survive and are extremely vulnerable due to small farm sizes, limited access to agricultural technology, and other non-climate stressors [3]. Moreover, the presence of disease, conflicts concerning natural resource scarcity, or ethnic strife may be intensified by migration away from climate-vulnerable areas and have further impacts on the rural poor [3,4].

The biophysical and biogeochemical effects of climate change on agricultural ecosystems have tremendous potential to result in positive feedback cycles, primarily through increased emissions. Agricultural activities currently contribute between 5-6 billion tons of GHG emissions annually (CO_2 equivalent) and together with deforestation account for a third of total human emissions [1]. It is responsible for 25%, 50%, and 75% of the global CO_2, N_2O, and methane emissions, respectively [1]. Although it is uncertain how these emission levels will change in the coming decades, the rising demand for food implies a likely increase. In fact, it is estimated that they could increase by as much as 40% [67], which would likely go on to exacerbate the effects of climate change, resulting in a positive feedback loop.

Soil degradation as a result of intensified land management can also result in higher respiration rates of carbon dioxide through depletion of soil organic carbon [28]. Land conversion from non-agricultural to agricultural land results in emissions of carbon lost in land clearing, reduced carbon storage ability, and increased emissions on that land over time [67]. Bajželj and Richards (2014) identified three major pathways for agriculture-climate change positive feedback loops regarding land conversion to exist:

1. Warming temperatures and other factors result in reduced yields, which incurs greater land conversion for agricultural purposes in order to supply global food demand
2. As existing cropland becomes less suitable for agricultural production farmers will seek out new land
3. Alterations in water supply and demand for irrigated agriculture result in cropland expansion to make up for reduced yields.

The above mentioned feedback loops will be likely to hit the poverty-stricken tropic nations the hardest. Growing populations in these areas will mean greater food demand in the future, but reduced yields from warming temperatures and other climate change effects will make it difficult to supply on their own. As a result, these nations would likely need to begin importing massive amounts of food. Doing so would require money they simply do not have. Thus, the pressure on the land would increase, resulting in a vicious cycle of hunger and environmental degradation. These potentially disastrous effects have inspired multiple governments, non-profits, and even private industries to seek out ways to mitigate climate change. Carbon sequestration, or the ability of plants to capture and store carbon from the atmosphere, is perhaps the most important tool we have for reducing atmospheric carbon and minimizing these negative effects in the long run [68]. As a result, much attention has been paid to the potential for mixed tree-cropping systems to sequester large amounts of carbon while producing other products, such as food. The Intergovernmental Panel on Climate Change has stated that agroforestry has the highest potential for carbon sequestration among land management regimes [6]. It offers particularly high potential in that it can create important synergies between mitigation and adaptation activities [69].

The importance of agroforestry to carbon-based climate change mitigation comes from variety of factors. Mbow et al. (2014) noted that its enormous potential comes from the combination of the ability of trees to sequester carbon in their biomass and the vast amount of land area that is suitable for agroforestry practices, including degraded land. This is true even in the modern agriculture systems of temperate regions. Recent research has estimated that the United States, for instance, could offset as much as a third of its carbon emissions by engaging in practices such as silvopasture and alley cropping on even a small portion of its agricultural land base [64]. This could sequester over 500 Tg of carbon annually. Global estimates suggest that over a 50-year period total carbon sequestration could range between 1.1-2.2 Pg of carbon annually, although this is based on limited information regarding total agroforestry land area [70]. Additionally, it can reduce extraction and deforestation pressures on existing natural forests [6]. This is particularly relevant to tropical regions.

Since carbon is stored in the biomass of trees for a limited time, there has been debate regarding the non-permanence of this carbon sink and its legitimacy as a climate change mitigation technique. However, capturing and storing atmospheric carbon for even a short period of time can be beneficial to mitigation, particularly if the biomass from these trees are turned into long-lasting wood products or burned to offset the use of fossil fuels. Additionally, a substantial effort has been put forth to understand the role of soil organic carbon (SOC) as an atmospheric carbon sink in both natural forests and agroforestry regimes. As it turns out, research has found that these systems are also capable of improving SOC pools. It is estimated that as much as 30-300 Mg of carbon per hectare can be stored in one meter of soil on which agroforestry practices are utilized [25]. This also meets the requirements of 10-15 g/kg of carbon described by Osman (2014) to feasibly restore soil quality on degraded lands. Additionally, enhanced nutrient cycling in these systems can result in greater productivity of both above and belowground biomass. Lastly, the generally low levels of soil disturbance common in agroforestry is likely to promote the stabilization of SOC for long term storage [25].

In addition, to the enormous carbon sequestration potential of agroforestry, the lack of required agricultural inputs makes them even more cohesive to climate change mitigation. Perhaps the most important GHG emitted by agricultural activities is nitrous oxide (N_2O). Despite its relatively minor abundance in the atmosphere compared to carbon dioxide, its warming potential makes it an emission of great concern. A major source of nitrous oxide emissions are soil microorganisms, which create N_2O gas during nitrification and denitrification. Large inputs of synthetic nitrogenous fertilizers common in modern agricultural systems lead to high emissions of nitrous oxide. Additionally, frequent tilling can also lead to higher biological activity and emissions [71]. Agroforestry not only reduces the need for fertilizer inputs but inherently involves minimal tilling activities in order to protect perennial tree roots, thereby mitigating nitrous oxide emissions.

In summation, there is a very close link between soil degradation, climate change, and poverty [72]. Agroforestry offers a pathway to not only mitigate climate change at the global scale but also to create locally adapted systems that are resilient to its ongoing effects. The systems designed in this research can specifically reduce nitrous oxide emissions, which will be discussed in later sections.

2.2.5 Land and Soil Degradation

Since expanding global agricultural area would be neither sufficient nor sustainable in meeting future food and fiber demands, enhancing yields on existing croplands is the most promising option. Over the last 50 years, the world's agricultural production has grown between 2.5 and 3 times while cultivated area has only increased by 12 percent [1]. Unfortunately, present widespread land degradation as a result of intensive agricultural practices has made improving yields on existing croplands risky.

Global estimates suggest that roughly 38% of the global land area could be considered degraded [2]. Soil resources are critical to reaching stable food production. The degradation of soil by improper agricultural and forestry practices can be tied to the failure of many past civilizations [73]. Recent developments in agriculture have made intensive and unsustainable land management more common. These developments include increased availability of synthetic fertilizers, herbicides, and pesticides, improvements in tillage, planting and harvesting equipment, and increased use of subsurface drainage and irrigation [28]. Although these developments have improved global crop yields and reduced hunger, they ultimately have led to widespread land degradation. The responses of soil health to these practices have been particularly overlooked in recent decades and little concern has been paid to long-term sustainability.

Land degradation usually refers to damage to a set of ecosystem services provided by a particular area, which are very often a direct result of soil degradation. In comparison, soil degradation refers specifically to the processes by which human actions cause a decline in soil quality and as a result it is less suited for achieving a particular goal, such as crop production [38]. Lal (2015) describes four types of soil degradation: physical, chemical, biological, and ecological.

Physical degradation typically refers to the destruction of soil structure, which can lead to compaction and increased erosion from wind and water [2,28]. This greatly compromises the ability of soil to support long-term agriculture. Soil compaction is the physical consolidation of soil through the application of a force which disrupts soil structure, reduces porosity, and limits movement of water and air within the soil. Compaction results in stunted plant growth, malformation of roots, formation of large soil clods, and often standing pools of water in agricultural fields [2]. Reported yield reductions due to compaction have been as high as 90% in areas of West Africa and 50% in Europe and North America [74,75]. Since it is directly tied to frequent use of heavy agricultural machinery, compaction is more likely to impact cropping systems in developed nations. On farm losses from compaction in the United States, for instance, were estimated at $1.2 billion annually [2].

Erosion is the process by which materials from earth surfaces (such as soil) are forcefully detached and transported elsewhere by a variety of forces, most notably wind and water. Currently erosion likely poses the greatest threat to the physical health of agricultural soils worldwide, mostly due to improper soil management. Land management practices that contribute to erosion include continuous mono-cropping cycles, cultivation on slopes, and lack of vegetation cover between growing seasons. While certain levels of erosion are natural and acceptable, many agricultural areas have levels of erosion well above normal levels. On-site effects of erosion are numerous and devastating. They include exposure of roots, deformation of terrain, decline in soil quality, and reduced capability of ecosystem functions. Loss of soil organic matter and nutrients is a significant contributor to soil quality decline. It is estimated that a 10mm loss of top soil can mean a loss of as much as 350 kg N, 90 kg of P, and 1000 kg of K per hectare [76]. As a result of this and the other effects of erosion on agricultural soils, yield reductions of 30-90% have been recorded in West Africa and 20-40% in the US Midwest [2]. Total land area that is prone to erosion is estimated at between 1.65 and 1.96 billion hectares, of which 50% is severely eroded and likely to be abandoned [2,77]. This represents roughly 15% of Earth's total land area.

Chemical soil degradation is characterized primarily by nutrient depletion, leaching, and acidification [28]. Other sources include contamination with industrial wastes or by-products and reduced cation exchange capacity (CEC). Estimates from the early 1990's suggest the total chemically degraded land area in the world is approximately 240 million hectares [2]. Of all the forms of chemical soil degradation, nutrient depletion and leaching have perhaps the largest implications for future food production. It is the process by which soil nutrient stocks gradually shrink as result of nutrient mining, primarily from agricultural activities [51]. The losses from over-cultivation, soil erosion, and leaching combined with the lack of nutrient replenishment result in severely degraded soils. Although leaching happens even under natural vegetation, agricultural activities greatly exasperate leaching losses [78]

Globally, only half of the nutrients removed by crop production are replaced by fertilizer or other nutrient addition process [8]. This results in an average of 50 kg of nutrients lost per hectare on an annual basis [2]. Nutrient depletion affects 136 million hectares worldwide, 97% of which occurs on developing and least developed countries [2,51]. This is extremely true in Sub-Saharan Africa, one of the few locations where yields have been unable to substantially increase over the last century of agricultural improvements. In countries like Rwanda, Tanzania, Mozambique and Niger, nutrient depletion has been responsible for losses that make up as much as 25% of their Agricultural Gross Domestic Product [79]. For the sub-continent as a whole, nutrient depletion has reduced AGPD by 7% for a total value of over 4 billion US dollars, an amount that greatly exceeds the external assistance received for agricultural development from 1990-2000 [79]. The continued nutrient depletion in these regions has damaged socio-economic welfare and environmental quality alongside crop yields [51]. Worldwide, deficits in NPK (nitrogen, phosphorous, potassium) resulted in a 27% reduction in yields on average [51]. Although the majority of severe chemical soil degradation from nutrient depletion obviously occurs in developing countries, at the turn of the last century there was no country that was not affected by nutrient problems [51].

Although to a much lesser degree, acidification of soil is another contributor to chemical soil degradation that is exasperated by agricultural activities [2]. Soil acidity refers to the concentration of hydrogen (H^+) and hydroxide (OH^-) ions in a soil solution and is measured as pH. A pH higher than 7 is considered basic (higher concentration of hydroxide ions) and a pH lower than 7 is acidic (higher concentration of hydrogen ions). The solubility of micronutrients is greatly affected by the acidity of soil, making it an important trait to consider for agricultural purposes. A slightly acidic soil of around 6.5 is optimum for most terrestrial plant species [2]. Agricultural practices can accelerate the acidification of soil as a result of nitrogenous fertilizer applications, leaching of nitrates, removal of produce, and to some extent, the build-up of organic matter [80]. The additions of ammonia-based nitrogenous fertilizers are particularly good at acidifying soil. The chemical process that converts ammonium to nitrate in the soil acidifies soil with bi-products. Under normal conditions, plants discharge an alkaline substance as they uptake the nitrogen compounds and the soil remains relatively balanced [2]. However, when nitrate is leached from the soil, which increases in the presence of agricultural activities, the balance of acidic and alkaline additions is broken in the favor of acidification [2]. There is little information regarding the direct effects of soil acidification on crop yields, but it is well known that extremely acid soils (below a pH of 4.8) are toxic and can lower production and even kill plants that are not adapted to them [81]. These soils in general have reduced nutrient recycling, particularly in terms of nitrogen. The nitrogen cycle depends on the activity of soil microbes which are negatively affected by soil acidity. The *Rhizobium* bacteria that are a responsible for biologically fixed nitrogen in legumes are particularly affected by altered reduced soil pH [81].

An important part of soil quality is sustaining biotic habitat. Soil biota are responsible for numerous biological, biogeochemical, and physical processes on which soil quality heavily depends. These processes include retention and delivery of nutrients to plants, maintenance of soil structure, pest and pathogen control, bioremediation of pollutants, and erosion control [24]. The positive link between soil biodiversity and the provision of terrestrial ecosystem services is well documented [82]. Of the three forms of soil degradation discussed thus far (physical, chemical, and biological), biological soil deficiencies are the most complex and suffer from large knowledge gaps [53]. The existing research suggests, however, that it is extremely important to sustainable agriculture and soil management. The estimated economic benefit of the ecosystem services it provides is estimated to be 1.5 quadrillion U.S. dollars globally [83].

Microbial populations are perhaps most critical to soil biological health and agricultural production as they are extremely important in nutrient provision for crops. Because of their ability to catalyze redox reactions, soil microbial organisms are critical mediators of the cycling of carbon, nitrogen, and other nutrients [53]. Unfortunately, modern industrial agriculture primarily focuses on chemical manipulation with fertilizers as opposed to improving soil biotic health [53]. Moreover, agricultural practices such as frequent tillage often have a negative impact on soil biota and the ecosystem services they can provide [84]. Tillage disturbs soil habitat, decreases soil structure, and temporarily increases the decomposition rates of organic matter on which microbial populations depend. Soil organic matter (SOM) is an important indicator of biological soil quality. Roughly half of SOM is carbon, and is referred to as soil organic carbon (SOC). Roughly 2% of SOC mass is attributed to soil organisms [53]. Despite the small proportion they make up, they ultimately determine the fate of most organic compounds. The depletion of the SOC pool via frequent tillage also results in diminished pools of plant available nutrients such as N,P, and S [28].

Ecological degradation is more accurately interchangeable with land degradation and refers to the process by which the ecosystem services provided by healthy soil (and other components of land) are diminished by human activities. These services include elemental cycling, water purification, and decline in net primary productivity. What is most alarming from the ecological perspective is the positive feedbacks between soil quality and diminished ecosystem services. For instance, depletion of soil organic matter reduces nutrient cycling, which in turn diminishes plant production of organic matter. Very often it can occur where once the process of soil degradation is started, it can perpetuate itself into a rapid decline or downward spiral [53]. Agriculture contributes to this through practices discussed in the previous sections such as mono-cropping, over-cultivation, frequent tilling, and application of pesticides and fertilizers [85]. Globally, ecosystem services have been reduced by as much as 60% as a result of intensive agriculture and deforestation [86].

Since soil health no longer speaks only in terms of production, it must be approached on multiple fronts. In order to address physical, chemical, biological, and ecological soil degradation effectively, a holistic approach utilizing agroecological concepts is required. Mixed systems promote healthy soil structure, chemistry, and biology through inter-specific relationships and occupation of varying ecological niches, creating highly efficient production systems. Rattan Lal (2015) described several important strategies to improve soil quality and reduce degradation, including (1) preventing losses from erosion, (2) encouraging the accrual of SOC, (3) improving availability of macro and micro-nutrients, (4) increasing soil biodiversity and enhancing microbial processes, and (5) enhancing rhizospheric processes. Through the integration of multiple species and enhancement of their relationships, agroforestry creates cascading positive feedback loops between soil biology, chemistry, and structure that ultimately address all of the aforementioned soil degradation issues.

A of the common trait of agroforestry systems is reduced tillage, largely due to the presence of perennial root systems susceptible to damage from such activities. Low-tillage agriculture has been hailed as a sustainable land management technique. This, when combined with the fact that tree root systems provide added structure, explain why agroforestry systems are remarkably good at reducing losses from soil erosion [43]. Besides erosion control, low-tillage agroforestry can help build organic matter and reduce the risk of root compaction from agricultural equipment [2]. A recent study in India found that across all agroforestry systems there was an average of 1.17 – 1.65-fold increase in organic carbon within a few years of establishment [87]. Soil organic matter and carbon content are extremely important to soil health. It is responsible for improving nutrient cycling efficiency, restoring physical soil quality, and increasing soil moisture, among multiple other benefits [43].

Buildup of organic matter is linked to increased fungal and microbial biomass, which in turn increase carbon use efficiency and soil carbon sequestration [88]. Moreover, soil organic matter has been proven to encourage arbuscular mycorrhizal fungi populations, which enhance plant uptake of phosphorous and water, and strengthen disease resistance [89]. Thus many of the benefits of improved soil organic matter content have to do with their effects on microbial and fungal populations, particularly when it comes to nutrient cycling. Inputs, such as leaf litter from trees, provide microbial communities with the carbon needed for energy. These microbes then go on to promote decomposition of nutrients into soil, nitrogen fixation, uptake of phosphorous, and promotion of plant growth [90]. Much of the nutrients in decaying leaf litter inputs from trees comes from deep layers of the soil that would otherwise be inaccessible to shallow-rooting crops, thereby adding to the overall nutrient budget of a system. Soil biota are even capable of influencing erosion by helping form stable aggregates, improving macroporosity, and reducing runoff [87]. Through constant inputs of organic matter from trees in agroforestry systems, both soil organic carbon and soil biota are significantly improved [91].

In a study in the hill-regions of Nepal, three systems were compared: a traditional monoculture, a fully developed agroforestry system, and a newly established agroforestry system (two years old). The results showed that agroforestry systems improved multiple soil health indicators such as cation exchange capacity, soil pH, organic matter and nitrogen content, and even aluminum content [92]. Data revealed that these indicators improved even in the two year old system, proving that the soil health improvement could be attributed to the differences in land management [92]. Agroforestry practices improve nutrient supply and cycling in a number of ways. The mechanisms we've discussed thus far, such as organic matter build up, access to nutrients deep in the soil by trees, and improved soil biota populations, all result in enhanced nutrient cycling. Moreover, the incorporation of nitrogen-fixing trees, which is very common to agroforestry, provides additional nutrients to the system budget. The transfer of this nitrogen to agricultural or forestry crops can occur in a few different forms, which will be the topic of discussion in following sections. In the tropics, the use of nitrogen fixing species (also known as fertilizer trees) has been critical to improving yields [93]. Low availability of nitrogen and phosphorous are major constraints on degraded land in these areas and utilizing the relationships between legumes, bacteria, and interspecies interactions are integral to restoring soil quality [86]. The ability of agroforestry practices to restore, improve, and maintain soil nutrient cycling will be the subject of the remainder of this review, dealing particularly with nitrogen relationships between species.

2.3 Belowground Species Interactions and Nutrient Cycling in Agroforestry

Agroforestry systems are capable of tremendous impacts on belowground ecosystems and the rhizospheres of individual species. Studies have shown that the addition of even a single tree species into agricultural systems can positively affect soil microbial communities and nutrient cycling [94]. Through various mechanisms, trees are capable of increasing soil carbon, capturing leached nutrients, and improving the physical characteristics of soil [95]. They are also capable of accessing resources in the soil that shallow-rooted agricultural plants are not, which leads to greater overall resource use efficiency [96].

Although trees have considerable potential to positively impact agricultural systems, there is perhaps equal potential for negative interactions. Positive relationships between species (both above and belowground) can be beneficial to both (mutualistic), beneficial to one and neutral to the other (facilitative), or beneficial to one and harmful to the other (parasitic) [96]. Competition refers to a relationships where both species are harmed. While plants can have multiple relationships in regards to different factors (water, nutrients, light, etc.), the emphasis of a relationship label is based on the overall effects on growth and yield of the species involved. Considerable research has concluded the most frequent limitation in agroforestry systems is competition for water [97,98]. However, there have been several instances recorded where hydraulically lifted water from trees can result in higher water use efficiency in soil [9,96,99,100]. As a result, it is critical to understand the varying niches and needs of the species being incorporated into the same space. Fernandez et al (2008), for instance, stresses the need for selecting tree species that are capable of reaching nutrients that adjacent agricultural crops would not be able to access, thereby reducing competition. Moving forward, we will examine in detail the potential effects of alley cropping, a common agroforestry practice, on nutrient cycling and use by various species combinations.

2.3.1 Alley Cropping and Nutrient Cycling

There are several potential mechanisms that trees utilize to alter nutrient cycles in agricultural systems. Perhaps the most important among these include: (1) accessing nutrients in the soil profile unavailable to shallow-rooted plants, (2) adding these nutrients to the soil through leaf litter, (3) adding C and other compounds to the soil through root turnover, (4) serving as catchment for leached nutrients from higher soil profiles, (5) improving microbial communities, and (6) improving soil structure [95,96,98]. Furthermore, nitrogen-fixing species have high potential to improve nitrogen contents in soil through a few different processes, which will be discussed in detail later on. All of these mechanisms can positively affect an agroecosystem. However, without careful planning and a developed understanding of individual species these relationships could lead to competition for resource use and result in lower yields and growth.

Alley cropping is a common agroforestry practice whereby agricultural crops are grown in the space (or "alleys") between organized rows of planted trees spaced at a pre-determined distance (Table 1) [32]. It is a widely common form of integrated agriculture in both temperate and tropical ecosystems and relies heavily on the idea of complimentary root patterns and resource use between species. The ability of multiple species to create beneficial relationships between one another and soil health in this practice has been proven in multiple studies. For instance, a long-term study on a silver maple and corn-soybean alley cropping system found that multiple soil parameters were signicantly improved over the course of the 20-year rotation, including water stable soil aggregates, soil C and N, and enzyme activity [101]. Additionally, alley cropping provides a method for year-round biomass production and results in a higher annual production level per unit area compared to mono-culture systems [10]. This is particularly important for tropical agriculture systems.

Alley cropping is particularly beneficial to soil nutrient cycling in part because of the improved soil microbial habitat it provides. Abundant carbon exudates from tree roots into the soil provide sources of food for microbe populations, which in turn improve microbe-dependent nutrient levels, such as nitrogen [102]. Additionally, a study on *L. leucocephala* hedgerows in Nigeria showed higher levels of larger decomposers like earthworms as well [103]. These changes tend to occur primarily in the higher organic matter layers of the soil, which activates the deposited nutrients from tree leaf litter, making them available to both trees and crops [104]. Provided that trees and row crops are not competing for the same resources, this practice has high potential to improve nutrient cycling in agroecosystems. Tree leaf litter and roots help to improve levels of nutrient-rich soil organic matter and microbial activities to maintain constant nutrient inputs. An alley cropping system studying multiple different potential trees in Nigeria showed that in all systems recycled N, P, K, Ca, and Mg were 12, 9, 11, 35, and 24 times higher than controls, respectively [105]. Its capacity for nutrient cycling makes no-tillage alley cropping an important practice for tropical systems, where fertilizers can be expensive and hard to find [106]. It is important to note that agroforestry and alley cropping in particular provide improved methods for producing not just agricultural crops but also tree products such as fruits, nuts, and woody biomass. Although much of the focus of agroforestry to date has been on the crops grown between alleys with tree-based products generally considered as secondary products, a trend towards equal focus has been seen in recent research. As mentioned before, it is estimated that plantation-grown trees will likely contribute to nearly 50% of the total global roundwood supply by 2050 [107]. Thus, the intensive production of wood fiber on plantations will take on a new importance in the years to come. Fortunately, as has been made clear throughout this report, agroforestry provides a prime opportunity to combine production of livestock, crops, and trees. Considerable studies have been produced regarding alley cropping in recent years with heavy emphasis on tree crops. Part of the reason for this is the ability of this practice to reclaim infertile and marginal lands, thereby restoring soil health and improving total land production for the farmer. In the Midwestern U.S., alley cropping using trees that produce both high quality lumber in the long-term and fruits or nuts in the short-term have gained considerable popularity

[108]. Common trees for this include black walnut, pecan, and chestnuts, all of which produce valuable nuts that can be sold prior to tree harvest for lumber. A few studies have set out to explore how herbaceous species planted between tree alleys could benefit the growth, yield, and nutrient status of the tree crops. For instance, a study on multiple herbaceous ground covers grown between rows black walnut were shown to improve walnut foliar nitrogen content [19]. In a pecan-kura clover alley cropping system in Missouri, soil organic carbon and soil enzyme activities were significantly higher when compared to controls [22]. Water-stable soil aggregation was also dramatically improved by the presence of both species [22].

2.3.2 Biological Nitrogen Fixation and Alley Cropping

Biological nitrogen fixation (BNF) refers to the process by which certain species of plants are able to transform atmospheric nitrogen (N_2 gas) into plant available forms for their own use. This transformation requires the enzyme nitrogenase, which is found only in certain soil microorganisms [11]. To achieve BNF, certain plants create symbiotic relationships with these bacteria by providing them with sugars for energy in exchange for the transformed nitrogen. The most common relationship of this kind found in nature is between legumes and Rhizobium bacteria [11]. Rhizobia infect the root hairs of leguminous plants which then form “nodules” where the bacteria are housed and used for BNF. Figure 1 describes the chemical process by which legumes transform this nitrogen.

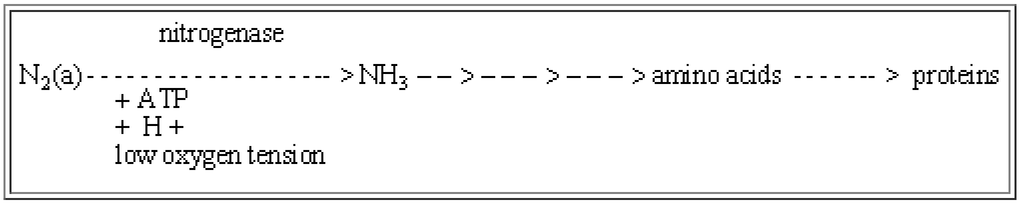


Figure 1. Chemical process for biological nitrogen fixation occurring in the root nodules of legumes[11]

Biological nitrogen fixation is critical to natural and productive soils. In agroforestry, nitrogen fixing trees are widely used in alley cropping systems to benefit associated agricultural crops. This is particularly true in the tropics, where the trees are utilized as wood fuel and for soil restoration [109]. The added carbon inputs through leaf litter in agroforestry systems encourages greater microbiological activity and diversity which synergizes the BNF process [90]. In temperate ecosystems, there are few options in terms of woody leguminous species and herbaceous species capable of BNF are more likely to be utilized. Clovers, vetches, and other herbaceous nitrogen fixers are grown and tilled into fields prior to planting of cash crops in order to improve soil nitrogen levels and yields of future crops [110]. In fact, cover crops have been used for soil conservation since ancient times in societies like Greece, Rome, and China [2]. However, leguminous cover crops have yet to be widely integrated into agroforestry, temperate or tropical. Until recently, little research has been done on the potential for herbaceous legumes to benefit agroforestry systems such as alley cropping. In a study on an alley cropping system of pecan and kura clover, results suggested that the intercropped legume was able to supplement the nitrogen needs of the pecan [22]. A system such as this is capable of improving soil organic matter, structure, and nutrient cycling while producing high quality forage for cattle-production. Furthermore, a study in a tropical maize alley cropping system found that when the maize was sequentially cropped with cowpea, fertilizer additions were no longer required to maintain yields

[105]. Despite the lack of research it is fairly clear that herbaceous cover crops have great potential to substantially agroforestry systems.

2.4 Modes of N-Transfer

Leguminous cover crops are extremely efficient at building soil organic matter which in turn has multiple benefits on nutrient cycling that can result in positive effects on the associated crops [25,28,53]. However, a perhaps more prominent service legumes provide is the ability to transfer biologically fixed nitrogen to other species incapable of BNF themselves. This nutrient relationship can occur through more than one mechanism. Research has identified three pathways for the transfer of nitrogen from legumes to non-nitrogen-fixing plants: (1) indirect transfer via accumulation and decomposition of biomass into the soil for future plant uptake, (2) direct transfer of mineralized nitrogen exudates from living root nodules, and (3) direct transfer via common mycorrhizal networks in the soil [12]. All three of these pathways represent important opportunities to utilize interspecies relationships in agroforestry.

2.4.1 Indirect Transfer

The process of indirect transfer requires a longer time-scale than direct transfer. It is considered indirect primarily because it requires the organic nitrogen from decaying plant material to undergo transformation into plant available forms before it can be received by another plant. An example of this is the common use of cover crops in modern agriculture. In this process, the species is grown for a period and then is destroyed, usually via tilling into the soil, where the organic plant tissue decays and its nutrients are transformed by soil microbes into plant available forms for the subsequently planted cash crop to use. By using legumes capable of BNF for this process, landowners can access a virtually unlimited pool of atmospheric nitrogen to be used for crop production. In agroforestry, indirect transfer often takes form in leaf litter from leguminous trees accruing on the soil where they then follow the same process.

The difficulties of this form of nitrogen transfer are largely due to timing and labor. For instance, using herbaceous legumes in an alley cropping system requires frequent cutting in order to continually return the nutrient rich biomass (known also as "green manure") to the soil. In tropical alley cropping systems with woody legumes, intensive tree trimming is often practiced for the same purpose [32]. Since the biomass must then undergo multiple chemical transformations before it becomes available to plants, inputs require timing in order allow plants to efficiently utilize the added nutrients. Timing, however, is difficult to achieve, as multiple and often immeasurable factors stand in the way of determining the appropriate time to cut. Nevertheless, indirect transfer today remains the major pathway through which nitrogen is passed from nitrogen fixing species to non-nitrogen fixing species. This is largely due to its extremely efficient ability to build soil organic matter which will help to supply nutrients to associated cash crops regardless of timing. As a result, this form of transfer may appear small in the short-term while organic matter is accruing but ultimately result in considerable ongoing transfer over the period of several years [13].

A considerable amount of research has been done on this form of nitrogen transfer. For instance, in a tropical alley cropping system of *G. sepium* and maize it was demonstrated that leaf litter from nitrogen fixing trees contributed as much as 10% of the maize final N content while increasing overall soil health through indirect transfer [12]. Between 3-102 kg N ha^{-1} yr^{-1} can potentially be transferred through the death and decay of leguminous root cells alone [111]. Examples of systems where cover crops were used in alley cropping to benefit tree crops are few and far between, but the results have shown potential. In a study that integrated five leguminous covers into a walnut plantation, it was found that cool-season legumes such as hairy vetch were capable of significantly improving soil nitrate levels and stimulating early walnut growth [19]. In a similar experiment in France, walnut foliar nitrogen content was enhanced when alley cropped with alfalfa and soinfoin [21]. In a study on *Abies frasieri* plantations in Michigan, it was found that trees intercropped with alfalfa combined with low fertilization were equally as productive as conventional systems at the recommended fertilizer rate [112].

The problem with many of these studies, however, is that indirect transfer is rarely isolated from direct transfer. While it is generally accepted that indirect mechanisms are largely responsible for transfer in these systems, it is likely that it is not sole source of nitrogen transfer. In a study on *P. avium* and alley cropped with various leguminous shrubs, direct transfer from root-to-root exchange were separated from transfer via leaf litter. The results revealed that the trees were capable of utilizing between 7.5% and 20% of leaf litter N, depending on the shrub species [14]. More importantly, however, the study indicated that direct nitrogen transfer also represented a substantial N source for the *P. avium* tree, which varied depending on the species paring [14]. As research goes forward, it will be important to make the distinction between modes of transfer in order to fully understand the benefits of species pairings within agroforestry systems. By gaining this better understanding of N transfer and how management may effect it, landowners may be able to apply practices that more efficiently utilize this mechanism, which could reduce labor and inputs.

2.4.2 Direct Transfer

While indirect N transfer requires the death and decay of organic plant material followed by the transformation of nutrients into plant available forms, direct transfer offers pathways for plant-available nitrogenous molecules to pass from the living root system of a nitrogen-fixing plant to an adjacent root system of another plant that is not capable of the fixation proccess [12]. Direct N transfer occurs through two belowground pathways: root exudation and shared mycorrhizal networks.

Root exudation refers to the secretion of compounds from the root systems of living plants. These compounds vary in chemical structure and have multiple impacts on the immediate rhizosphere. They can also be assimilated by other plant roots in the immediate area. The mechanisms behind root exudation remain somewhat poorly understood but existing research has attributed it to three passive pathways: diffusion, ion channels, and vesicle transport (Figure 2)[113].

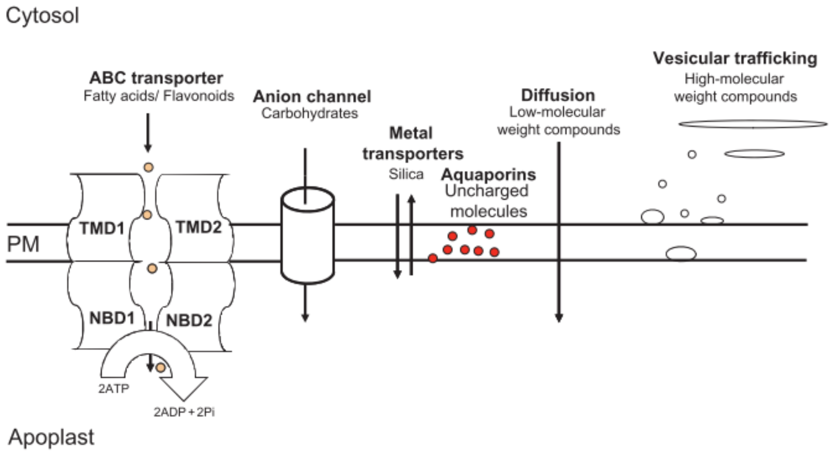


Figure 2. Description of mechanisms behind root exudation of compounds through a plant cell membrane[113]. 'PM' refers to plasma membrane, 'TMD' refers to transmembrane domain, and 'NBD' refers to nucleotide-binding domain.

Most plant species deposit a range of molecules into the rhizosphere through this process, including organic acids, sugars, secondary metabolites, and allelopathic compounds [114]. However, recent research has focused specifically on the potential for nitrogen-rich exudates from leguminous roots to benefit other species. These nitrogenous compounds are typically soluble, low-molecular weight, and primarily occur through diffusion channels [115]. Biologically fixed N is the main source of exuded nitrogen. It's estimated that about 3% of N fixed during this process is secreted from the roots through exudation [116]. Nitrogenous exudates from temperate legumes primarily take form as ammonium and amino acid molecules, both of which are capable of uptake by plants [13,117].

Direct N transfer from root exudates occurs belowground, is relatively constant, and eliminates the need for any additional labor or timing. As a result, exudates from legumes present a potentially significant source of nitrogen to target species that would be slow, but constant throughout the growing season. Multiple studies have shown that exudate-based transfer of nitrogen can provide adjacent crops with a substantial portion of their nitrogen requirements [14–16,118]. White and red clovers (*T. repens* and *T. pratense*), for instance, were found to transfer as much as 7.5 and 3.6 $g^{-1}m^{-2}$ N in one growing season, respectively, to adjacent forage grasses through belowground transfer [17]. Existing research on exudate transfer will be discussed in more detail in Chapter 4.

The second mechanism through which direct transfer can occur is through shared mycorrhizal networks in the soil. Roughly 70-90% of all plant species are known to form symbiotic relationships with mycorrhizal fungi [119]. These fungal species generally show low host specificity, resulting in belowground web of shared fungal connections through which a variety of nutrients can pass, including nitrogen [12]. Mycorrhizal transfer is bidirectional, meaning that nutrients can be passed in both directions between connected species [120]. However, fungal mediated transfer of this kind is driven by source-sink relationships and N transfer primarily occurs from nitrogen-dense species (such as legumes) to less nitrogen-dense species [15]. Less than 10% of N transfer is from non-legumes to legumes, while as much as 80% of transfer occurs in the reverse direction [121].

Multiple studies have confirmed the transfer of fixed N through shared fungal networks. Recorded instances of this include berseem clover (*Trifolium alexandrinum L.*) to maize (*Zea mays L.*) where around 4% of maize-N was derived from this form of transfer [122] and pea (*Pisum sativum L.*) to barley (*Hordeum vulgare L.*) where 15% of donor N was transferred to the barley [123]. Fungal relationships have also been recorded between white clover to perennial ryegrass, soybean to maize, and mung bean to rice [13,124]. Jalonen el al. (2009) distinguished mycelial transfer from exudate transfer in an experiment on *G. sepium* trees and *D. aristatum* grass. This study, similar to other mycorrhizal transfer studies, found that while N transfer through this mechanism is noteworthy, it is often insignificant in comparison to the other forms of N transfer [12,13,15].

3 *Contributions of Trifolium pratense to soil health and hybrid poplar growth in temperate alley cropping systems*

3.1 Abstract

Due to limitations in land availability and a desire to protect remaining natural forests, much of the future global wood demand will be met by intensively managed plantation forests. These plantations will also become particularly important to the biofuel industry in the growing years via short-rotation woody crops (SRWC's), or fast-growing hybrid trees intended solely for biomass production. However, while these plantations are effective, they can pose problems to sustainable land management, primarily regarding nutrient depletion. Agroforestry practices such as alley cropping provide a feasible solution to issues regarding the sustainability of SRWC's. In particular, there is potential for leguminous cover crops to replace the need for fertilizers and enhance multiple soil health parameters in tree plantations while sustaining or improving productivity. Here, I examined the potential for red clover (*Trifolium. pratense)* to benefit the growth and nutrition of a hybrid poplar (*Populus nigra L.×P. maximowiczii* A. Henry ('NM6')) as well as soil carbon and nitrogen in a temperate alley cropping system. This was done using a random-block design with four vegetation treatments: (1) *T. pretense* at a full seeding rate, (2) *T. pretense* at half seeding rate, (3) natural vegetation, and (4) and bare-soil (maintained via pesticide). Additionally, two spacing treatments were applied to poplar tree rows: a narrow spacing (1.82 x 1.82m) and a wide spacing (1.82 x 3.05m). The results showed mixed signs of competition and benefits, with most of the positive effects being seen in the second year. In the wide spacing, the presence of red clover resulted in higher soil nitrate levels at both full and half seeding rates by the second growing season of cover cropping. This resulted

in higher leaf nitrogen concentrations by the end of the second growing season at both seed rates in the wide spacing and at the half seed rate in the narrow spacing. However, the positive effects on poplar and soil nutrition were not reflected in tree growth at either spacing after just two growing seasons with the cover crop application.

3.2 Introduction

Globally, industrial forest plantation area is estimated to be as much as 264 million hectares [36,125]. While the actual area is disputed based on changing definitions of forest plantations and methods of estimation, the critical role that they will play in future global fiber production is certain [126–128]. Today the majority of plantation area is focused on creating roundwood for lumber or pulpwood [36]. However, the establishment of short-rotation woody crops (SRWC's) such as hybrid poplars and willows for the purpose of producing biofuels has gained considerable attention in recent years.

Despite the controversy regarding their benefits to climate change mitigation, biofuels do offer an opportunity to replace fuel requiring a long carbon cycle (coal, natural gas, oil, etc.) with energy that could be carbon neutral in a much shorter term. As, Ceotto and Candillo (2011) put it, ". . . society cannot accept the undesirable effects of biofuels when they are done wrong, but also cannot afford to miss the benefits of biofuels when they are done right" [129]. Since these forest plantations are grown in short cycles (4-10 years) and are extremely low-cost, they are ideal for marginal lands. In fact, it is estimated that over 5.5 billion gallons of renewable fuels could be produced on marginal lands in the U.S. Midwest alone [130]. Additionally, if agroforestry practices were applied to these plantations, they could reduce the need for fertilizer inputs while providing multiple other ecosystem services to landowners, including carbon sequestration, soil restoration, and wildlife habitat, without reducing yields [7,131,132]. Practices such as alley cropping can also provide opportunities to produce crops or livestock between rows of SRWC's, which could increase and diversify landowner income using land that was formerly unproductive. However, doing so without an increase in synthetic nitrogenous fertilizer use is critical, as these emit high levels of GHG's during both production and application [49,129].

Cover crops have significant potential for adding nitrogen to the soil as well as improving overall nutrient cycling [110]. Multiple studies have shown the ability of leguminous cover crops to transfer biologically fixed nitrogen to non-leguminous trees through indirect or direct means [19–22]. Dupraz et al. (1999), for instance, found that nitrogen content of leaves in black walnut were improved by the presence of adjacently planted *Medicago sativa L.* and *Onobrychis sativa L.* (both nitrogen-fixers). In a similar study on a black walnut and *Vicia villosa* Roth. alley crop, resulted in significantly increased soil nitrate and walnut foliar N concentrations [19]. Furthermore, Waring & Snowdon (1985) also found that nitrogen fixed by clover improved growth and nutrition of *Pinus radiata* seedlings after 7 years. In addition to nitrogen transfer, the ability of cover crops to improve soil organic matter means potentially long-term benefits for nutrient cycling. Kremer and Kussman (2011), for instance, found that alley cropping kura clover between rows of pecan increased both soil organic C and enzyme activity after an 8-year rotation. Some studies, however, have shown signs of significant competition between cover crops and trees that ultimately reduced yields [133].

The mixed results from past studies makes it important to establish further research to examine the potential of other species combinations. Furthermore, there is little research regarding the utilization of biological nitrogen fixation to benefit SRWC species. To fill this gap, I investigated the benefits of alley cropping leguminous cover crops into SRWC plantations. The species selected for this research was *Populus nigra L.×P. maximowiczii* A. Henry (‘NM6’), a hybrid poplar with great potential in biofuel production and documented success in loamy soils[131,134,135] and *T. pratense*, a common leguminous cover crop. Specifically, I examined the contributions of *T. pratense* to soil organic carbon, organic nitrogen, and plant-available nitrogen, and the subsequent effects on poplar growth and foliar nitrogen. Due to the high phosphorous requirements for biological nitrogen fixation in legumes [90], foliar phosphorous levels were also monitored to determine if there is competition between trees and cover crops. The success of this study will inform the potential for similar alley cropping systems to improve sustainable production of woody biofuels and other tree products.

3.3 Methods

The field experiment was conducted on a 65 x 78 m plot from 2014 – 2016 for three growing seasons and was located at Sandhill Farms of the Tree Research Center (long. 42°65’N, lat. 84°42’ E) on Michigan State University’s campus in East Lansing, MI. Average summer and winter temperatures are 15.5 and -6.6°C, respectively. Annual average precipitation in the area is 853 mm with rainfall distributed fairly evenly throughout the year. The soil at Sandhill is a Mariette fine sandy loam, which is classified as moderately well drained with high available water capacity and medium surface runoff. The species chosen for this experiment were a hybrid poplar (*Populus nigra L.×P. maximowiczii* A. Henry ('NM6')) and red clover (*Trifolium pretense*), a common legume and temperate cover crop with high nitrogen-fixing potential [17,136].

3.3.1 Experimental Design

To accomplish the study goals I used a 4 x 2 factorial with a random block design to mitigate any potential biases in the field. The field was broken into six blocks stretching north-south, each consisting of four rows of *Populus nigra L.×P. maximowiczii* A. Henry ('NM6'). Blocks alternated between two spacing treatments (1.82 x 1.82 and 1.82 x 3.05 meters) moving from east to west. Each of the blocks were then divided into four plots for vegetation treatments with sizes of 9.14 x 5.49 m and 9.14 x 9.14m for the narrow and wide tree spacing, respectively, each with 24 trees per plot. A buffer space of approximately 5.5 meters was allotted between blocks and plots in which no treatment was applied to avoid effects between treatments. Four vegetation regimes were assigned to plots in both spacing treatments: (1) red clover at a full seeding rate (Treatment RCF), (2) red clover at half seeding rate (Treatment RCH), (3) natural vegetation (Treatment NV), and (4) a bare soil control (Treatment C).

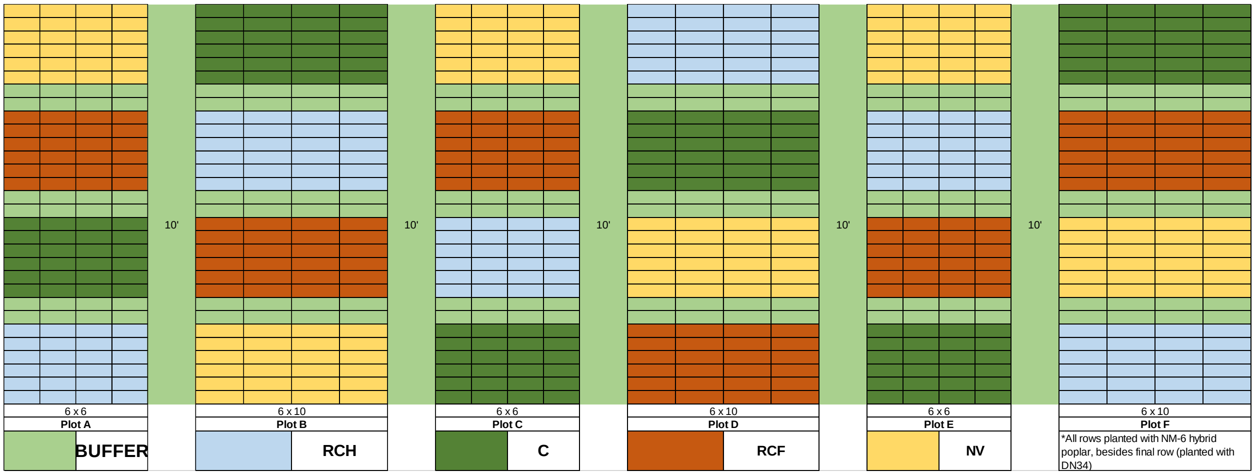


Figure 3. Plot layout of field study at the Tree Research Center in East Lansing. Individual squares correspond to a single tree. Relative width of "blocks" corresponds to narrow and wide spacing treatments and colors indicate vegetation treatments.

3.3.2 Field Methods

In May of 2014, *Populus nigra L.*× *P. maximowiczii* A. Henry ('NM6') cuttings were planted in rows of 30 at the aforementioned spacing treatments. During the first growing season, no cover crop treatments were applied. The field was maintained via regular mowing and periodic herbicide treatments of glyphosate (250ml/L) within two feet of either side of tree rows to allow trees to establish. The experimental field was surrounded by a three-wire electric fence to prevent damage from deer browse.

At the end of the first season soil and leaf samples were taken, as well as the height and diameter of each tree in order to establish baseline data. Soil samples were taken using a stainless steel tubular soil sampler which was placed into the ground roughly at approximately 15cm in depth. The soil was collected, bagged, and stored at -18°C until further analysis. Samples were taken at five random points in each of the six blocks and separated by spacing treatment. Leaves were sampled by taking one healthy leaf from each tree, which were then bagged, weighed, oven-dried, and ground for further analysis. Height of the leading shoot of each tree was taken using a field ruler and diameter of each shoot was taken at approximately 5cm above its connection to the cutting with an electronic caliper. Since hybrid poplars are primarily utilized for the creation of biofuels, diameters were applied to the following equation:

$$Biomass\ (\frac{lbs}{tree}) = Basal\ Area\ (square\ feet)\ x\ 562.089$$

Equation 1. Allometric equation for determining biomass of hybrid poplar[137].

to provide biomass values, which is the most appropriate value for poplar productivity. This particular equation was chosen because it was derived using the same poplar hybrid (NM6) under similar climatic conditions in Escanaba, Michigan [137].

At the beginning of the 2015 season a low mortality rate was observed among the cuttings (less than 1%) and the vegetation treatments were put in place. Prior to seeding, a motorized rotor tiller was used to scarify the earth (5-7.5 cm depth) and break up the sod between rows, allowing for two feet of untilled soil on each side of the trees that would be treated with glyphosate throughout the season to control weeds. This was done in all plots, including the natural vegetation and bare earth treatments, in order to kill existing vegetation and maintain similar starting points within all treatments. After tilling, *T. pratense* seed was applied to the appropriate plots (Figure 3) via a hand-operated seed distributor in April of 2015. Red clover was seeded at 13.5 kg/ha for the full rate and 6.73 kg/ha for the half rate. Seed for treatments was sourced from LaCrosse Seed of Lansing, Michigan. The natural vegetation treatment was thereafter allowed to grow uninhibited (besides routine mowing), while the bare-earth treatment was treated every six weeks with glyphosate (7.8 mL/L) to remove vegetation.

Starting in June of 2015, after six weeks to allow the cover crop treatments to effectively establish, the alley cropped areas were routinely mowed every 21 days in order to regularly return the biomass to the soil. Prior to each mowing, samples of the biomass from *T. pratense* (RCF and RCH) and natural vegetation (NV) treatments were collected to be analyzed for N and P concentrations as well as total weight to determine total nutrient additions throughout the season. Samples of cover crop biomass were taken using a push-mower with a bushel bagger attached. A square meter was mowed at three random points in each plot. Each square meter of biomass was then bagged, dried, weighed, and ground for analysis. The field was mowed and sampled every three weeks during the growing season on four sample dates (6/11/15, 7/2/15, 7/23/15, and 8/13/15).

Soil samples were taken as in the previous year with a tubular soil sampler by hand at mid-Summer (July) and early fall (September) in 2015 and 2016. The soil sample pattern was similar to cover crop sampling with three samples taken at random points in each plot. Soil was bagged and stored at -18°C until further analysis. At the end of the season, leaf samples were taken by plucking one healthy leaf from each tree in all plots. Leaves were bagged, dried, weighed, and ground for nutrient analysis. All tree heights and diameters (excluding trees located inside buffers) were observed and recorded using a field ruler and electronic caliper as in the previous season. Cover crops were not tilled and allowed to go to seed at the end of the growing season of 2015. As a result, similar cover crop densities were observed in the 2016 growing season. The same management methods were applied, with regular cutting (every 21 days) and herbicide applications to control treatments and tree rows. Additionally, the same processes for sampling soil, poplar leaf, height, and diameter was repeated in 2016. However, the cover crop biomass was not sampled in the second season, and return was assumed to be proportionate, if not the same as the previous year.

3.3.3 Sample Analysis

Soil samples were mixed thoroughly and a subsample of 5 grams was taken and mixed with 50 mL of potassium chloride (KCl). Samples were then placed in a shaker and mixed for 1 ½ hours for nutrient extraction. After shaking, samples were filtered using Whatman No. 1 filters pre-washed in potassium chloride solution to remove soil sediment. The resulting extracted nutrient solution was analyzed for nitrate and ammonium levels using a SAN++ segmented flow analyzer (Skalar Inc., Atlanta, GA) measured at 540 and 660 nm, respectively. An additional 10 grams of soil were placed in an oven at 65°C for 72 hours and thereafter weighed for determining soil moisture content.

All leaf and cover crop biomass samples dried for 72 hours at 65°C, weighed, and ground to a fine powder prior to analysis for total N and P. A subsample of 0.3 grams (± 0.005) was then weighed and placed in 100 mL test tube, mixed with 4.5 mL of sulfuric acid (H_2SO_4) in a vortex and allowed to sit overnight for pre-digestion. Then, 1.5 mL of hydrogen peroxide (30%) was added to each tube and then placed in a block digester (AIM600 Block Digesting System) at 340 ± 10°C for one hour under a programed temperature schedule. After digestion, sample tubes were filled to 100 mL with deionized water and mixed with a vortex. The digested sample mixture was then buffered and chlorinated after dialysis to form a chemical complex measured 660 and 880 nm for total N and P, respectively, on a SAN++ segmented flow analyzer (Skalar Inc.).

3.3.4 Data Analysis

Results from nutrient analysis of poplar leaves were expressed as concentrations (mg/g). To obtain nutrient return from cover crops, concentrations of N and P from analysis were applied to the dry biomass weight to find total nutrient content. Results from soil nitrate and ammonium analysis were returned from analysis in mg/L and were transformed to concentrations (µg/mg).

The above values, along with values regarding green manure biomass and nutrient return, poplar nutrient concentrations, height, and diameter were compared for significant differences between treatments using an analysis of variance or a Student's T-Test depending on the number of treatments being compared. Means were then separated using a Tukey's test of honestly significant difference. Linear correlations were tested using Pearson's correlation analysis. All tests used values of *P* lower than 0.05 as an indicator of statistically significant difference. All data analyses were performed in R 3.3.1 software, as was the creation of graphs and tables [138].

3.4 Results

3.4.1 Cover Crop Nutrient Concentrations

Nutrient analysis of the sampled green manure revealed different concentrations of nitrogen and phosphorous between treatments involving clover (RCF and RCH) and the natural vegetation treatment (NV) (Table 2). Treatment RCH had significantly higher N concentrations than NV at both narrow and wide spacings (p = 0.02 and 0.00), while treatment RCF had significantly higher N concentrations than NV only at the wide spacing (p = 0.0), however the narrow spacing exhibited strong differences (p = 0.06). No differences in N concentrations were observed between different spacing treatments in red clover treatments. However, in treatment NV the biomass in the narrow spacing had higher N concentrations (p = 0.02).

Phosphorous concentrations showed a reverse trend, with natural vegetation plots being generally higher. Treatment NV had higher P concentrations than treatment RCF (wide and narrow spacing) and treatment RCH (narrow spacing) (p = 0.01, 0.00, 0.00). No differences between spacing treatments were observed under similar cropping treatments.

Nitrogen/phosphorous ratios were also analyzed to determine potential effects on soil nutrients and health (Figure 5). Ratios were significantly higher in treatment RCF at both wide and narrow spacings (p = 0.00 and 0.00). The same was observed in treatment RCH for wide and narrow spacings (p = 0.00 and 0.00).

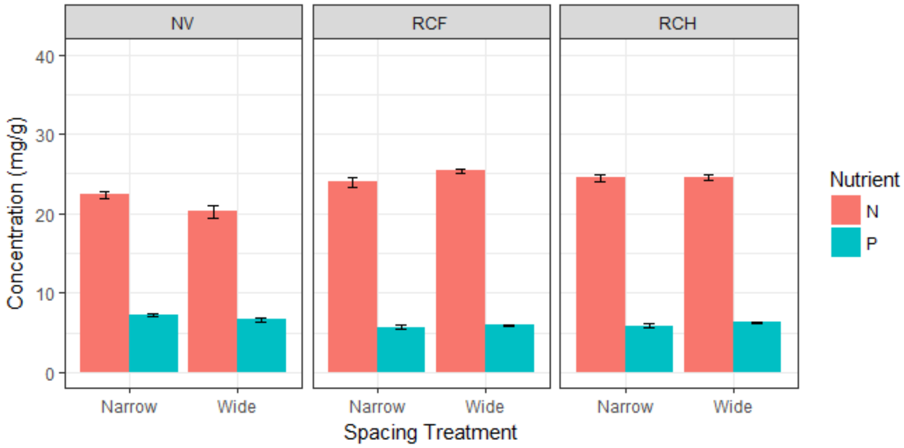


Figure 4. Average nutrient concentrations of biomass returned during routine cuttings from natural vegetation (NV), red clover at a half seed rate (RCH), and red clover at a full seed rate (RCF).

Table 2. Average nutrient concentrations returned during cutting from natural vegetation (NV), red clover at a half seed rate (RCH), and red clover at a full seed rate (RCF).

	N (mg/g)	P (mg/g)	N/P Ratio
Wide Spacing			
RCH	24.60 (0.33) Aa	6.0 (0.11) Aab	4.27 (0.39) Aa
RCF	25.36 (0.31) Aa	5.93 (0.09) Aa	4.54 (0.34) Aa
NV	20.24 (0.74) Ab	6.66 (0.26) Ab	3.41 (0.36) Ab
Narrow Spacing			
RCH	24.46 (0.44) Aa	5.91 (0.33) Aa	4.55 (0.34) Aa
RCF	24.00 (0.63) Aa	5.68 (0.24) Aa	4.52 (0.36) Aa
NV	22.35 (0.38) Bb	7.22 (0.17) Bb	3.41 (0.33) Ab

Values are mean (± standard error). For each value, small letters indicate significant differences between vegetation treatments within the same spacing treatment while capital letters indicate significant differences within vegetation treatments between spacings ($P < 0.05$, $n = 9$)

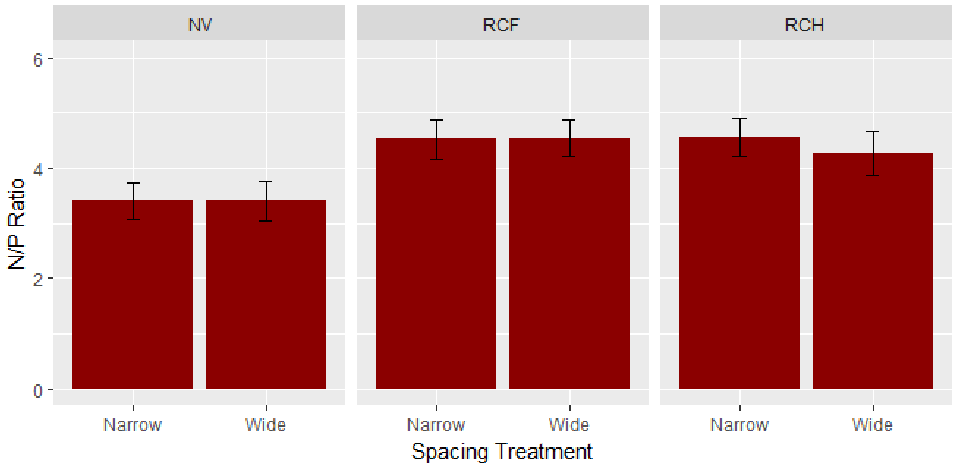


Figure 5. Average nitrogen-to-phosphorous ratios based on nutrient concentrations of cover crop biomass return from natural vegetation (NV), red clover at a half seed rate (RCH), and red clover at a full seed rate (RCF).

3.4.2 Cover Crop Nutrient Return

Treatments involving clover (RCH and RCF) had much higher biomass return compared to in the natural vegetation treatment (Figure 6). At both wide and narrow spacing types, treatments RCH (p = 0.00 and 0.00) and RCF (p = 0.00 and 0.00) had higher dry weight returns than treatment NV. Additionally, treatment RCF at the wide spacing had higher biomass production than the narrow spacing (p = 0.01).

Total nutrient return from green manure over the 2015 season showed similar trends to green manure nutrient concentrations (Table 3). Treatments RCH and RCF had significantly higher N content return values at wide (p = 0.00 and 0.00) and narrow spacing treatments (p = 0.00 and 0.00) compared to treatment NV. Treatment RCF also had significantly higher N content at the wide spacing than at the narrow spacing (p = 0.00). Analysis for phosphorous revealed that despite lower P concentrations than treatment NV, treatments RCH and RCF showed higher values of total P content at both wide (p = 0.00 and 0.00) and narrow spacings (p = 0.00 and 0.00). No differences were observed between spacing types within vegetation treatments.

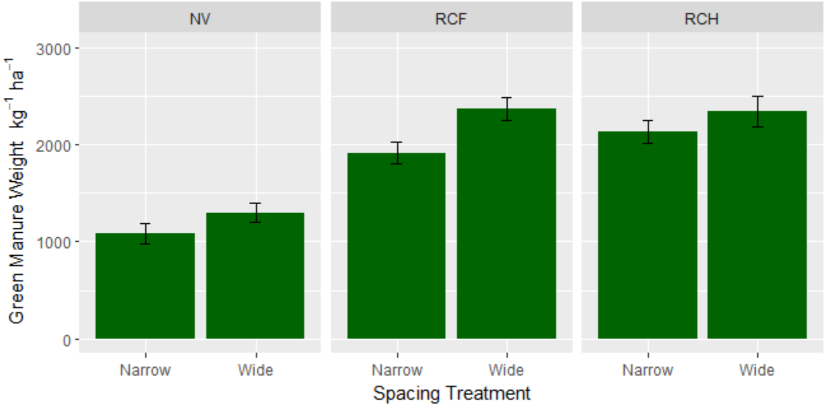


Figure 6. Total dry weight of biomass returned from natural vegetation (NV), red clover at a half seed rate (RCH), and red clover at a full seed rate (RCF) during the 2015 season.

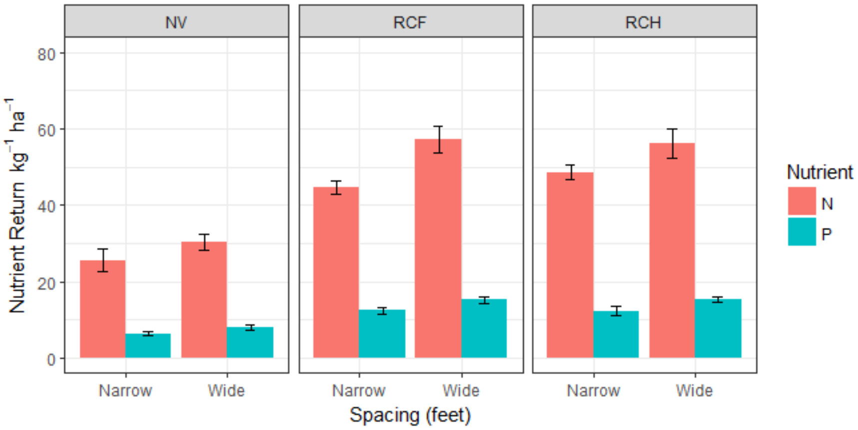
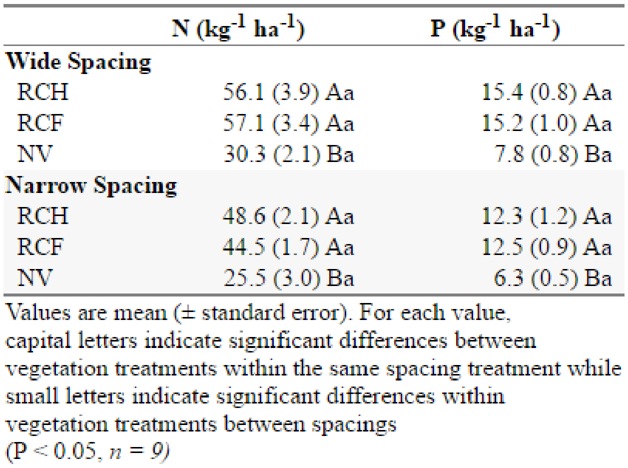


Figure 7. Total nutrient return in biomass from natural vegetation (NV), red clover at a half seed rate (RCH), and red clover at a full seed rate (RCF) during the 2015 season.

Table 3. Total nutrient return via green manure by the end of the 2015 season from natural vegetation (NV), red clover at a half seed rate (RCH), and red clover at a full seed rate (RCF).



	N (kg^{-1} ha^{-1})	P (kg^{-1} ha^{-1})
Wide Spacing		
RCH	56.1 (3.9) Aa	15.4 (0.8) Aa
RCF	57.1 (3.4) Aa	15.2 (1.0) Aa
NV	30.3 (2.1) Ba	7.8 (0.8) Ba
Narrow Spacing		
RCH	48.6 (2.1) Aa	12.3 (1.2) Aa
RCF	44.5 (1.7) Aa	12.5 (0.9) Aa
NV	25.5 (3.0) Ba	6.3 (0.5) Ba

Values are mean (± standard error). For each value, capital letters indicate significant differences between vegetation treatments within the same spacing treatment while small letters indicate significant differences within vegetation treatments between spacings ($P < 0.05$, $n = 9$)

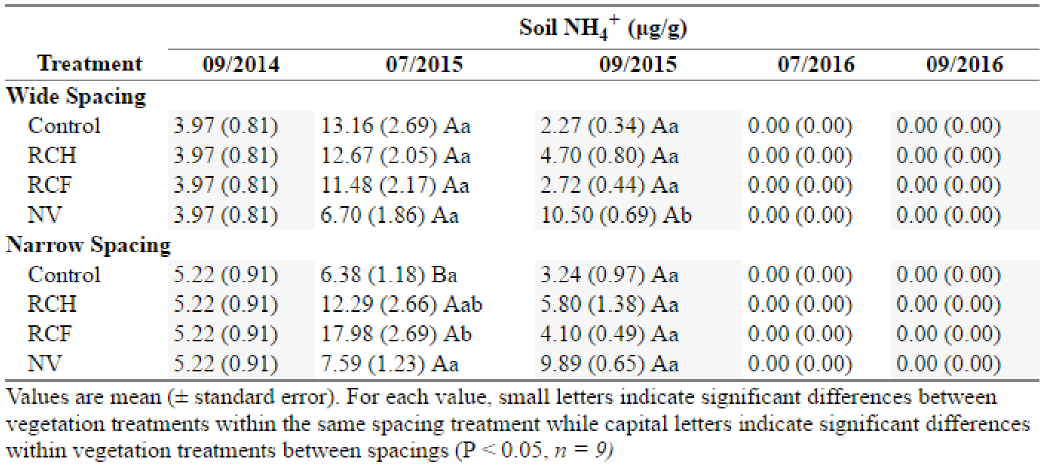
3.4.3 Plant-Available Soil Nitrogen

There was a notable lack of differences in soil nutrient parameters between spacing treatments (Tables 4 & 5). The only differences existed in the 2016 mid-season nitrate levels in treatment RCF where the nitrate in wider spacing was significantly higher than in the narrow spacing ($p = 0.00$) and RCH where the narrow spacing was higher than the wider spacing ($p = 0.00$). No other differences existed between spacing treatments at the other four sampling dates over the three-year experiment.

Soil nitrate increased in most treatments (including controls) up to the late-season sample in 2016, while soil ammonium showed a decreasing trend after the end of the first growing season onward. Mid-season estimates of soil nitrate in 2016 (07/2016) showed that treatment RCF had significantly higher levels than treatments C and NV ($p = 0.00$ and 0.00) at the wide spacing while RCH had significantly higher nitrate than the same treatments ($p = 0.00$ and 0.00) at the narrow spacing. However, no significant differences were seen in soil nitrate by the end of the final season. Furthermore, no differences were found between treatments in 2016 at mid or late-season estimates regarding ammonium. This was due to an apparent lack of soil ammonium in all treatments.

Due to the lack of differences between spacing treatments, analysis was also done removing spacing treatments which revealed somewhat similar results (Figure 8). Mid-season 2016 nitrate levels were significantly higher in both treatments RCH and RCF compared to the control (p = 0.00 and 0.00) and treatment NV (p = 0.00 and 0.00). No significant differences were found among vegetation treatments by the end of the experiment. Ammonium levels showed no differences after removing spacing.

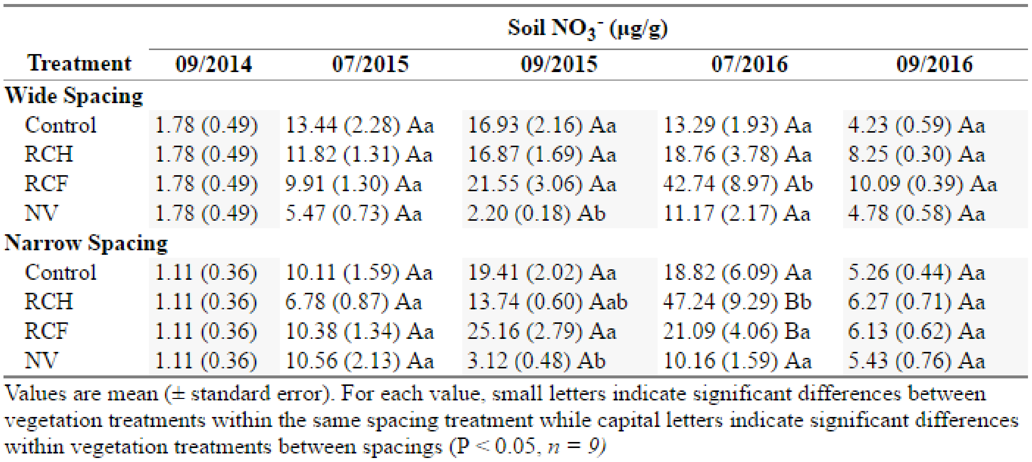
Table 4. Summary of soil ammonium in treatments prior to cover crop establishment (09/2014) and through two subsequent growing seasons for natural vegetation (NV), red clover at a half seed rate (RCH), red clover at a full seed rate (RCF), and control treatments.



	Soil NH_4^+ (µg/g)				
Treatment	**09/2014**	**07/2015**	**09/2015**	**07/2016**	**09/2016**
Wide Spacing					
Control	3.97 (0.81)	13.16 (2.69) Aa	2.27 (0.34) Aa	0.00 (0.00)	0.00 (0.00)
RCH	3.97 (0.81)	12.67 (2.05) Aa	4.70 (0.80) Aa	0.00 (0.00)	0.00 (0.00)
RCF	3.97 (0.81)	11.48 (2.17) Aa	2.72 (0.44) Aa	0.00 (0.00)	0.00 (0.00)
NV	3.97 (0.81)	6.70 (1.86) Aa	10.50 (0.69) Ab	0.00 (0.00)	0.00 (0.00)
Narrow Spacing					
Control	5.22 (0.91)	6.38 (1.18) Ba	3.24 (0.97) Aa	0.00 (0.00)	0.00 (0.00)
RCH	5.22 (0.91)	12.29 (2.66) Aab	5.80 (1.38) Aa	0.00 (0.00)	0.00 (0.00)
RCF	5.22 (0.91)	17.98 (2.69) Ab	4.10 (0.49) Aa	0.00 (0.00)	0.00 (0.00)
NV	5.22 (0.91)	7.59 (1.23) Aa	9.89 (0.65) Aa	0.00 (0.00)	0.00 (0.00)

Values are mean (± standard error). For each value, small letters indicate significant differences between vegetation treatments within the same spacing treatment while capital letters indicate significant differences within vegetation treatments between spacings ($P < 0.05$, *n = 9)*

Table 5. Summary of soil nitrate in treatments prior to cover crop establishment (09/2014) and through two subsequent growing seasons for natural vegetation (NV), red clover at a half seed rate (RCH), red clover at a full seed rate (RCF), and control treatments.



	Soil NO_3^- (µg/g)				
Treatment	**09/2014**	**07/2015**	**09/2015**	**07/2016**	**09/2016**
Wide Spacing					
Control	1.78 (0.49)	13.44 (2.28) Aa	16.93 (2.16) Aa	13.29 (1.93) Aa	4.23 (0.59) Aa
RCH	1.78 (0.49)	11.82 (1.31) Aa	16.87 (1.69) Aa	18.76 (3.78) Aa	8.25 (0.30) Aa
RCF	1.78 (0.49)	9.91 (1.30) Aa	21.55 (3.06) Aa	42.74 (8.97) Ab	10.09 (0.39) Aa
NV	1.78 (0.49)	5.47 (0.73) Aa	2.20 (0.18) Ab	11.17 (2.17) Aa	4.78 (0.58) Aa
Narrow Spacing					
Control	1.11 (0.36)	10.11 (1.59) Aa	19.41 (2.02) Aa	18.82 (6.09) Aa	5.26 (0.44) Aa
RCH	1.11 (0.36)	6.78 (0.87) Aa	13.74 (0.60) Aab	47.24 (9.29) Bb	6.27 (0.71) Aa
RCF	1.11 (0.36)	10.38 (1.34) Aa	25.16 (2.79) Aa	21.09 (4.06) Ba	6.13 (0.62) Aa
NV	1.11 (0.36)	10.56 (2.13) Aa	3.12 (0.48) Ab	10.16 (1.59) Aa	5.43 (0.76) Aa

Values are mean (± standard error). For each value, small letters indicate significant differences between vegetation treatments within the same spacing treatment while capital letters indicate significant differences within vegetation treatments between spacings ($P < 0.05$, $n = 9$)

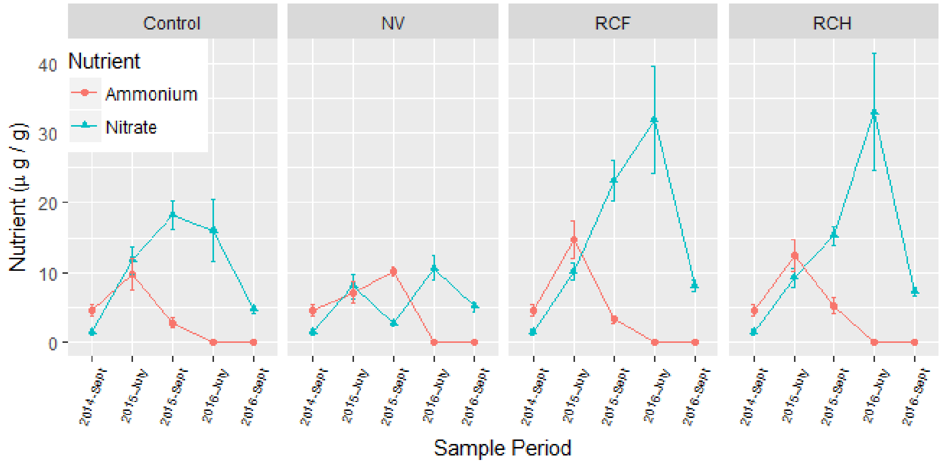


Figure 8. Soil mineralized nitrogen concentrations after combining spacing treatments across natural vegetation (NV), red clover at a half seed rate (RCH), red clover at a full seed rate (RCF), and control treatments.

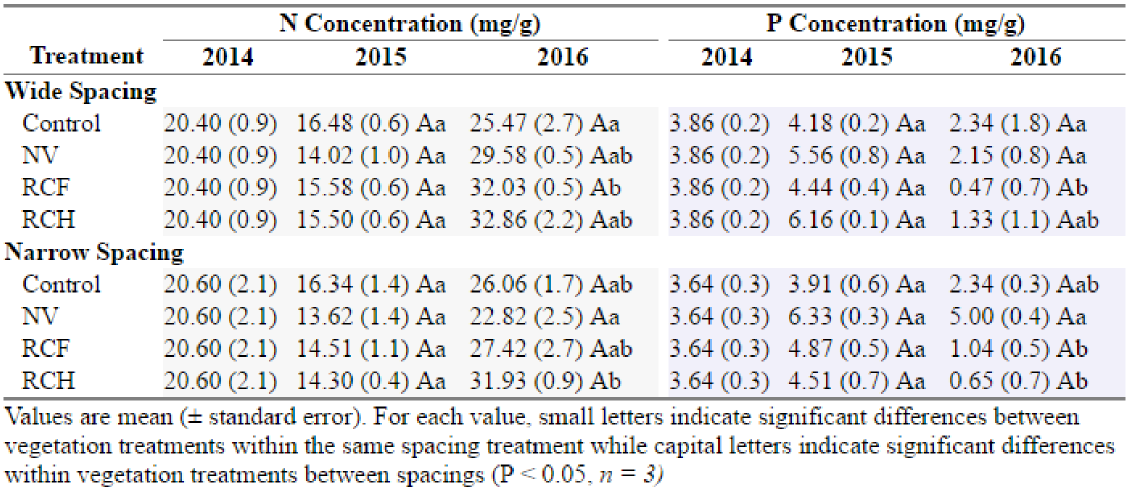
3.4.4 Poplar Leaf Nutrients

Samples of poplar leaves were also analyzed for N and P concentrations at the end of all three seasons (Table 6). No significant differences were found between wide and narrow spacing treatments in 2014, which was prior to the establishment of cover crop treatments. Additionally, no significant differences were found between spacing or vegetation treatments by the end of the

2015 growing season. There were also no significant differences between spacing treatments within the same vegetation treatment in either phosphorous or nitrogen. The small sample size in this experiment likely played a part in the small amount of significant differences.

In 2016, poplar leaves within clover treatments exhibited as much as 25% higher levels of nitrogen in comparison to bare-soil controls in the wide spacing, and similar results compared to the natural vegetation control at the narrow spacing. However, these differences were only significant at the full seed rate (RCF) at the wide spacing (p = 0.04) and at the half seed rate (RCH) at the narrow spacing (p = 0.05). Concerning phosphorous, vegetation treatments with clover generally had lower numbers. Red clover at a full seed rate (RCF) was significantly lower compared to treatments NV and C (p= 0.05 and 0.03) at the wide spacing, while at the narrow spacing red clover at the half seed rate (RCH) was lower only compared to treatment NV (p = 0.00).

Table 6. Average poplar leaf nitrogen and phosphorous concentrations between spacing treatments over the course of the experiment for natural vegetation (NV), red clover at a half seed rate (RCH), red clover at a full seed rate (RCF), and control treatments.



	N Concentration (mg/g)			P Concentration (mg/g)		
Treatment	**2014**	**2015**	**2016**	**2014**	**2015**	**2016**
Wide Spacing						
Control	20.40 (0.9)	16.48 (0.6) Aa	25.47 (2.7) Aa	3.86 (0.2)	4.18 (0.2) Aa	2.34 (1.8) Aa
NV	20.40 (0.9)	14.02 (1.0) Aa	29.58 (0.5) Aab	3.86 (0.2)	5.56 (0.8) Aa	2.15 (0.8) Aa
RCF	20.40 (0.9)	15.58 (0.6) Aa	32.03 (0.5) Ab	3.86 (0.2)	4.44 (0.4) Aa	0.47 (0.7) Ab
RCH	20.40 (0.9)	15.50 (0.6) Aa	32.86 (2.2) Aab	3.86 (0.2)	6.16 (0.1) Aa	1.33 (1.1) Aab
Narrow Spacing						
Control	20.60 (2.1)	16.34 (1.4) Aa	26.06 (1.7) Aab	3.64 (0.3)	3.91 (0.6) Aa	2.34 (0.3) Aab
NV	20.60 (2.1)	13.62 (1.4) Aa	22.82 (2.5) Aa	3.64 (0.3)	6.33 (0.3) Aa	5.00 (0.4) Aa
RCF	20.60 (2.1)	14.51 (1.1) Aa	27.42 (2.7) Aab	3.64 (0.3)	4.87 (0.5) Aa	1.04 (0.5) Ab
RCH	20.60 (2.1)	14.30 (0.4) Aa	31.93 (0.9) Ab	3.64 (0.3)	4.51 (0.7) Aa	0.65 (0.7) Ab

Values are mean (± standard error). For each value, small letters indicate significant differences between vegetation treatments within the same spacing treatment while capital letters indicate significant differences within vegetation treatments between spacings ($P < 0.05$, $n = 3$)

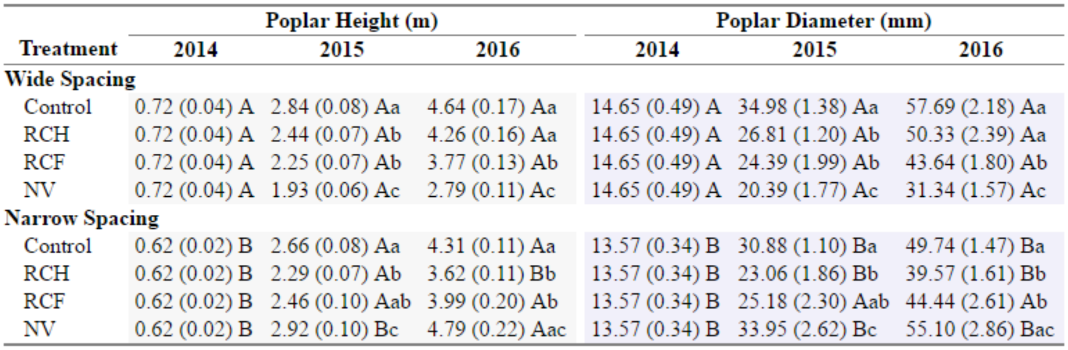
3.4.5 Poplar Height and Diameter

Measurements of height and diameter were taken at the end of all three growing seasons. Data was recorded and averaged by treatment for analysis. Measurements from 2014, prior to cover crop planting, revealed a slight difference between wide and narrow spacings in both height and diameter. Poplar heights were roughly 0.1 meter higher and diameters roughly 1 millimeter larger in the wide spacing compared to the narrow spacing (p = 0.00 and 0.00).

At the end of 2015, heights in treatment RCH were lower than in controls at both wide and narrow spacing (p = 0.01 and 0.03). Treatments RCF and NV also had lower heights compared to controls at the wide spacing (p = 0.00 and 0.00). The 2016 growing season showed slightly different results. At the wide spacing, treatments RCF and NV had lower heights than control treatments (p = 0.00 and 0.00), as did treatment RCH at the narrow spacing (p = 0.02).
In terms of differences between spacing treatments, within treatment NV, the narrow spacing had lower average heights than the narrow spacing (p = 0.00) in 2015. In 2016, treatment RCH had higher heights at the wide spacing than in the narrow spacing (p = 0.00).

Similar trends were seen in the 2015 season in terms of diameter. Treatment RCH had lower diameters compared to control in both wide and narrow spacing treatments (p = 0.00 and 0.03). Treatments RCF and NV also had lower diameter than in controls, but only in the narrow spacing (p = 0.00 and 0.00). More differences between spacing treatments within vegetation treatments existed in diameters than in heights. Control treatments and treatment RCH both had larger diameters at the wider spacing (p = 0.00 and 0.00), while treatment NV was higher in the narrow spacing (p = 0.00). By 2016, the only difference in diameter within the narrow spacing treatment was in treatment RCH, which was lower than controls (p = 0.01). At wide the spacing RCF and NV were both lower than controls (p = 0.00 and 0.00). Since the biomass measurements were based on the diameters retrieved from field measures, the same differences were reflected (Table 8). The only difference occurred when determining biomass in Mg ha^{-1}, where narrow spacing treatments had higher productivity compared to the wider spacing treatments, due to a higher number of trees per acre. Interestingly, differences between spacing treatments were only significant in RCF and NV (p = 0.00 and 0.00) by the end of the experiment in 2016.

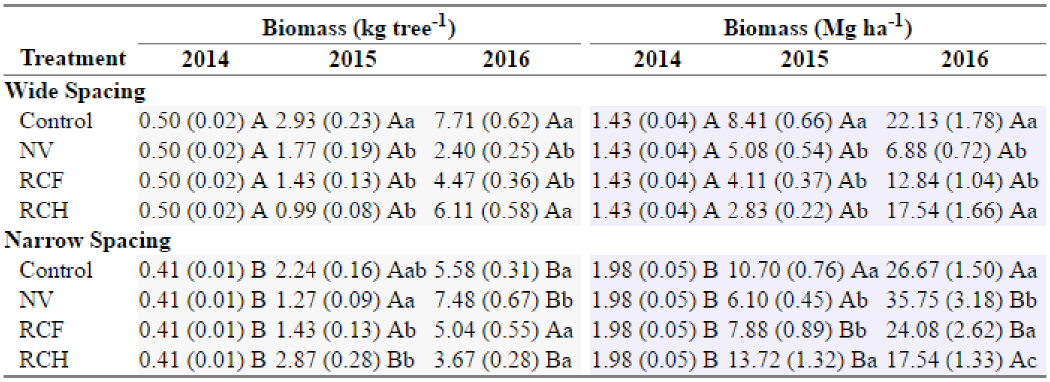
Table 7. Average height and diameters of poplar at the end of each growing season for natural vegetation (NV), red clover at a half seed rate (RCH), red clover at a full seed rate (RCF), and control treatments.



	Poplar Height (m)			Poplar Diameter (mm)		
Treatment	**2014**	**2015**	**2016**	**2014**	**2015**	**2016**
Wide Spacing						
Control	0.72 (0.04) A	2.84 (0.08) Aa	4.64 (0.17) Aa	14.65 (0.49) A	34.98 (1.38) Aa	57.69 (2.18) Aa
RCH	0.72 (0.04) A	2.44 (0.07) Ab	4.26 (0.16) Aa	14.65 (0.49) A	26.81 (1.20) Ab	50.33 (2.39) Aa
RCF	0.72 (0.04) A	2.25 (0.07) Ab	3.77 (0.13) Ab	14.65 (0.49) A	24.39 (1.99) Ab	43.64 (1.80) Ab
NV	0.72 (0.04) A	1.93 (0.06) Ac	2.79 (0.11) Ac	14.65 (0.49) A	20.39 (1.77) Ac	31.34 (1.57) Ac
Narrow Spacing						
Control	0.62 (0.02) B	2.66 (0.08) Aa	4.31 (0.11) Aa	13.57 (0.34) B	30.88 (1.10) Ba	49.74 (1.47) Ba
RCH	0.62 (0.02) B	2.29 (0.07) Ab	3.62 (0.11) Bb	13.57 (0.34) B	23.06 (1.86) Bb	39.57 (1.61) Bb
RCF	0.62 (0.02) B	2.46 (0.10) Aab	3.99 (0.20) Ab	13.57 (0.34) B	25.18 (2.30) Aab	44.44 (2.61) Ab
NV	0.62 (0.02) B	2.92 (0.10) Bc	4.79 (0.22) Aac	13.57 (0.34) B	33.95 (2.62) Bc	55.10 (2.86) Bac

Values are mean (± standard error). For each value, small letters indicate significant differences between vegetation treatments within the same spacing treatment while capital letters indicate significant differences within vegetation treatments between spacings ($P < 0.05$, $n = 72$)

Table 8. Average biomass productivity of poplars at tree and stand levels for natural vegetation (NV), red clover at a half seed rate (RCH), red clover at a full seed rate (RCF), and control treatments.



	Biomass (kg tree^{-1})			Biomass (Mg ha^{-1})		
Treatment	**2014**	**2015**	**2016**	**2014**	**2015**	**2016**
Wide Spacing						
Control	0.50 (0.02) A	2.93 (0.23) Aa	7.71 (0.62) Aa	1.43 (0.04) A	8.41 (0.66) Aa	22.13 (1.78) Aa
NV	0.50 (0.02) A	1.77 (0.19) Ab	2.40 (0.25) Ab	1.43 (0.04) A	5.08 (0.54) Ab	6.88 (0.72) Ab
RCF	0.50 (0.02) A	1.43 (0.13) Ab	4.47 (0.36) Ab	1.43 (0.04) A	4.11 (0.37) Ab	12.84 (1.04) Ab
RCH	0.50 (0.02) A	0.99 (0.08) Ab	6.11 (0.58) Aa	1.43 (0.04) A	2.83 (0.22) Ab	17.54 (1.66) Aa
Narrow Spacing						
Control	0.41 (0.01) B	2.24 (0.16) Aab	5.58 (0.31) Ba	1.98 (0.05) B	10.70 (0.76) Aa	26.67 (1.50) Aa
NV	0.41 (0.01) B	1.27 (0.09) Aa	7.48 (0.67) Bb	1.98 (0.05) B	6.10 (0.45) Ab	35.75 (3.18) Bb
RCF	0.41 (0.01) B	1.43 (0.13) Ab	5.04 (0.55) Aa	1.98 (0.05) B	7.88 (0.89) Bb	24.08 (2.62) Ba
RCH	0.41 (0.01) B	2.87 (0.28) Bb	3.67 (0.28) Ba	1.98 (0.05) B	13.72 (1.32) Ba	17.54 (1.33) Ac

Values are mean (± standard error). For each value, small letters indicate significant differences between vegetation treatments within the same spacing treatment while capital letters indicate significant differences within vegetation treatments between spacings ($P < 0.05$, $n = 68\text{-}72$)

3.4.6 Inter-Variable Correlations

Extensive analysis was completed to find possible linear correlations between variables that might give insight into how the cover crop treatments might have affect soil health and poplar growth and nutrition. However, few valuable inter-variable relationships were revealed. No correlations were found between green manure N return and poplar height or diameter in either 2015 or 2016. Green manure N return was positively correlated to poplar leaf N in the narrow spacing of treatment NV ($p = 0.01$, Adj $r^2 = 0.62$). Dry biomass weight return in the narrow spacing of treatment RCF was positively linked to soil organic C ($p = 0.01$, Adj $r^2 = 0.54$).

3.5 Discussion

The objectives of this study were to determine the effects, be they positive or negative, of alley cropping leguminous cover crops into a hybrid poplar biofuel plantation, and how management techniques may influence those effects. After three years of data (including a baseline year without alley cropping), the results gave mixed signs of competition and benefits between *T. pratense* and the hybrid poplar, but ultimately the study objectives were achieved. Soil and leaf nitrogen responses to the alley cropping treatments were clear, although they were not reflected in growth measurements by the end of the experiment. Additionally, evidence suggests that management methods can have impacts on mitigating negative competitive effects in such systems. These details will be discussed in the following sections.

3.5.1 Biomass Return

Cover crop analysis revealed significantly higher amounts of biomass, organic N, and organic P being returned to the soil in *T. pratense* treatments regardless of seeding rate. This was to be expected, as red clover is very productive in terms of biomass and is an extremely productive nitrogen-fixer [139,140]. Field observations revealed little visual difference between treatments red clover seeded at the full rate versus at the half rate (RCF vs RCH), which was reflected in both biomass and nutrient return. Additionally, the natural vegetation treatment consisted primarily of forbs and grasses, while treatment C remained free of vegetation.

Higher P concentrations in the alley crop biomass from treatment NV compared to other treatments could have been due to the lack of competition for P resources within these plots. This is supported by the slightly smaller margins of difference in P concentrations between vegetation treatments at the wider spacing (Table 2), where competition would be less significant. Additionally, lower P concentrations in treatments RCH and RCF reinforce this idea. Leguminous species require large amounts of phosphorous to sustain biological nitrogen fixation [90]. Thus, it follows that clover P concentrations might be lower in the absence of sufficient available phosphorous, particularly between narrow tree rows. However, total phosphorous return by the end of the 2015 season was higher in treatments RCH and RCF compared to natural vegetation at both spacing treatments. This likely reflects a greater overall efficiency at utilizing soil P by red clover plots.

As was expected, nitrogen returns were far greater in red clover treatments compared to treatment NV. Values of N return did not differ significantly between spacing treatments but a trend of lower N return was seen in the narrow row spacing, which indicates higher competition for soil N between poplar and clover roots.

3.5.2 Soil

Soil nitrate and ammonium levels were fairly consistent between treatments RCF and RCH, which falls in line with the trends seen in cover crop nutrient return (Figure 8). Both red clover treatments had generally higher plant available nitrogen levels compared to other treatments, particularly in terms of nitrate, although significant differences didn't occur until 2016. This is indicative of the "delayed effect" that is associated with indirect transfer, due to the required decay and chemical transformations that need to take place for the nitrogen to become available [12]. Up to July of 2016, there was a steady rise in soil nitrate in clover treatments as that nitrogen-rich biomass began to decay and become available to the poplar trees. By the end of the 2016 growing season, however, nitrate levels declined sharply. Since these samples were taken after a period of heavy rain, the reason for this decline is most likely nitrate leaching. Although there was no significant difference, there was also generally higher ammonium levels seen in the red clover treatments compared to controls. This may reflect some form of direct N transfer occurring, most likely through root exudates. The primary compound released by temperate herbaceous legumes through this pathway is ammonium, due to its low-molecular weight and ability to "leak" out of the root membrane, particularly in the early months of its life-cycle [13,141].

Soil parameters in treatment NV reflect the lack of nutrient rich green manure returns, with lower nitrate levels throughout the experiment, even compared to treatment C. Bare-soil control plots were regularly treated with herbicide to remove plant growth between rows and the only organic matter inputs to them was leaf litter at the end of the growing season. Thus the higher nitrate availability in these plots compared to treatment NV would most likely be due to a lack of competition between trees and forbs.

3.5.3 Foliar Nutrient Concentrations

Differences in soil parameters between treatments were reflected in poplar leaf nutrient concentrations. Leaf N concentrations in treatments RCF and RCH were not statistically higher than other treatments until 2016. This is also a strong indication of the "delayed effect" of indirect nitrogen transfer pathway [12]. Transferring N through biomass, which was likely the primary form of transfer in this study, requires the decomposition of the biomass followed by mineralization of organic N into plant available forms by microbes. Completing this process and successfully transferring N indirectly may still take more than one season to complete. The longer periods required by similar studies to see the benefits of legumes to tree species reflects this finding [20–22].

Regardless of timescale, the inputs from the previous season (2015) obviously benefited poplar nutrition, particularly at the wider spacing treatment. At the narrow spacing, only treatment RCH had higher poplar leaf N concentrations by 2016, indicating higher competition for N between poplar trees and clover at the full clover seeding rate (RCF). This could be a direct result of greater root interaction between roots and competition for plant-available N. It could also be caused by greater competition for P resources, resulting in lower biological nitrogen fixation by red clover and thus limited indirect (or direct) N transfer. This is greatly reinforced by the results of poplar leaf P analysis, which were consistently lower than treatments C and NV by 2016. This competition for P resources could hinder growth in the long-term, although other studies have shown that competition for soil resources tends to decline over time in these systems [19–22]. However, phosphate fertilizers could assist growth in the early years of a rotation. Additionally, it is possible that some of the competition for phosphorous could be mitigated in the short term through management methods. This is particularly evident in Table 6, where at the wide spacing and at a lower seeding density (RCH), phosphorous levels were higher compared to other red clover treatments. Moreover, these management methods did not result in any limitations in N transfer. Without using isotopic tracing methods, it is difficult to discern the relative importance of direct and indirect N transfer within this system. However, higher poplar leaf N in treatments RCH and RCF immediately after the first season of high inputs from cover crop biomass indicate that indirect transfer was likely the most important transfer mechanism.

3.5.4 Poplar Growth

Growth measurements revealed puzzling and inconsistent results. Heights and diameters of poplars within treatments RCH and RCF were not higher than treatment C at any point, but were higher than treatment NV at the wide spacing in both 2015 and 2016. Natural vegetation treatments varied widely with spacing while bare-soil controls were relatively steady. Regardless, there were no observable trends in terms of height or diameter and there were no linear correlations between nutrient return or soil parameters and growth. Similar results were seen in biomass, where bare-soil controls were consistently higher than red clover treatments, but treatment NV ranged largely between wide and narrow spacing. Additionally, there was a lack of consistent trends within the red clover treatments, with half seed rates being more productive at the wide spacing compared to the narrow spacing. In summation, neither spacing nor vegetation treatments showed consistent effects on productivity of poplar throughout the experiment. However, it is certain that the benefits seen in soil and poplar leaves in terms of nitrogen were not yet reflected in growth measures at the end of two seasons with the cover crop applications.

This study did not look at soil moisture or competition for water resources in the field. Since hybrid poplars have high water requirements for growth, competition for water resources between the cover crop and trees was very possible. The NV plots also had regular vegetation in the alleys between trees and competition for water should not differ significantly between these treatments. In bare-soil plots (treatment C), however, it is likely that water competition was reduced by the lack of vegetation. This likely explains why this treatment had consistently higher productivity compared to treatments RCH and RCF at both spacing treatments. It is unclear, however, why treatment NV had such variation, with the highest measurements of any treatment at the narrow spacing and the lowest measurements at the wide spacing. Nevertheless, the high water demand by hybrid poplars does make them more susceptible to competition in alley cropping systems such as these. This can reduce poplar growth, biological nitrogen fixation, and nutrient return from cover crop biomass. Similar to with phosphorous competition, it is possible to mitigate some of these competitive effects with management methods. The RCH treatment (half clover seed-rate) at the wide spacing seemed to do better than the full seed rate, indicating a possible reduction in resource competition, including water.

The mixed signs of benefits and competition brought by the presence of cover crops, particularly in red clover treatments, has been demonstrated in similar studies. Wilson et al (2011) found similar signs of competition in a short-term study on cover crops and *Abies fraseri* trees. However, other studies have found the benefits of cover crops eventually outweigh symptoms of competition in the long term [19,20,22]. Waring et al (1985) studied the effects of clover on *P. radiate* in comparison to urea fertilization (and controls). By the end of the second year, significant signs of competition were seen between clover and tree seedlings [20]. However, by the third year the competition had subsided. By the end of the seven-year study, seedlings in clover treatments had significantly higher growth measurements than controls and were the same as seedlings in the fertilized treatment, indicating that clover could serve as a substitute for heavy fertilization [20]. Since the benefits of red clover to soil and poplar nutrition were seen as early as the second season in this study, it is reasonable to believe that similar benefits to growth would be seen over a longer period. Long-term studies such as these are difficult and cumbersome but ultimately may be needed to clarify the real potential of cover crops to tree plantations over time.

3.6 Conclusion

The purpose of this study was to determine the potential benefits of regularly returned green manure from red clover (*T. pratense*) to hybrid poplar (*Populus nigra L.×P. maximowiczii* A. Henry ('NM6')) growth and soil nutrient parameters at different tree row spacing treatments. Cover crop nutrient returns of both phosphorous and nitrogen were higher in red clover treatments at both the full and half seed rate compared to plots with natural vegetation. This generated a positive response in soil nitrate within the same plots in the subsequent growing season, indicating a delayed effect to complete indirect nitrogen transfer. The effects of the cover crop biomass return and soil nutrient response were reflected in poplar leaf nutrition by the second growing season. At narrow spacing treatments, competition for other soil resources, including water and phosphorous, may have resulted in limited N transfer. As a result, poplar leaf N concentrations were significantly higher only in plots where clover was seeded at the half-rate by the second year, where competition for soil resources was assumed to be weaker. At the wide spacing, the presence of red clover resulted in significantly higher poplar leaf N by the second growing season only at the full seed rate compared to controls. Similar N concentrations were seen at the half-seed rate, but were not significant due to higher variability and limited sampling power. However, competition for soil phosphorous was apparent, with generally lower concentrations found in treatments involving red clover. Additionally, no benefits to poplar growth were seen by the presence of red clover.

In summation, this study revealed mixed signs of competition and benefits of cover crops to hybrid poplar trees over the two-year experimental period. Red clover (*T. pretense*) has potential to improve soil nitrogen, nitrogen cycling, and poplar leaf N concentrations but could out-compete trees for phosphorous, particularly in the short term. However, evidence suggests that these competitive effects could be at least partially mitigated through management methods such as wider tree row spacing and cover crop seeding density. Additionally, this study falls in line with similar alley cropping experiments where signs of competition were more prominent in early years but were generally diminished with time as beneficial relationships became more prominent and positive influences on tree growth were seen. Longer-term studies are needed to see how these dynamics might change throughout the life-cycle of a plantation.

4 *Quantifying belowground nitrogen transfer between Trifolium repens and agroforestry tree species in temperate alley cropping systems via root exudates*

4.1 Abstract

Direct nitrogen transfer via root exudates represents a potentially significant source of plant-available nitrogen to agroforestry systems. Nitrogen-fixing species, such as legumes, are capable of capturing and assimilating atmospheric nitrogen. In the process, low molecular-weight nitrogenous compounds such as ammonium and amino acids are secreted from roots into the rhizosphere where they can be readily assimilated by the root systems of other species. Utilizing this source of N transfer could be beneficial to the growth and nutrition of targeted species. To date, existing research on this subject has focused primarily on transfer of N from nitrogen-fixing trees to herbaceous crops or herbaceous legumes to associated grasses. To take existing research further, this experiment was established to quantify how mineralized nitrogen is directly transferred from herbaceous nitrogen-fixing legumes to recipient tree species in alley cropping systems. Specifically, this experiment focused on the direct transfer of fixed nitrogen from an herbaceous legume (*Trifolium repens*) to two tree species (*Castanea sativa x C. crenata* ("Colossal") and *Populus nigra* L.×*P. maximowiczii* A. Henry 'NM6') in a simulated alley cropping system. To accomplish this I utilized the ^{15}N nitrogen tracing method that has been used in similar studies in a greenhouse setting. In this process, the donor species (nitrogen fixer) is fertilized with $K^{15}N0_3$ via foliar application and the recipient crop species is then analyzed for nitrogen isotope ratios after a designated growth period. The values of percent nitrogen derived from transfer in both poplar 12.1% (±1.1) and chestnut 33.3% (±7.4) revealed the potential importance of N transfer to alley cropping systems. The majority of transfer was likely to have

taken place via the production of nitrogenous root exudates from white clover, of which 2.62 (±1.24) mg N was produced daily in each pot (or 0.04g N m^{-2} day $^{-1}$). Previous research indicates that the role of direct N transfer increases in field settings over time. Since this study took place in a greenhouse, similar studies in the field over longer periods are needed to understand how this potential is reflected in actual production systems.

4.2 Introduction

There is considerable existing research indicating the indirect benefits provided by legumes to non-legumes in agroforestry systems. However, until recently it was rare to find studies that sought to explore the specific mechanisms that make this possible. We know that the presence of legumes in agricultural and natural systems can often mean the provision of atmospherically fixed nitrogen to species that cannot achieve this process themselves, either indirectly or directly. However, there is still much research needed to gain a better understanding of these transfer mechanisms so that they can be utilized in sustainable agriculture and forestry.

Within the small pool of research that has explored direct nitrogen transfer, the large portion of it has focused on the pathway of nitrogen from leguminous trees to herbaceous crops in tropical climates or herbaceous legumes to grasses in temperate zones. For example, in a field experiment on conventionally and organically grown grass-clover leys in Switzerland, an average of 51% of total grass nitrogen was found to have been transferred from nearby clover species, the majority of which was assumed to have been through root exudates [142]. Furthermore, in a long-term field study on a silvopastoral site in Central America, transfer from nitrogen-fixing *Gliricidia sepium* trees to *Dichantium aristatum* grass swards via leaf litter (indirect transfer) was shown to only account for one-third of total nitrogen transfer, implying that the direct forms of transfer have higher relative importance [143]. The same species were later used in a greenhouse experiment that discerned between direct transfer pathways (root exudates and common mycorrhizal networks). The results showed that root exudates alone were capable of supplying as much as 63% of total grass nitrogen [15]. Although grass only absorbed roughly 22% of the available *G. sepium* exudates, it proved to be an important source of nitrogen[15].

Despite a recent growth in research on direct N transfer, there is still little research focusing on herbaceous legumes as N donors to trees. Dupraz et al. (1999) showed that the presence of alfalfa planted between rows of black walnut improved walnut foliar nitrogen content but did not distinguish the mode of transfer. In 2011, a study was published showing that kura clover benefited soil and tree nitrogen in a pecan alley cropping system, but this study also did not identify or distinguish how nitrogen was transferred [22]. Roggy et al (2004) focused on trees as the recipient species of nitrogen transfer but did not use herbaceous species as the donor. This study found that *E. augustifolia*, was capable of transferring as much as 25% of its fixed nitrogen to *Prunus* trees via root exchange, while other species contributed lower amounts [14] To summarize, some studies exist that focus on trees as the beneficiaries of adjacently planted herbaceous legumes, but it appears that few, if any, have actually quantified and distinguished direct nitrogen transfer from indirect transfer in such a system.

Here I quantified direct nitrogen transfer from Dutch white clover (*Trifolium repens*) to a hybrid chestnut species (*Castanea sativa x C. crenata* (“Colossal”)) and a hybrid poplar species

(*Populus nigra* L.×*P. maximowiczii* A. Henry 'NM6') in a greenhouse setting. Dutch white clover was chosen as the donor species because of its high nitrogen fixing and transfer potential [13,17]. Chestnuts and poplars were chosen as recipient tree species to highlight two important tree plantation industries: fruit/nut production and biomass energy production. The results of this research have potential to benefit both industries and improve the sustainability of their production.

While the distinction between direct and indirect forms of transfer does not mean from the perspective of the recipient plant, the more efficient utilization of direct transfer mechanisms could mean less inputs and labor for the landowner. Since indirect transfer requires the turnover of plant biomass, continuous mowing and re-seeding is required to maintain legumes in cover cropped tree plantations. However, if direct transfer is found to be an equal or perhaps even more substantial source of N for the trees, these labor and inputs would be unnecessary. Furthermore, a landowner could take this a step further by utilizing the above-ground biomass for raising livestock while continuing substantial N transfer through belowground mechanisms.

The objectives of this study were to (1) determine the nitrogen transferred from *T. repens* to the two chosen species, (2) examine the potentially different ability of the two species to absorb available clover exudates in the soil, (3) determine the exudate potential of *T. repens*, and (4) compare the photosynthetic, nutrient dynamics, and growth to trees grown in interaction treatments to those grown in ideal conditions (controls). These values will be achieved by growing these species together in a greenhouse setting and utilizing isotopic tracing methods over a short (63 day) experimental timeline. These findings will inform decision making for tree plantation owners and help lay a foundation for future research.

4.3 Materials and Methods

4.3.1 Experimental Design

This study consisted of two experiments:

1. Transfer Experiment (two treatments):
    a. Interaction (IR): Trees and ^{15}N labelled white clover were grown together in large pots allowing for uninhibited root interaction to study direct nitrogen transfer
    b. Control (C): Clover, chestnut, and poplar grown in individual pots to compare growth and nutrient dynamics with interaction treatment.
2. Root Exudate Experiment (EX): Strips of white clover (same size as in interaction treatment) grown individually in pots, labelled with ^{15}N, and then transplanted to hydroponic tanks for one week to study total exudate potential throughout the course of the transfer experiment

The interaction treatment was replicated four times for both tree species and were divided into four sample dates. An initial sample was taken immediately prior to applying the isotopic tracer (5/2/16) and was followed by three more samples after application in three week increments (5/23/16, 6/13/16, and 7/4/16). Following the initial sample, samples in the exudate experiment and Treatment (C) were harvested at the same time as Treatment (IR). Treatment (C) consisted of three replicates of unlabeled white clover, chestnut, and poplar grown alone in pots. Similarly, pots in the exudate experiment, which consisted of isotopically labelled white clover grown alone in pots, was replicated three times at each sampling date. All sampling in this experiment was destructive, meaning the whole plant was harvested and separated by compartment for analysis. The experimental model and sampling pattern is depicted in Figure 9.

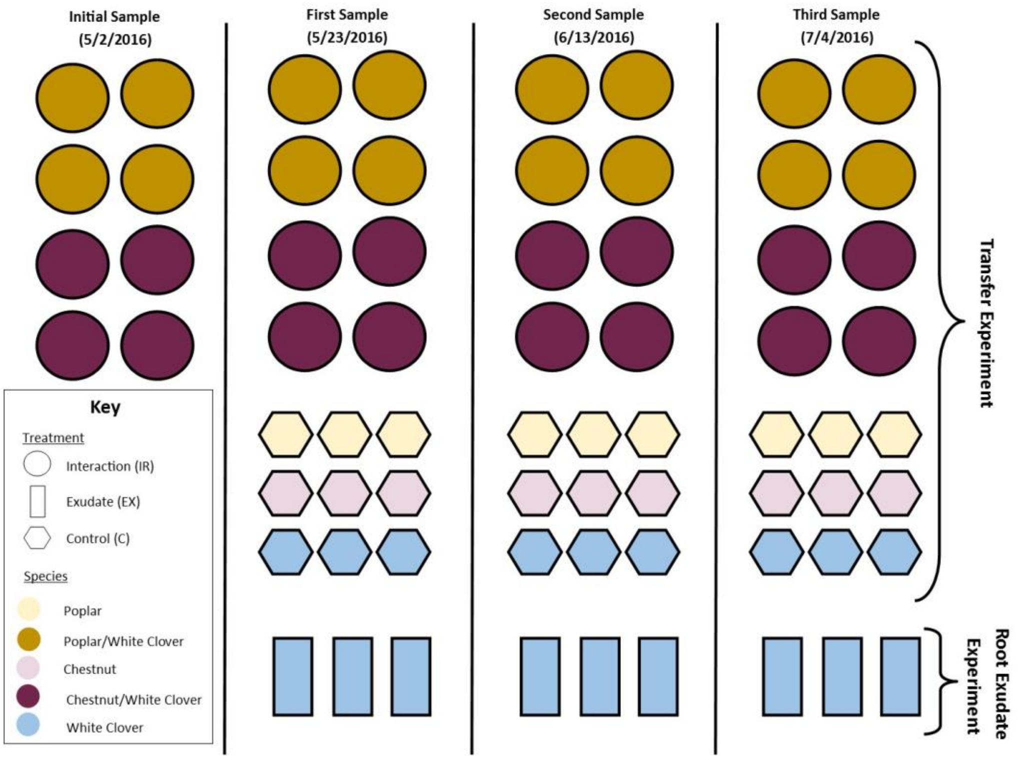


Figure 9. Greenhouse study experimental model and sampling design.

These experiments took place in a greenhouse running east-west, located at the Tree Research Center (TRC) of Michigan State University's campus in East Lansing, Michigan (42°40'N, 84°27'W) from April 2016 to early July 2016. The average temperature during the course of the experiment was 21.1°C (71°F) with maximum temperatures ranging between 21-29°C (70-85°F) and minimum temperatures ranging between 15-22°C (60-72°F). The greenhouse was well ventilated and mimicked external environmental conditions for the majority of the experimental period (with the exception of precipitation).

4.3.2 Materials

Soil used for the experiment was a coarse perennial potting mix from Al-Par Peat Company in Elsie, Michigan and was composed of 33% pine bark fines, 33% reed sedge peat, 3% vermiculite, and 1-3% sand. Containers used for (IR), (EX), and (C) had soil volumes of 21, 10, and 14 liters, respectively, with the exception of units containing white clover in (C), which also used the 10L containers from (EX).

Cuttings of *Populus nigra* L.×*P. maximowiczii* A. Henry (‘NM6’) were obtained from 2-year coppice fields at the Tree Research Center on MSU’s campus. Cuttings were harvested one month prior to the beginning of the experiment (March 2016) and cold-stored at 18°C prior to planting. Chestnut saplings (*Castanea sativa x C. crenata* (“Colossal”)) were obtained from Nash Nurseries in Owosso, Michigan. Saplings were one year old, propagated by seed in the spring of 2015 and treated with three applications of 20-10-20 (NPK) solution in the first growing season but were not fertilized in the nursery during the months of 2016 prior to purchase. The chestnut saplings were purchased and planted prior to leafing out for the season. White clover (*Trifolium repens*) seed was obtained from LaCrosse Seed in Lansing, Michigan and was not inoculated at purchase. Inoculant to be used prior to seeding was also purchased from LaCrosse Seed and was the recommended strain for true clovers (*Rhizobium leguminosarum biovar trifolii*). The isotopic tracer used for this study was purchased from Sigma-Aldrich (St. Louis, MO) and contained 98% atom-enriched $K^{15}N0_3$.

4.3.3 Propagation and Seeding

Saplings, cuttings, and white clover seed were sown or transplanted four weeks prior to the beginning of the experiment to allow root systems to establish. A few days prior to this planting, soil in all pots were fertilized with non-nitrogenous fertilizer to avoid nutrient deficiencies without tampering with nitrogen dynamics. Fertilizers used were 0-45-0 triple super phosphate (45% P_2O_5, 15% calcium) and 0-0-50 sulfate of potash (50% potash, 18% sulfate, 2% chloride). Pots were given fertilizers in amounts proportionate to size. In Treatment (IR) were given 1.5 grams of each fertilizer dissolved in water and pots in Experiment (EX) and Treatment (C) were given one gram of each.

When transplanting chestnuts, special care was taken to shake off as much pre-existing soil in the root ball as possible, so as not to influence the soil composition in the experiment. Since the previous soil was mostly mulch, it was not difficult to shake or brush off the soil. Poplar cuttings were inserted, buds facing upward, into the soil until only one bud was showing above the surface to maintain one new stem per planted cutting. In treatment IR, trees were planted in pots first, prior to seeding the white clover. White clover was seeded in all treatments using the same method. Prior to sowing, all seeds were manually inoculated with *Rhizobium leguminosarum biovar trifolii*. Seeds were spread on a large plastic tray and sprayed gently with a mixture of whole milk with a small amount of molasses to make sure that the inoculant would stick to the seed and provide sugar for microbial growth. The inoculant was then sprinkled heavily onto the seed and mixed by hand. The seeds were spread out on the tray and left in a shaded area to dry for 10-15 minutes before sowing. Once dry, clover was sown at the USDA recommended full seeding rate of four pounds per acre [144].

To keep the observational units of clover uniform in size and density between both experiments, seed was spread to create “strips” of clover. One strip was approximately 10 x 30 cm (or 4 x 11”) and required approximately 2.46mL of white clover seed. Seed was spread evenly over the designated strip area and covered with 0.3-0.5 cm of soil. In Treatment (IR) two strips of clover were sewn on both sides of the planted tree. In Experiment (EX) and Treatment (C), single strips were planted in each pot. All pots were well watered after propagation and watered daily until plants were well established.

4.3.4 Watering

Throughout the course of the experiment pots were watered to minimize competition for moisture. All water used for the pots was de-ionized to prevent contamination with external nutrient inputs. Smaller pots, such as in (EX) and (C), typically required watering every day, especially for pots that had clover in them. Pots in (IR) were watered at least every other day. After the isotopic tracer had been applied to the clover, all watering going forward was done at or below the soil surface using a special nozzle for gentle water flow to avoid water coming in contact with the isotopic label on the clover leaves and contaminating the soil. Additionally, pots were never watered above the saturation point. By doing this, leaching of the isotopic tracer was kept to a minimum.

4.3.5 Isotopic Tracer Application

Labelling of herbaceous species using a ^{15}N foliar brushing method has been demonstrated in multiple studies [14,16,142,145,146] and has been proven to be the most effective process for exudate N labelling [147]. Putz et al (2010) effectively labelled red clover (*T. pratense*) N using a 98atom% ^{15}N urea and Jalonenen et al (2009) labelled *G. sepium* trees using 99atom% $K^{15}NO_3^-$, both using foliar application methods. I used the same process to label mobile and immobile nitrogen in the plant with the ^{15}N tracer. Four weeks after propagation, the isotopic tracer was applied to strips of clover in Treatments IR and EX after taking the initial sample to ascertain natural ^{15}N abundance. This marked the beginning of the experimental period, which would last nine weeks in total, divided into three sampling periods. Tracing methods were modeled after methods utilized by Jalonen et al. (2009) on *G. sepium* trees in a similar greenhouse experiment. Total fertilizer applied to each strip of clover accounted for less than 0.01% of total nitrogen content after four weeks of growth, which was determined by a pilot study where clover was grown alone in the same soil and analyzed for nitrogen content.

The tracer was applied in three applications, or every two days over the course of one week. In each application, each individual “strip” of clover received 2.5 mg of $K^{15}N0_3$ diluted in 5mL of deionized water with 1% v/v Brij-35 (30%) to act as a surfactant and ensure fertilizer was absorbed onto the leaf surface. The tracer solution was carefully applied to clover leaves manually with a small paintbrush. This totaled to 15 mg $K^{15}N0_3$ in treatment IR pots (two strips per pot) and 7.5 mg $K^{15}N0_3$ in Treatment EX pots over the course of the week. To avoid contamination of soil or recipient species leaves with the tracer, the soil was lined with a thin plastic and trees were covered with aerated plastic bags briefly during the application process.

4.3.6 Photosynthesis and Growth Measurements

Photosynthesis measurements were conducted on each of the trees in the IR and C treatments using a LICOR (LI6400XT, Lincoln, NE). The instrument was calibrated before each measurement and the photosynthesis photon flux density (PPFD) was set at 1800 µmol m^{-2} s^{-1}, reference CO2 was maintained at 400 µmol, and the air flow rate was held at 500 µmol s^{-1}. These measurements were taken three times at the midpoint of each of the three experimental periods (days 11, 32, and 53).

Growth measurements were taken immediately prior to destructive sampling. Using a standard meter stick, the height of the tallest living bud in each tree was recorded in centimeters. Diameters were retrieved using an electronic caliper and recorded in millimeters. In chestnuts, diameter was taken exactly one inch above the root collar. Since poplars are prolific at sprouting and they were propagated from cuttings, several samples had more than one shoot. As a result, diameter was taken from the tallest shoot at exactly one inch above its base in the cutting. Data for height and diameter in each observational unit were subtracted from the initial measurements to be displayed as growth during data analysis.

4.3.7 Sampling

At each sampling date, all plant material was separated by compartment (leaf, stem, shoot, root), weighed, and stored in a cooler at 4-6°C until they were taken back to the lab and immediately placed in an oven for drying. All soil samples were bagged and stored at -18°C until further analysis. Special care was taken for samples from (EX) to obtain exudate samples, and will be described in detail in the next section.

In (IR) where clover and tree species were grown simultaneously, delicate handling was required to obtain samples, particularly with respect to roots. In pots with chestnuts, trees were first cut at the base to separate roots from above ground biomass. In pots with poplar, new shoots were removed from the shoot first. Biomass were then separated into stems and leaves, bagged, and stored in a cooler. Aboveground biomass of clover was harvested using a pair of hand-shears. Clover was cut at the base of the stem and the resulting sample was then bagged and stored in a cooler.

To obtain root samples, the soil (which had become an aggregated root ball by the first experimental sample) was gently removed from the container and place in a large, shallow plastic bin. Then, the roots were slowly and gently separated by shaking and brushing off excess soil. Once separated, the roots of the two clover strips and tree roots in each pot were rinsed with deionized water to remove any residual soil. Both tree and clover roots were thereafter bagged and stored in a cooler. Poplar roots were separated from the shoot (cutting) before bagging.

Obtaining plant samples in treatment C was done in the same way as IR but since all species were grown alone, root retrieval was considerably easier. All compartmentalization of plant parts remained the same in all treatments (see Table 9). Soil samples were taken after the removal of roots from the soil aggregates. Remaining soil in the bin was mixed thoroughly by hand and a plastic bag was filled with approximately 0.16 L of soil, which was then placed in a cooler until it could be stored in a freezer in the lab.

Table 9. Description of sample compartmentalization for each species in greenhouse experiment.

Plant Sampling Compartmentalization	
Poplar	Leaf, Stem, Shoot, Root
Chestnut	Leaf, Stem, Root
Clover	Leaf and Stem, Root

4.3.8 Exudate Experiment (EX) Sampling

One week prior to each sample date (after the initial sample), three replicates were transplanted to aerated hydroponic tanks to study total exudate potential. Samples were removed from their pots and root balls were gently shaken to remove as much soil as possible. To further remove any soil from the roots of the clover, samples were placed on a wire screen with 1.3cm gaps and rinsed with deionized water until only bare roots were left. The aerated tanks were filled with approximately 6 liters of deionized water. Clover strips were laid into the tanks on a plastic mesh lining that allowed roots to be submerged in water but held the aboveground portion of the plant above the waterline. Once strips were stable in the tanks, the top of the tank was lined with a plastic wrap to prevent airborne contaminants from getting into the water and effecting analysis. Tanks were aerated via a single 0.42 amp air pump (max PSI – 3.48) with multiple hose attachments. Hoses (0.635cm diameter) were attached to O^2 diffusing stones which were placed beneath the roots in each tank (one for each replicate). Tanks were allowed to sit in the greenhouse under the same light conditions as the other treatments for one week to allow exudate to collect in the water solution, and this was assumed to be representative of exudate production in treatment IR.

At the end of the week samples in aerated tanks were harvested alongside samples from the other two treatments. Strips of clover were gently lifted from aerated tanks and allowed to drain of water for a few minutes. They were then placed on a plastic tray and cut at the base of the stem to separate aboveground from belowground biomass (stem/leaves vs roots). Biomass samples were bagged, weighed, and refrigerated prior to drying and further analysis. All exudate solution volume was measured and placed into multiple 1-liter glass bottles which were then stored in a refrigerator at 4°C. To obtain exudate, exudate solution was first filtered using Whatman #1 filters to remove any dead plant cells. The remaining solution was then poured in Pyrex glass beakers (500 mL) and placed in an oven with plenty of air circulation at 50°C to slowly evaporate water from the solution until only the dry exudate remained. To avoid volatilization of NH_3, solution in all containers was maintained at a pH of 4. This was done via daily monitoring with an electrode (calibrated daily) and adding sulfuric acid (H_2SO_4) with a pipette until pH was less than 4. Total additions of sulfuric acid to solution amounted to less than 0.5 mL in each sample. Evaporation of all water from the solution typically took between 8-12 days. Once this process was finished, dried exudate was collected from Pyrex containers, weighed, ground to a fine powder using a pestle and mortar, and stored in a 25mL plastic Nalgene bottle at room temperature until analyzed for ^{15}N and total nitrogen. Since the collected samples only represent the middle week of each sample period, N content values were multiplied by three to extrapolate exudation to cover the entire sample period.

4.3.9 Laboratory Analysis

Soil samples were mixed thoroughly and a subsample of 5 grams was taken and mixed with 50 mL of potassium chloride (KCl). Samples were then placed in a shaker and mixed for 1 ½ hours for nutrient extraction. After shaking, samples were filtered using Whatman No. 1 filters pre-washed in potassium chloride solution to remove soil sediment. The resulting extracted nutrient solution was analyzed for nitrate levels using a SAN++ segmented flow analyzer (Skalar Inc., Atlanta, GA) measured at 540nm. An additional 10 grams of soil were placed in an oven at 65°C for 72 hours and thereafter weighed to retrieve soil moisture content. Results of the analysis were expressed in mg/L and were transformed to concentrations (µg/mg) using Equation 1.

All compartmentalized plant material obtained from sampling was weighed before and after drying to determine green and dry weights. Material was dried in a ventilated oven at 65°C for 72 hours and then ground to a fine powder using a coffee grinder. The grinder was cleaned after each sample using a mixture of 200 proof ethyl alcohol and deionized water (4:1) to avoid cross-contamination. Poplar shoots were too large to grind with a coffee grinder and had to be processed using an electric wood mill that ground it to 0.2 mm or less. Dried, ground samples were then funneled into small 25 mL plastic Nalgene vials and stored at room temperature until further analysis.

To obtain measurements of nutrient concentrations in plant material, a subsample of 0.3 grams (± 0.005) was weighed and placed in 100 mL test tube mixed with 4.5 mL of sulfuric acid (H_2SO_4) in a vortex and allowed to sit overnight for pre-digestion. Then, 1.5 mL of (30%) hydrogen peroxide (H_2O_2) was added to each tube and then placed in a block digester (AIM600 Block Digesting System) at 340 ± 10°C for one hour under a programed temperature schedule. After digestion, sample tubes were filled to 100 mL with deionized water and mixed well with a vortex. To determine N and P concentrations, the digested sample mixture was then buffered and chlorinated after dialysis to form a chemical complex measured at 660nm and 880nm on a SAN++ segmented flow analyzer (Skalar Inc., Atlanta, GA). Measurements of K and Ca were obtained using the digested solution in an atomic absorption spectrometer (AAnalyst 400, Perkin Elmer). Values were expressed in parts per million (ppm) or mg/L and were transformed into % nutrient and nutrient content using the sample dry weights. Measurements of nitrogen and phosphorous were taken in all treatments (IR, EX, C) in both clover and tree species. Potassium and calcium measurements were taken only in trees. Nutrient analysis was done by plant compartment and as plant totals. Values were expressed as both concentrations and content.

4.3.10 Isotopic Analysis

Isotopic analysis of nitrogen content was done at the UC-Davis Stable Isotope Facility in Davis, California via PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotpe ratio mass spectrometer (Sercon LTD., Cheshire, UK). Plant samples were combusted at 1000°C in a reactor packed with chromium oxide and silvered copper oxide. After combustion, the oxides were removed in a reduction reactor (reduced copper at 650°C) and a helium carrier flowed through a water trap (magnesium perchlorate) and a CO_2 trap. N_2 and CO_2 were then separated on a Carbosieve GC column (65°C, 65 mL/min) before going into an IRMS [148].

Soil samples were analyzed for isotope ratios using an Elementar Vario EL Cube (Elementar Analysensysteme GmbH, Hanau, Germany) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercond Ltd., Cheshire, UK). Samples were combusted at 1080°C in a reactor with copper copper oxide and tungsten (VI) oxide. After combustion, oxides were removed in a reduction reactor (reduced copper at 650°C) and the helium carrier then flowed through a water trap (magnesium perchlorate). N_2 and CO_2 were separated using a molecular sieve adsorption trap before entering the IRMS [148].

Throughout the analysis are interspersed replicates of no less than two different laboratory standards that have been calibrated against NIST Standard Reference Materials [148]. Final values are expressed in delta form which are expressed relative to international standards VPDB (Vienna PeeDee Belemnite) and Air for carbon and nitrogen, respectively. Delta values were found using Equation 2.

$$\delta 15N(sample) = \frac{atom\%15N(sample) - atom\%15N(atmosphere)}{atom\%15N(atmosphere)}\ x\ 100$$

Equation 2. Equation used by UC-Davis Isotope Lab to determine **δ**15N values from isotopic analysis. Here, “atom%15N(sample)” refers the percentage of **δ**15N atoms in sample and “atom%15N(atmosphere)” refers to the atmospheric **δ**15N concentration (0.3663%).

4.3.11 Data and Statistical Analysis

The $\boldsymbol{\delta}^{15}$N values received from isotopic analysis were then applied to Equation 4 to determine the percent of nitrogen derived from transfer (%Ndft) in each tree. Using this formula, %Ndft can be found in both trees at each sampling period. Since **δ**^{15}N values were compartmentalized into the different parts, **δ**^{15}N were averaged across all compartments in each observational unit to be applied to the formula.

$$\%Ndft(t) = \frac{\delta 15Nr(0) - \delta 15Nr(t)}{\delta 15Ns(0) - \delta 15Nd(t)}$$

Equation 3. Equation to determine the percent nitrogen derived from transfer (%Ndft) for each tree species at each sampling period. Where "r" denotes recipient, "d" denotes donor, "s" denotes soil, "0" denotes the beginning of the experiment, and "t" denotes the sample period.

Values of transferred milligrams of nitrogen in each tree, percentage of clover exudate that was absorbed by each tree, and the percentage of total clover nitrogen content that was exuded by the end of the experiment, were determined using Equations 4,5, and 6, respectively.

$$\mathrm{N(Transfer)} = \mathrm{\%Ndft(F)} \ \mathrm{x} \ \mathrm{N(Tree)}$$

Equation 4. Conversion of %Ndft into actual N transfer (mg). Where "%Ndft(F)" refers to the final value of %Ndft for each tree at the end of the experiment and "N(Tree)" refers to the total estimated N content of each tree in milligrams.

$$\mathrm{\%ExNAbs(Tree)} = \frac{\mathrm{N(Transfer)}}{\mathrm{ExN(F)}}$$

Equation 5. Equation for calculating the proportion of available exudate from clover actually absorbed by each tree. Where "N(Transfer) denotes the milligrams of N sourced from transfer in each tree and "ExN(F) denotes the total N exuded over the course of the experiment in milligrams.

$$\%\mathrm{NEx(Clover)} = \frac{\mathrm{N(Transfer)}}{\mathrm{(N(Transfer) + N(Clover))}}$$

Equation 6. Calculation for determining the proportion of clover nitrogen lost through exudation. Where “N(Transfer)” refers to tree N derived from transfer at the end of the experiment and “N(Clover)” refers to total N content of the clover at the end of the experiment.

The above values along with values regarding soil nitrate, plant nutrient concentrations and content, photosynthetic activity, height, and diameter were compared for significant differences between treatments using an analysis of variance. Means were then separated using a Tukey’s test of honestly significant difference. Linear correlations were tested using Pearson’s correlation analysis. All tests used values of P lower than 0.05 as an indicator of statistically significant difference. All data analyses were performed in R 3.3.1 software, as was the creation of graphs and tables [138].

4.4 Results

4.4.1 Nitrogen Transfer

Parameters describing transfer are expressed in δ^{15}N, percent of tree nitrogen derived from transfer (%Ndft), tree N content (mg) derived from transfer (mgNdft), and percent of available clover exudate absorbed by trees (%ExAbs).

4.4.1.1 δ15N Values

Clover in treatments EX and IR were successfully and uniformly labelled by the K15NO3- fertilizer. Table 10 shows the δ^{15}N values of the clover and trees in treatment IR by plant compartment. Clover leaf δ^{15}N in both poplar and chestnut pots increased significantly three weeks after the isotope application at day 0 (p = 0.00 and 0.00), as did clover roots (p = 0.02 and

0.03) and then decreased over the next six weeks as the 15N was diluted from continued growth, N assimilation, and N transfer.

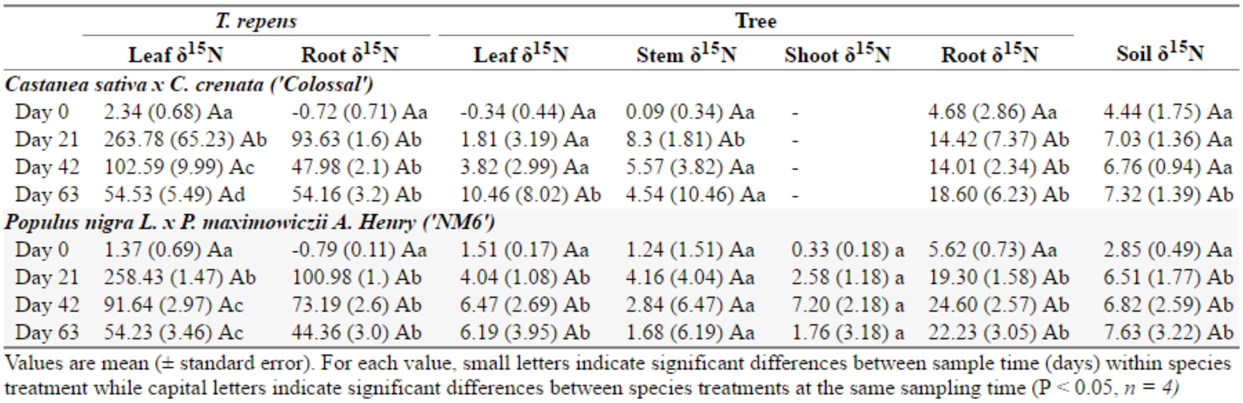
In chestnut, **δ**^{15}N values in roots and leaves differed significantly at the end of the experiment from Day 0 (p = 0.00 and 0.01). Chestnut stem **δ**^{15}N increased after the first sample period but slowly decreased over the next six weeks.

Poplar leaf and root **δ**^{15}N were also significantly higher at the end of the experiment than the beginning (p = 0.00 and 0.00).

There were no significant differences in tree leaf or stem **δ**^{15}N values between poplar and chestnut at any of the sample periods. Nor were there significant differences between clover roots or leaves between poplar and chestnut treatments. Clover leaf **δ**^{15}N in treatment EX were lower than those in treatment IR at day 21 of the experiment, but did not differ at day 42 or at the end of the experiment. More importantly, clover roots in treatment EX did not differ from clover roots in IR.

The **δ**^{15}N values of both species within treatment IR were positively and significantly correlated with days since the isotopic labelling of the clover. Poplar trees showed a Pearson Correlation Index (PCI) of 0.69 and an adjusted r^2 of 0.44 (p = 0.00). Chestnut trees had a PCI of 0.69 and an adjusted r2 of 0.44 (p = 0.00).

Table 10. Summary of $\delta^{15}N$ values in treatment IR by species and compartment over the course of the 63-day experiment.



	T. repens		Tree				
	Leaf $\delta^{15}N$	**Root $\delta^{15}N$**	**Leaf $\delta^{15}N$**	**Stem $\delta^{15}N$**	**Shoot $\delta^{15}N$**	**Root $\delta^{15}N$**	**Soil $\delta^{15}N$**
Castanea sativa x C. crenata ('Colossal')							
Day 0	2.34 (0.68) Aa	-0.72 (0.71) Aa	-0.34 (0.44) Aa	0.09 (0.34) Aa	-	4.68 (2.86) Aa	4.44 (1.75) Aa
Day 21	263.78 (65.23) Ab	93.63 (1.6) Ab	1.81 (3.19) Aa	8.3 (1.81) Ab	-	14.42 (7.37) Ab	7.03 (1.36) Aa
Day 42	102.59 (9.99) Ac	47.98 (2.1) Ab	3.82 (2.99) Aa	5.57 (3.82) Aa	-	14.01 (2.34) Ab	6.76 (0.94) Aa
Day 63	54.53 (5.49) Ad	54.16 (3.2) Ab	10.46 (8.02) Ab	4.54 (10.46) Aa	-	18.60 (6.23) Ab	7.32 (1.39) Ab
Populus nigra L. x P. maximowiczii A. Henry ('NM6')							
Day 0	1.37 (0.69) Aa	-0.79 (0.11) Aa	1.51 (0.17) Aa	1.24 (1.51) Aa	0.33 (0.18) a	5.62 (0.73) Aa	2.85 (0.49) Aa
Day 21	258.43 (1.47) Ab	100.98 (1.) Ab	4.04 (1.08) Ab	4.16 (4.04) Aa	2.58 (1.18) a	19.30 (1.58) Ab	6.51 (1.77) Ab
Day 42	91.64 (2.97) Ac	73.19 (2.6) Ab	6.47 (2.69) Ab	2.84 (6.47) Aa	7.20 (2.18) a	24.60 (2.57) Ab	6.82 (2.59) Ab
Day 63	54.23 (3.46) Ac	44.36 (3.0) Ab	6.19 (3.95) Ab	1.68 (6.19) Aa	1.76 (3.18) a	22.23 (3.05) Ab	7.63 (3.22) Ab

Values are mean (± standard error). For each value, small letters indicate significant differences between sample time (days) within species treatment while capital letters indicate significant differences between species treatments at the same sampling time ($P < 0.05$, *n = 4)*

Soil $\delta^{15}N$ values were higher at day 63 than at day 0 in chestnut pots (p=0.049). In poplar pots, soil $\delta^{15}N$ was higher in each successive sample period than at day 0 (p = 0.02, 0.01, 0.00). Lastly, exudate $\delta^{15}N$ in treatment EX differed significantly from day 21 to day 42 (p=0.02), day 21 to day 63 (p=0.00), and strongly from day 42 to day 63 (p=0.07). Additionally, soil $\delta^{15}N$ was positively correlated with both chestnut and poplar roots (p = 0.00 and 0.00, r^2 = 0.58 and 0.72, and PCI = 0.78 and 0.86).

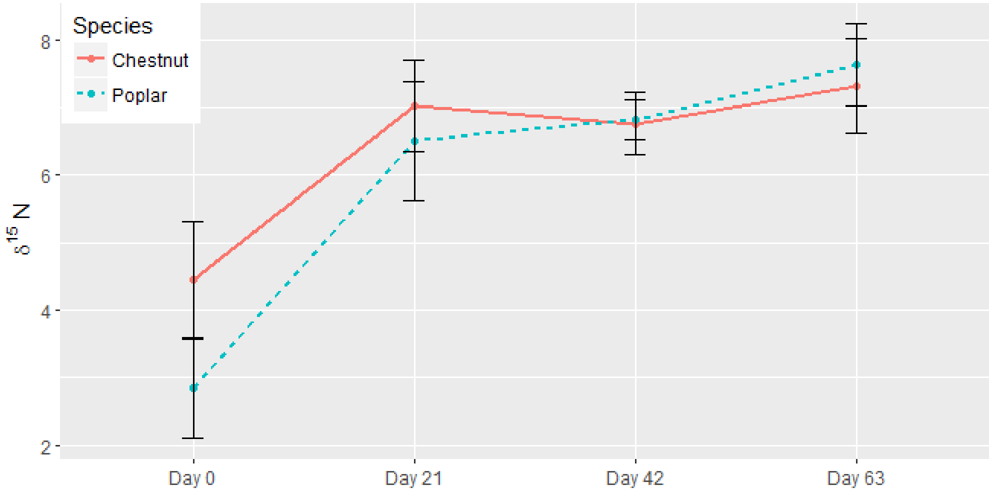


Figure 10. Average $\delta^{15}N$ values for poplar and chestnut soils in treatment IR over the course of the experiment.

Clover root $\boldsymbol{\delta}^{15}$N and exudate $\boldsymbol{\delta}^{15}$N in treatment EX was positively correlated (p=0.00) with an adjusted r^2 = 0.84 and a correlation of 0.93. Clover roots $\boldsymbol{\delta}^{15}$N and tree root $\boldsymbol{\delta}^{15}$N were positively correlated in both chestnut and poplar pots in treatment IR (p = 0.03 and 0.00). Additionally, poplar and tree average $\boldsymbol{\delta}^{15}$N were both positively correlated over successive sample periods (p = 0.00 and 0.00).

4.4.1.2 Exuded Nitrogen

Table 11 describes nitrogen parameters recovered from treatment EX. Exuded N amounts remained steady throughout the experiment and did not differ significantly between periods. Daily exudation throughout the experiment averaged at 2.62 (±1.24) mg N. Total exudation over 63 days amounted to 172.8 (±78.5) mg N.

The percent of clover total N content that was exuded did not significantly differ between experimental periods. Daily exudation averaged at 0.16% (±0.04) of total clover N. Total experimental exudation amounted to 3.4% (±0.85) of total clover N.

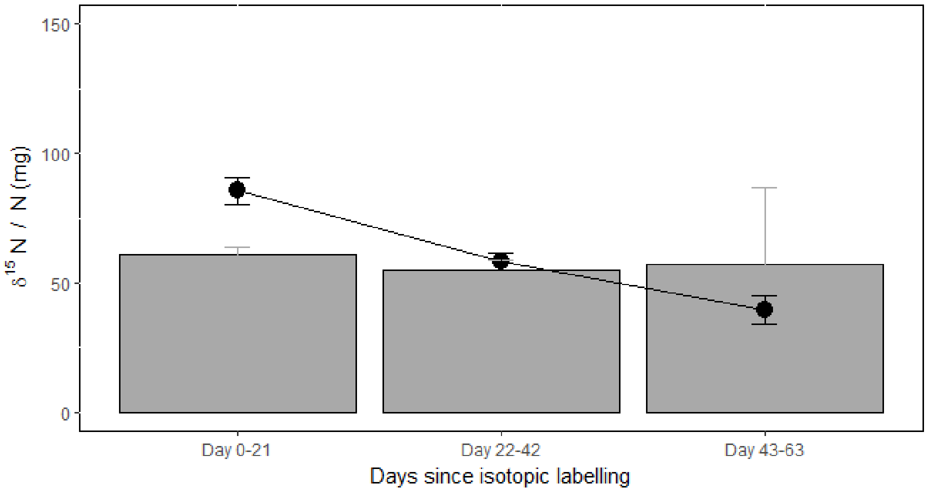
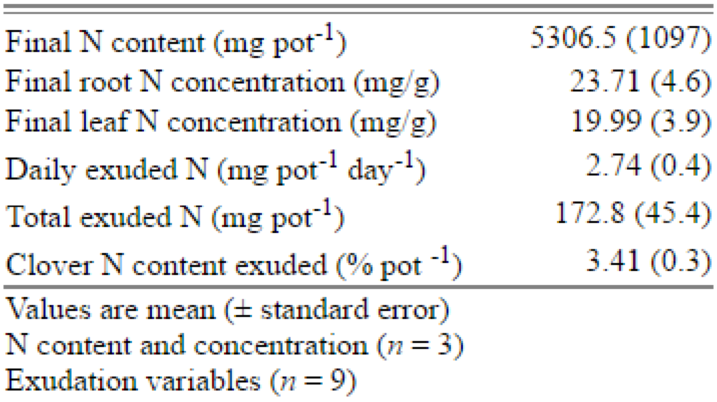


Figure 11. Average $\boldsymbol{\delta}^{15}$N values (line) and N content in milligrams (bars) of white clover exudate in exudate experiment (EX). Horizontal bars represent standard error.

Table 11. Table summary of parameters from clover exudate experiment (EX).



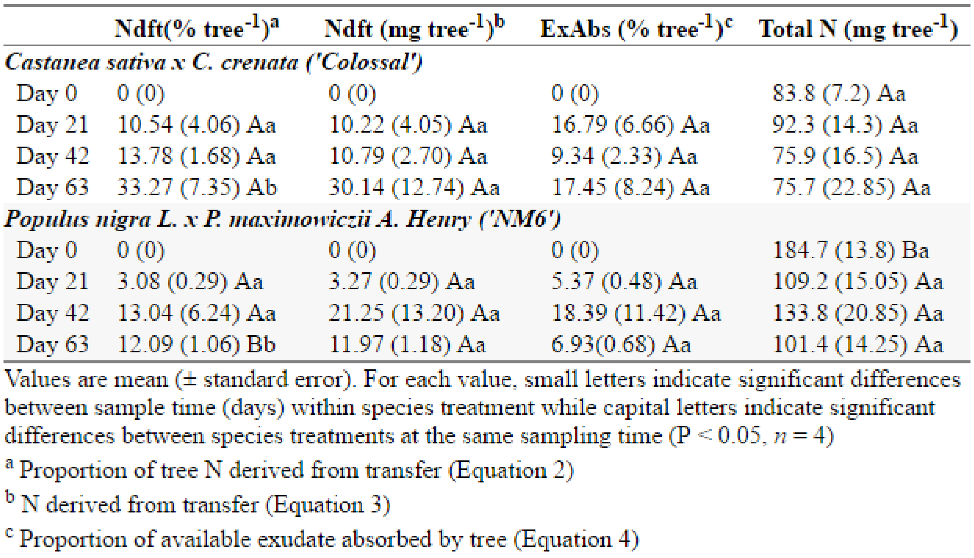
Final N content (mg pot^{-1})	5306.5 (1097)
Final root N concentration (mg/g)	23.71 (4.6)
Final leaf N concentration (mg/g)	19.99 (3.9)
Daily exuded N (mg pot^{-1} day^{-1})	2.74 (0.4)
Total exuded N (mg pot^{-1})	172.8 (45.4)
Clover N content exuded (% pot $^{-1}$)	3.41 (0.3)

Values are mean (± standard error)
N content and concentration ($n = 3$)
Exudation variables ($n = 9$)

4.4.1.3 Percent N Derived from Transfer

The δ^{15}N values were averaged by individual trees within pots and applied to equations 3, 4, and 5 to determine %Ndft, mgNdft, and %ExAbs, which are shown in Table 12.

Table 12. Summary of N transfer parameters from isotopic labelling experiment.



	Ndft(% $tree^{-1}$)^{a}	**Ndft (mg $tree^{-1}$)^{b}**	**ExAbs (% $tree^{-1}$)^{c}**	**Total N (mg $tree^{-1}$)**
Castanea sativa x C. crenata ('Colossal')				
Day 0	0 (0)	0 (0)	0 (0)	83.8 (7.2) Aa
Day 21	10.54 (4.06) Aa	10.22 (4.05) Aa	16.79 (6.66) Aa	92.3 (14.3) Aa
Day 42	13.78 (1.68) Aa	10.79 (2.70) Aa	9.34 (2.33) Aa	75.9 (16.5) Aa
Day 63	33.27 (7.35) Ab	30.14 (12.74) Aa	17.45 (8.24) Aa	75.7 (22.85) Aa
Populus nigra L. x P. maximowiczii A. Henry ('NM6')				
Day 0	0 (0)	0 (0)	0 (0)	184.7 (13.8) Ba
Day 21	3.08 (0.29) Aa	3.27 (0.29) Aa	5.37 (0.48) Aa	109.2 (15.05) Aa
Day 42	13.04 (6.24) Aa	21.25 (13.20) Aa	18.39 (11.42) Aa	133.8 (20.85) Aa
Day 63	12.09 (1.06) Bb	11.97 (1.18) Aa	6.93(0.68) Aa	101.4 (14.25) Aa

Values are mean (± standard error). For each value, small letters indicate significant differences between sample time (days) within species treatment while capital letters indicate significant differences between species treatments at the same sampling time ($P < 0.05$, $n = 4$)

[a] Proportion of tree N derived from transfer (Equation 2)

[b] N derived from transfer (Equation 3)

[c] Proportion of available exudate absorbed by tree (Equation 4)

Transfer was measured as cumulative throughout the successive sample periods. In chestnuts, %Ndft values were higher than day 21 at day 63 (p = 0.01). In poplar pots, estimates were not significantly different from day 21 to the end of the experiment. Total estimated N transfer for chestnut treatments at the end of the experiment was 33.3% (±7.4) of total tree N. In poplars, estimated N transfer ranged between 12.1% (±1.1) of total tree N. Percent transferred N differed significantly between poplar and chestnut at the end of the experiment (p = 0.04).

Chestnut Ndft% was positively correlated with days since the beginning of the experiment (p= 0.00) with an adjusted r^2 of 0.63. The same was true in poplar treatments (p = 0.00), with an adjusted r^2 value of 0.78.

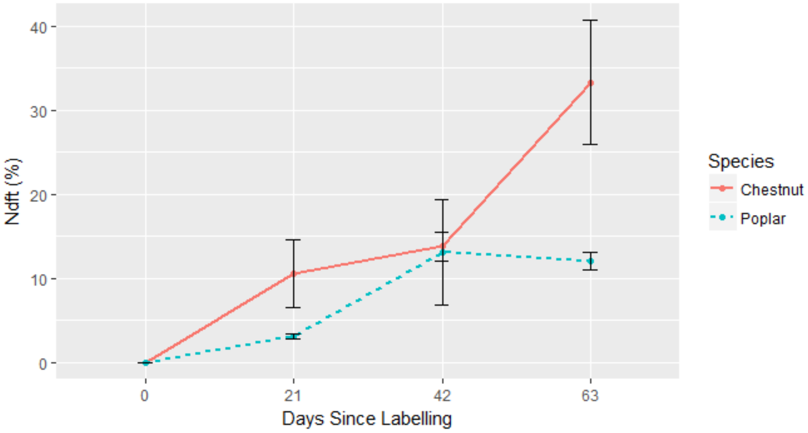


Figure 12. Percent of nitrogen derived from transfer (%Ndft) by species over the course of the experiment.

4.4.1.4 Tree N Content Derived from Transfer (mgNdft)

Nitrogen content derived from transfer within trees showed similar patterns to %Ndft, as is to be expected. However, there were no significant differences. Positive and significant correlations were found between mgNdft and days since the beginning of the experiment in chestnut (p = 0.01) and poplar (p = 0.00). Total N transfer in chestnut pots was 30.14(±12.7) mg N per pot and

11.97(±1.8) mg N per pot in poplars. No significant differences existed between species treatments.

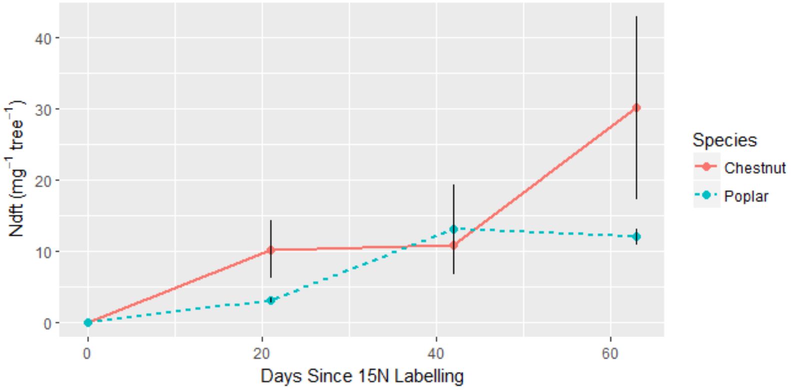


Figure 13. Nitrogen content derived from transfer by species over the course of the experiment

4.4.1.5 Percent of Exuded N Absorbed (%ExAbs)

Percent of total clover-exuded N absorbed into the trees were produced on the assumption that all transfer occurred via root exudates and had the same trends as N content transfer. There were no statistically significant difference between sample periods in poplar or chestnut pots. No significant correlations were found between the percent of exudate absorbed and days since the beginning of the experiment. Percent exuded absorbed in chestnut pots at the end of the experiment was 17.4% (±8.2) per pot by the end of the experiment and 6.9% (±0.7) per pot in poplars. No significant differences existed between species treatments.

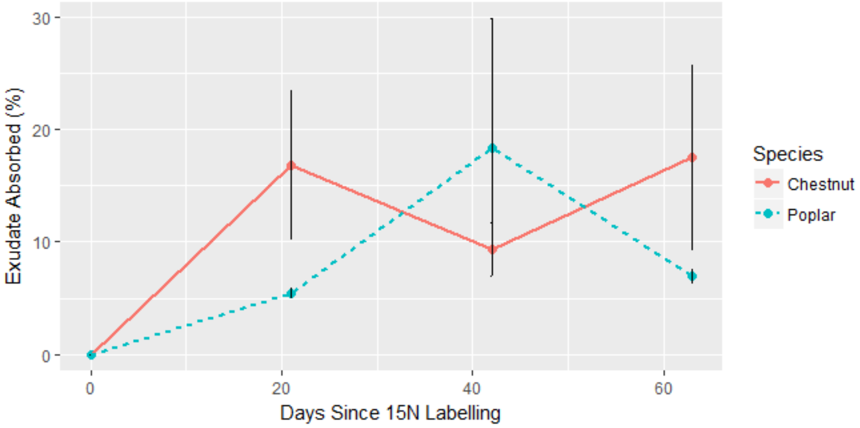
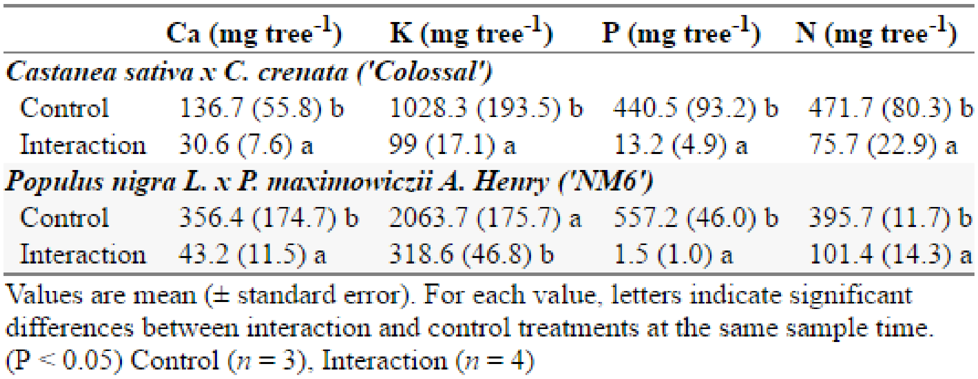


Figure 14. Percentage of available exudate absorbed by each tree species over the course of the experiment.

4.4.2 Tree Nutrients (N, P, K, and Ca)

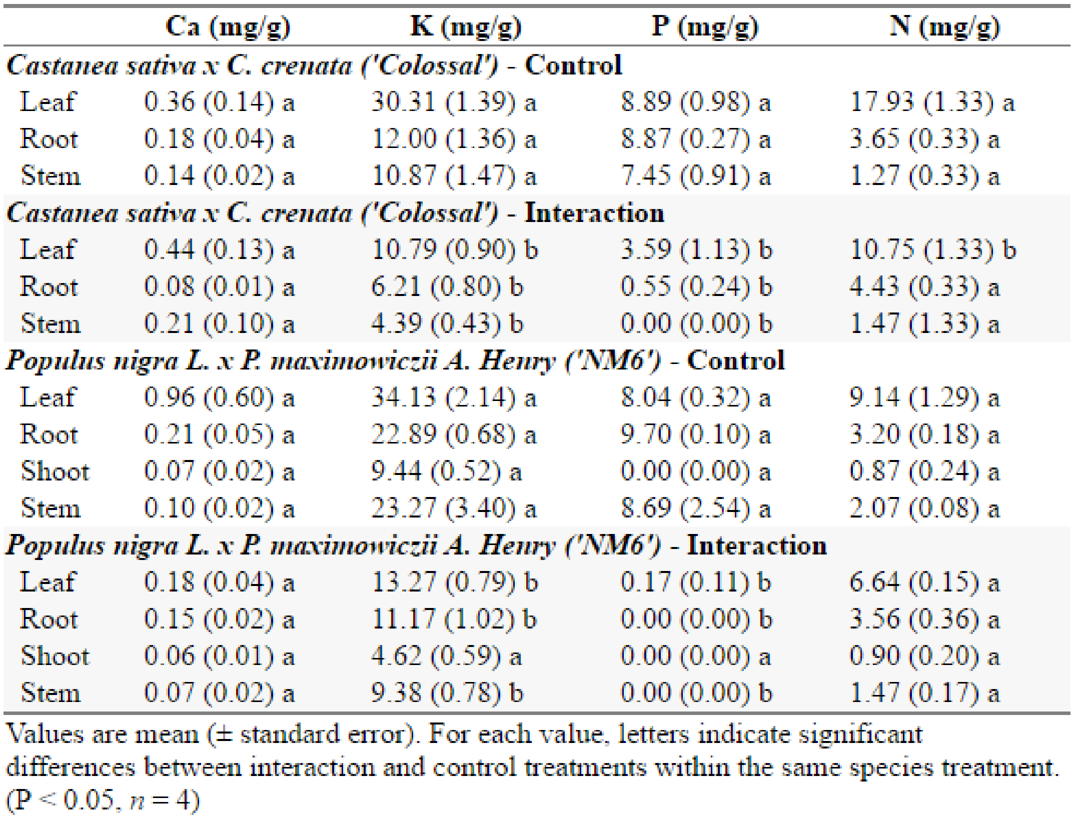
Table 13. Summary of nutrient content per tree between treatments IR and C at the end of the experiment.



	Ca (mg tree^{-1})	K (mg tree^{-1})	P (mg tree^{-1})	N (mg tree^{-1})
Castanea sativa x C. crenata ('Colossal')				
Control	136.7 (55.8) b	1028.3 (193.5) b	440.5 (93.2) b	471.7 (80.3) b
Interaction	30.6 (7.6) a	99 (17.1) a	13.2 (4.9) a	75.7 (22.9) a
Populus nigra L. x P. maximowiczii A. Henry ('NM6')				
Control	356.4 (174.7) b	2063.7 (175.7) a	557.2 (46.0) b	395.7 (11.7) b
Interaction	43.2 (11.5) a	318.6 (46.8) b	1.5 (1.0) a	101.4 (14.3) a

Values are mean (± standard error). For each value, letters indicate significant differences between interaction and control treatments at the same sample time. ($P < 0.05$) Control (*n* = 3), Interaction (*n* = 4)

Table 14. Summary of average nutrient concentrations between treatments IR and C at the end of the experiment.



	Ca (mg/g)	K (mg/g)	P (mg/g)	N (mg/g)
***Castanea sativa x C. crenata ('Colossal')* - Control**				
Leaf	0.36 (0.14) a	30.31 (1.39) a	8.89 (0.98) a	17.93 (1.33) a
Root	0.18 (0.04) a	12.00 (1.36) a	8.87 (0.27) a	3.65 (0.33) a
Stem	0.14 (0.02) a	10.87 (1.47) a	7.45 (0.91) a	1.27 (0.33) a
***Castanea sativa x C. crenata ('Colossal')* - Interaction**				
Leaf	0.44 (0.13) a	10.79 (0.90) b	3.59 (1.13) b	10.75 (1.33) b
Root	0.08 (0.01) a	6.21 (0.80) b	0.55 (0.24) b	4.43 (0.33) a
Stem	0.21 (0.10) a	4.39 (0.43) b	0.00 (0.00) b	1.47 (1.33) a
***Populus nigra L. x P. maximowiczii A. Henry ('NM6')* - Control**				
Leaf	0.96 (0.60) a	34.13 (2.14) a	8.04 (0.32) a	9.14 (1.29) a
Root	0.21 (0.05) a	22.89 (0.68) a	9.70 (0.10) a	3.20 (0.18) a
Shoot	0.07 (0.02) a	9.44 (0.52) a	0.00 (0.00) a	0.87 (0.24) a
Stem	0.10 (0.02) a	23.27 (3.40) a	8.69 (2.54) a	2.07 (0.08) a
***Populus nigra L. x P. maximowiczii A. Henry ('NM6')* - Interaction**				
Leaf	0.18 (0.04) a	13.27 (0.79) b	0.17 (0.11) b	6.64 (0.15) a
Root	0.15 (0.02) a	11.17 (1.02) b	0.00 (0.00) b	3.56 (0.36) a
Shoot	0.06 (0.01) a	4.62 (0.59) a	0.00 (0.00) a	0.90 (0.20) a
Stem	0.07 (0.02) a	9.38 (0.78) b	0.00 (0.00) b	1.47 (0.17) a

Values are mean (± standard error). For each value, letters indicate significant differences between interaction and control treatments within the same species treatment. ($P < 0.05$, $n = 4$)

Nitrogen

Chestnut leaf N concentrations were significantly higher in the control throughout all four sample periods (p = 0.00, 0.00, 0.00, 0.02). Chestnut roots in the interaction treatment were higher than in the control at day 0 (p = 0.00). Besides these, no other differences in N concentrations were observed in chestnut pots. Poplar shoots in the interaction treatment had higher N concentrations at day 0 (p = 0.00). While poplar leaves in the control treatment had significantly higher N concentrations at days 21 and 42 (p = 0.00, 0.00), N concentrations did not differ in any compartment between poplar treatments by day 63. In both tree species, N concentrations between interaction and control treatments differed more in early sample periods but became more similar towards the end of the experiment.

Chestnut leaf N content was higher in controls at days 21, 42, and 63 ($p = 0.00, 0.00, 0.00$). Poplar leaf N content showed the same trend with controls being higher in the last three sample periods ($p = 0.00, 0.00, 0.00$). Total tree N content values were higher in chestnut controls at days 42 and 63 ($p = 0.00, 0.00$) and higher in poplar controls at days 21, 42, and 63 ($p = 0.00, 0.00, 0.00$). Poplar N content was higher than chestnut N at day 0 ($p = 0.00$) but no differences occurred between species treatments in subsequent sampling periods.

Clover N contents among treatments did not differ at day 0 of the experiment. At day 21, clover grown in control treatments was higher than in treatment EX ($p = 0.02$), but did not differ from clover grown in treatment IR. At day 42, control treatments had higher N content than in treatment EX, IR (chestnut), and IR (poplar) ($p = 0.00, 0.00, 0.00$). By day 63, treatment EX had lower N contents than in C, IR (chestnut), and IR (poplar) ($p = 0.00, 0.00, 0.01$), but there were no differences between control and interaction treatments. Clover N content and tree N content showed no correlation in chestnut pots from treatment IR. However, poplar pots showed a negative correlation between the two factors ($p = 0.04$) with an adjusted r^2 of 0.19 and a Pearson's Correlation Index of (-0.499).

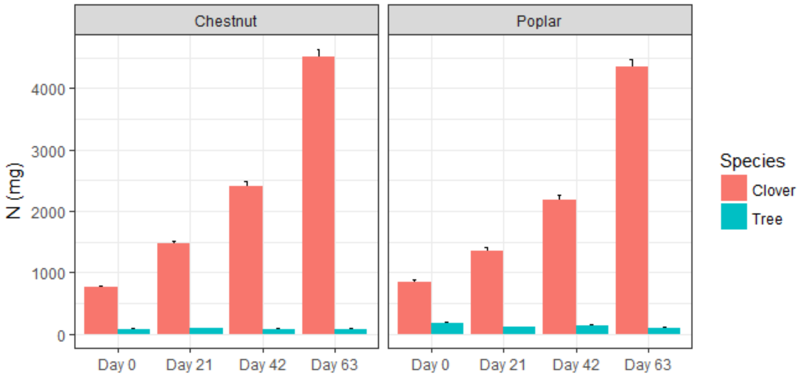


Figure 15. Changes in nitrogen content over time in clover and trees within treatment IR.

<u>Phosphorous</u>

Chestnut leaf P concentrations were higher in controls at days 42 and 63 (p = 0.04 and 0.00). Root concentrations were higher in controls at days 21, 42, and 63 (p = 0.00, 0.00, and 0.00). Stem concentrations were higher in controls at days 42 and 63 (p = 0.03 and 0.00).

Poplar leaf P concentrations were higher in controls at day 21, 42, and 63 (p = 0.03, 0.00, and 0.00), poplar stem concentrations were higher at days 42 and 63 (0.03 and 0.00), and poplar roots had higher P concentrations at day 63 (p = 0.00).

Total chestnut tree phosphorous was significantly higher in controls by day 63 (p = 0.00) and total poplar tree phosphorous was higher in controls at days 42 and 63 (p = 0.00 and 0.00). No differences in total P content were observed between poplar and chestnut pots throughout the experiment.

There were no differences in clover P content between poplar and chestnut interaction treatments at any point. At the end of the experiment, clover in treatment EX had a higher P content than both interaction treatments (p = 0.00 and 0.00). Clover grown in controls had higher P content than EX, IR (chestnut), and IR (poplar) (p = 0.046, 0.00, and 0.00).

Clover P content and tree P content showed no linear correlations in chestnut pots from treatment IR. However, poplar pots in this treatment did show a significant positive correlation between clover and tree P (p = 0.02) with an adjusted r^2 of 0.31 and a Pearson's Correlation Index of 0.595.

Potassium

Potassium was measured in both chestnuts and poplars from treatments IR and C. Chestnut leaf K concentrations were higher in control treatments from day 21 until the end of the experiment (p = 0.01, 0.00, and 0.00). In poplars, both leaves and stems in control treatments had higher concentrations from day 21 onward (p = 0.00, 0.00, 0.00, and 0.00). Additionally, control poplar roots were higher at day 63 (p = 0.00). The same differences were observed in both trees in regards to K content by plant compartment. Total tree K content in chestnut controls were found to higher at days 42 and 63 (p = 0.00 and 0.00). In poplars, control tree K content was higher at days 21, 42, and 63 (p = 0.01, 0.00, 0.00)

Calcium

Fewer significant differences were found in terms of calcium concentrations and contents. No differences in Ca concentrations were found amongst different plant components between control and interaction treatments in poplar or chestnut plots. However, chestnut and poplar leaf Ca contents were higher in controls than in interaction treatments at day 63 (p = 0.00 and 0.00).

Similarly, both poplar and chestnut total tree Ca contents were higher in controls by the end of the experiment (p = 0.00 and 0.01).

4.4.3 Soil Nitrogen

In control and interaction treatments, soil nitrate levels declined from day 0 to day 63 of the experiment, with a levels being significantly lower at the end than at the beginning (see Figure 16).

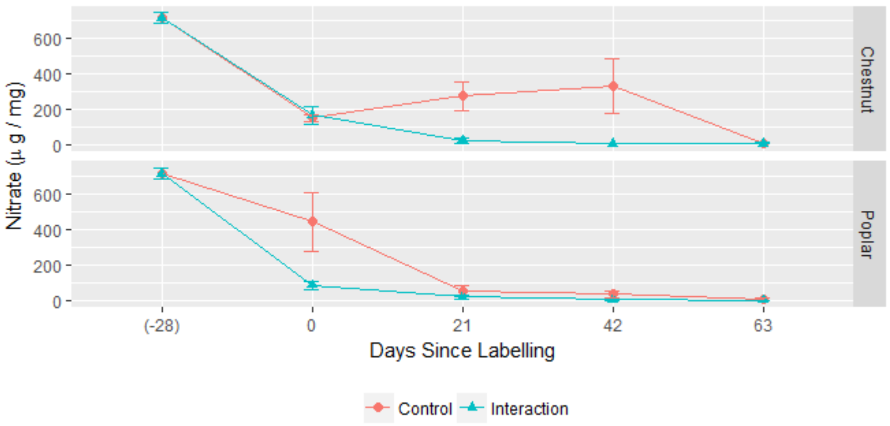
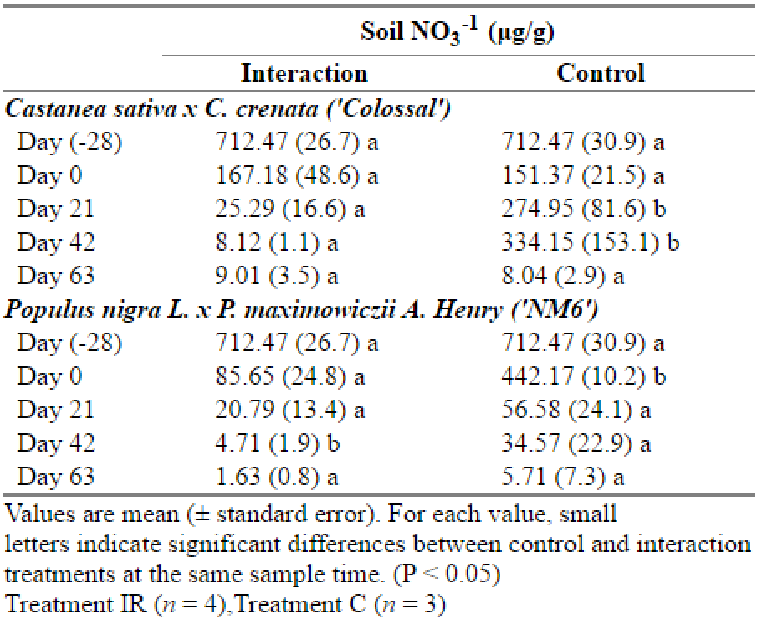


Figure 16. Average soil nitrate concentrations in treatments IR and C over the course of the experiment, including initial soil samples from bagged soil (-28).

At day 0, poplar controls showed higher nitrate levels than chestnut control pots (p = 0.00), this reversed by day 21, with chestnut controls being higher (p = 0.00). Curiously, chestnut soil nitrate in control pots rose slightly between days 0 and 42, before declining to levels similar to other treatments by day 63. As a result, chestnut control pots had higher nitrate levels than chestnut interaction pots at days 21 and 42 (p = 0.00 and 0.00). However, by the end of the experiment, there were no significant differences in soil nitrate concentrations between all treatments.

Table 15. Summary of average soil nitrate concentrations in treatments IR and C.



	Soil NO_3^{-1} (µg/g)	
	Interaction	**Control**
Castanea sativa x C. crenata ('Colossal')		
Day (-28)	712.47 (26.7) a	712.47 (30.9) a
Day 0	167.18 (48.6) a	151.37 (21.5) a
Day 21	25.29 (16.6) a	274.95 (81.6) b
Day 42	8.12 (1.1) a	334.15 (153.1) b
Day 63	9.01 (3.5) a	8.04 (2.9) a
Populus nigra L. x P. maximowiczii A. Henry ('NM6')		
Day (-28)	712.47 (26.7) a	712.47 (30.9) a
Day 0	85.65 (24.8) a	442.17 (10.2) b
Day 21	20.79 (13.4) a	56.58 (24.1) a
Day 42	4.71 (1.9) b	34.57 (22.9) a
Day 63	1.63 (0.8) a	5.71 (7.3) a

Values are mean (± standard error). For each value, small letters indicate significant differences between control and interaction treatments at the same sample time. ($P < 0.05$)
Treatment IR ($n = 4$), Treatment C ($n = 3$)

Soil organic N measurements were taken in treatment IR pots as a result of the isotopic analysis. An increasing trend was seen from day 0 to day 63 in percent soil N in both chestnut and poplar pots (see Figure 21). However, no significant differences were found between sample periods or tree species.

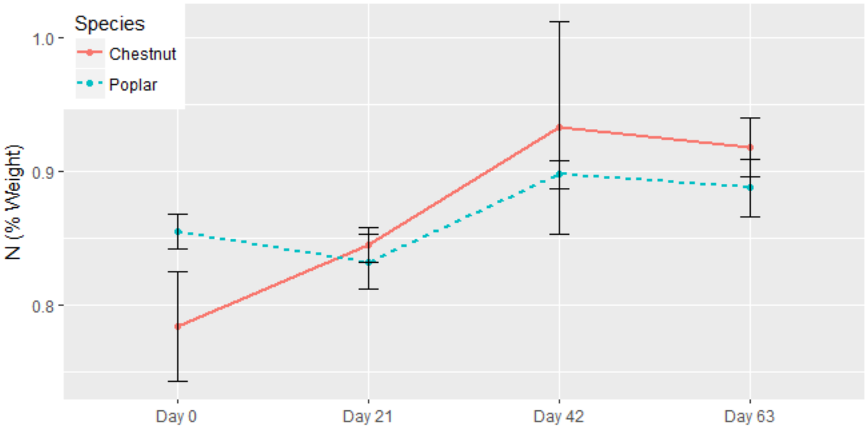


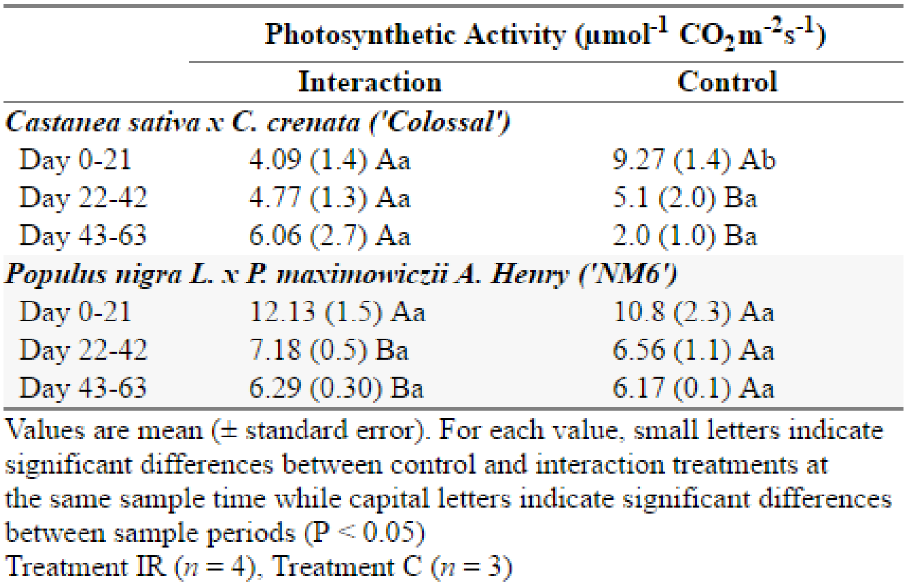
Figure 17. Changes in average soil organic nitrogen in treatment IR over time.

4.4.4 Photosynthesis

Poplars in both treatments IR and C declined in photosynthetic activity from day 0 to day 63, and did not differ between treatments at any point. Both treatments were significantly and inversely correlated with days since the beginning of experiment Pearson Correlation Indexes of (-0.56) and (-0.73) for treatments C and IR, respectively.

Chestnut controls showed higher photosynthetic activity than chestnuts in treatment IR in the first sample period ($p = 0.01$) but declined over the next two periods, while treatment IR grew (see Figure 25). Chestnuts in treatment IR did not have a significant linear correlation, while controls showed a significant negative correlation ($p = 0.00$) with a Pearson's Correlation of (-0.74).

Table 16. Summary of photosynthesis measurements in treatments IR and C



	Photosynthetic Activity (µmol^{-1} CO$_2$m^{-2}s^{-1})	
	Interaction	**Control**
Castanea sativa x C. crenata ('Colossal')		
Day 0-21	4.09 (1.4) Aa	9.27 (1.4) Ab
Day 22-42	4.77 (1.3) Aa	5.1 (2.0) Ba
Day 43-63	6.06 (2.7) Aa	2.0 (1.0) Ba
Populus nigra L. x P. maximowiczii A. Henry ('NM6')		
Day 0-21	12.13 (1.5) Aa	10.8 (2.3) Aa
Day 22-42	7.18 (0.5) Ba	6.56 (1.1) Aa
Day 43-63	6.29 (0.30) Ba	6.17 (0.1) Aa

Values are mean (± standard error). For each value, small letters indicate significant differences between control and interaction treatments at the same sample time while capital letters indicate significant differences between sample periods ($P < 0.05$)
Treatment IR ($n = 4$), Treatment C ($n = 3$)

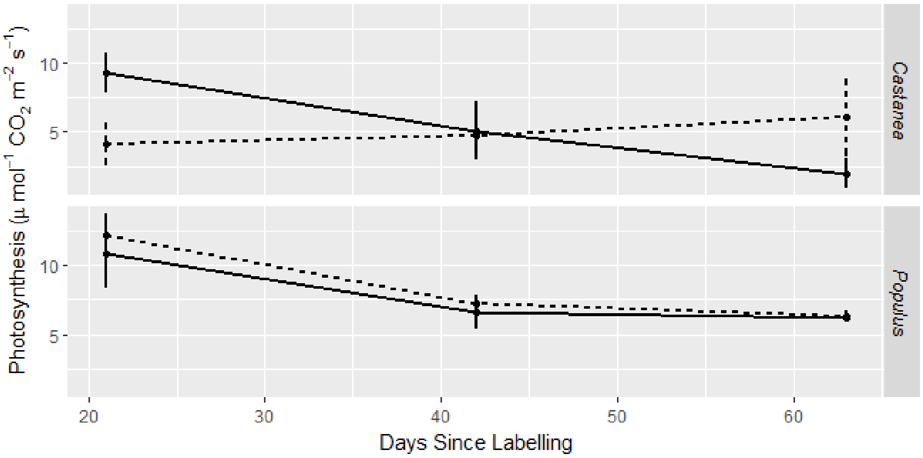
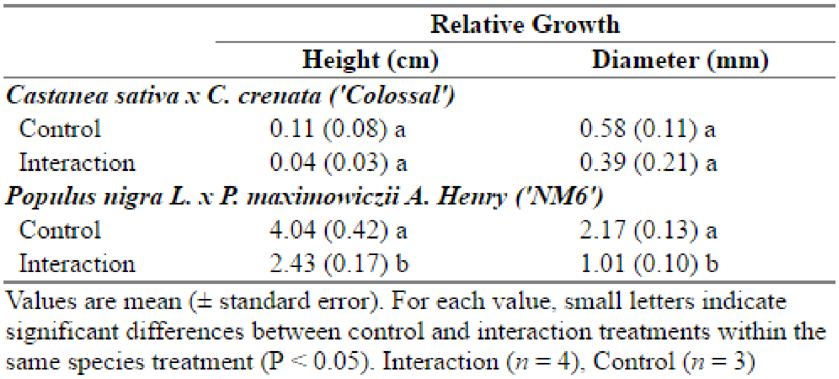


Figure 18. Photosynthetic activity in treatment IR (dotted line) and C (solid line)

4.4.5 Height and Diameter

Since chestnut trees were purchased as one-year saplings, there was an existing degree of morphological variability going into the experiment. To eliminate the effect of this, values of height and diameter were expressed in relative growth compared to the initial sample (shown in Table 17). Analysis of chestnut height growth showed no significant differences between sample dates or treatments. In poplar pots, which were started from cuttings, control treatments had both higher relative height ($p = 0.00$) and diameter ($p = 0.00$) compared to the interaction treatment by the end of the experiment.

Table 17. Summary of relative tree growth at the end of the experiment.



	Relative Growth	
	Height (cm)	Diameter (mm)
Castanea sativa x C. crenata ('Colossal')		
Control	0.11 (0.08) a	0.58 (0.11) a
Interaction	0.04 (0.03) a	0.39 (0.21) a
Populus nigra L. x P. maximowiczii A. Henry ('NM6')		
Control	4.04 (0.42) a	2.17 (0.13) a
Interaction	2.43 (0.17) b	1.01 (0.10) b

Values are mean (± standard error). For each value, small letters indicate significant differences between control and interaction treatments within the same species treatment ($P < 0.05$). Interaction ($n = 4$), Control ($n = 3$)

4.4.6 Other Correlations between Variables

Percent nitrogen derived from transfer and N content derived from transfer were positively and significantly correlated in both chestnuts and poplars (p = 0.00 and 0.00). Furthermore, N content derived from transfer was also positively and significantly correlated with total tree N content in both species (p = 0.01 and 0.00)

In both poplar and chestnut pots of treatment IR, there was a negative correlation between soil nitrate and percent nitrogen derived from transfer (%Ndft). As soil nitrate declined throughout the experiment, nitrogen transfer continued despite the lack of resources. Chestnuts had a PCI of (-0.55) and poplars had a PCI of (-0.86) (p = 0.02 and 0.00).

Photosynthetic activity was not correlated with leaf N content in poplar controls or interaction treatments. In chestnuts, photosynthesis was positively and significantly correlated with leaf N content (p = 0.00, r^2 = 0.67) but not in the control (p = 0.08).

Regardless of exudate or root estimates, photosynthetic activity was positively correlated with N content derived from transfer (mgNdft) within leaves of chestnuts ($p = 0.04$, PCI = 0.86, $r^2 = 0.72$). Conversely, poplar trees showed a significant negative correlation for the same variables ($p = 0.00$, PCI = (-0.62), $r^2 = 0.32$).

4.5 Discussion

The goal of this study was to establish the existence of and quantify the direct nitrogen transfer mechanism between herbaceous legumes and tree species. Due to limitations in resources, this project did not separate nitrogen transfer between mycorrhizal and exudate sources. However, since existing research has shown that mycorrhizal transfer typically makes up a relatively small portion of total transfer [15,16], this did not harm the integrity of the study.

The use of ^{15}N leaf-feeding techniques allowed us to discern between the relative contributions of the soil and clover exudate to tree N content. Labelling techniques in tracing experiments similar to these have been shown to be reliable in multiple studies [14,16,17,121,141,146]. After labelling, the $\boldsymbol{\delta}^{15}N$ values of clover were similar to those found in previous studies on this species (*T. repens*), confirming that the clover was effectively labelled [141]. The added N to the clover that occurred through labelling accounted for less than 0.1% of the total clover nitrogen by the end of the experiment. As a result, leaf labelling did not likely physiologically affect clover growth or exudation and therefore would not influence estimates of N transfer. The decrease in $\boldsymbol{\delta}^{15}N$ values in clover biomass compartments and exudates over time was a result of biomass accrual and subsequent ^{15}N dilution.

4.5.1 Clover Exudates

Clover N content in the exudate experiment (EX) was lower than that in the interaction treatment (IR) by the end of the experiment, however this was the only difference seen between these treatments and could have been due to the week spent in hydroponic tanks. As a result, the lack of statistically significant differences in either N content or $\boldsymbol{\delta}^{15}$N between these treatments throughout the majority of the experiment implies that exudate values obtained from treatment EX were representative of transfer in interaction treatments. It is important to note, however, that exudation potential is often positively influenced by other root systems within the rhizosphere [115]. Estimates for exudation potential came from clover grown alone, thus, the lack of interaction with other roots in this treatment could result in different exudate estimates.

The exudate was clearly labelled by the ^{15}N isotope, meaning a portion of the exudate came from mobile N within the plant. The relatively lower $\boldsymbol{\delta}^{15}$N values seen in the exuded N compared to clover biomass, particularly towards the end of the experiment, supports the fact that exuded mobile N are usually recently fixed compounds within root nodules [116,146].

Exudation potential of white clover (*T. repens*) amounted to roughly 2.74 mg N day^{-1} pot^{-1}. Few studies have explored the exudation potential of this species. Høgh-Jensen and Schjoerring et al (2000) revealed that white clover exuded 4.5-7.5 g N year^{-1} m^{-2} to rye grass in mixed clover-grass leys in Copenhagen. When extrapolated to a square meter, the exudation potential of white clover in this study would be 0.04 g N m^{-2} day^{-1}, which would put it within a similar rate, given similar growing season lengths (around 150 days). Additionally, the percent of total clover nitrogen that was exuded by the end of the experiment (3.4%) falls in line with estimates from previous research, which suggest about 3% of N captured during biological nitrogen fixation is secreted from the roots through exudation [116].

4.5.2 N Transfer

Values of nitrogen transfer in this study were retrieved using only exudate $\delta^{15}N$ as the "donor" source in equation 3. However, since the ^{15}N label could be unevenly portioned within the clover, $\delta^{15}N$ values of tree roots and exudates could differ [16]. The $\delta^{15}N$ values of the clover roots represent both mobile N and N bound in radical structure within total root N content [15]. These values are used in situations where decaying root cells are the main form of transfer. Because of this, a similar study by Jalonen et al. (2009) used the same equation to determine the contributions of N from *G. sepium* trees to *D. aristatum*, but also substituted the $\delta^{15}N$ values of tree roots to account for this potential difference.

In this study, $\delta^{15}N$ values did not differ significantly between exudate and clover roots. Isotopic analysis of cover in (IR) and (EX) revealed that the clover leaves, roots, and exudates were all effectively labelled and showed similar patterns to what has been seen in previous ^{15}N transfer experiments [15,16]. Also it is extremely unlikely that the young clover roots (111 days old by the end of the experiment) would have had any significant issues with senescence or root decay [141]. Clover roots and stolons have high longevity, averaging 290 and 411 days before showing signs of decay, respectively [149]. Moreover, it has been suggested by multiple publications that the majority of transfer takes place through exudates during early growth stages of legumes [13,141]. Considering also that soil organic N did not significantly rise during the experiment, it can be safely assumed that the majority of transfer taking place in this experiment was through direct transfer pathways. As a result, this study did not use $\boldsymbol{\delta}^{15}$N values from clover roots in transfer estimates.

This study did not attempt to differentiate between direct transfer through exudates or through common mycelial networks as other studies have [146]. However, the use of potting soil as the growth medium in this study would likely have hampered mycelial growth due to a lack of natural fungal populations. Additionally, other studies, which used natural soils, reported a low contribution to overall transfer (<1%) through fungal connections [146]. As a result, it can then be assumed the majority of direct transfer in this study likely occurred via living root exudates.

Estimates of nitrogen transfer were statistically higher in chestnuts compared to poplars at the end of the experiment. Since $\boldsymbol{\delta}^{15}$N values between clover from poplar and chestnut pots in treatment IR were very similar, differences in transfer could be due to varying abilities to capture exudate between species or differing rhizospheric interactions between root systems, which can greatly affect exudation [13]. The difference in transfer between species could also be attributed to differing root patterns. Since concentrations of N exudates are higher closer to the roots, transfer is greater when roots of the two species come in close proximity [13]. No measures of rooting patterns were taken during the experiment but an observed difference between the two species revealed that chestnuts likely had a wider-spreading root system and likely came in closer contact with clover roots. Poplar roots tended toward the bottom of the pots they were in, and had few lateral roots in contact with clover.

The final estimates of transfer for poplar (12%) and chestnut (33%), are within range of previous estimates in other studies. For instance, a similar study quantifying transfer from clover to grass had estimates as high as 51% [142]. Transfer rates increased throughout the experiment in both species. Since exudation remained relatively constant throughout the experiment, with no real differences between sample periods, the observed increases were likely due to an increased ability to absorb available exudate by the end of the experiment. Figure 15 shows the percent of exudate absorbed remaining relatively constant for poplars, which explains the lower transfer values. In chestnuts, however, ability to absorb the available exudate ranged considerably, being highest at day 63, where the largest boost in transfer was observed. Ultimately, estimates of the proportion of available exudate absorbed for poplar (7%) and chestnuts (17.5%) were quite low, although a similar study found similar absorption rates at 22%[15]. The higher values seen in chestnuts were likely due to a wider spreading root crown that would be more capable of capturing exudates. Considering that nitrogen transfer increases in field settings over time [16,21,146,150], these are promising figures for such a short period. Furthermore, since a considerable amount of the exudate ends up in the soil, this could facilitate long-term soil nutrient cycling and greater indirect transfer [146]. Root exudates are also known to positively influence soil microbial activity and can stimulate symbiotic relationships with rhizobia and mycorrhizal fungi, which could encourage transfer through fungal networks and indirect pathways, creating a positive feedback between nitrogen fixation, transfer, and exudation [113].

Since these estimates came from a controlled greenhouse study using potting soil and pots, they may not be entirely representative to what would occur in a field setting over the same period of time. The nature of the pots likely influenced the root structure of both plants in treatment IR, and could mean there was more root-to-root contact. As a result, transfer estimates from this study could be higher than what they would be in a field in the same time frame where roots would have more space to grow and possibly avoid contact. However, as was just mentioned and proved in other studies, nitrogen transfer in field settings increases with time and root growth even in field settings, indicating that these estimates could be representative of long-term nitrogen transfer potential. Additionally, a field setting may actually increase transfer through mycelial networks due to a higher natural abundance of fungal populations.

3.5.3 Nutrient Dynamics, Photosynthesis, and Growth

Both poplar and chestnut trees had higher concentrations and contents of N, K, and Ca by the end of the experiment. The largest differences were seen in the potassium contents of trees between treatments. There was significant evidence for competition over all four of these nutrients between trees and clover, particularly nitrogen. The decrease in soil nitrate over the course of the experiment in interaction treatments indicates a priority use of soil N resources over biologically fixed nitrogen by clover and trees. Additionally, the lack of significant differences between clover N content in controls and in treatment IR indicates that clover was able to outcompete the tree for this plant-available soil N. Due to the limited available soil N sources early on in the experiment, it is reasonable to believe a large portion of the N after that point was biologically fixed by the clover. Since exuded N is often recently fixed, it is likely that N transfer became a more important resource for the trees in interaction treatments, as soil resources were depleted.

Despite the obvious signs of competition for nutrients between clover and trees in interaction treatments, these surprisingly did not seem to affect photosynthetic activity. In poplar pots, photosynthesis was practically the same between control and interaction treatments, with treatment IR being slightly higher throughout. Perhaps even more surprising were the results of chestnut photosynthesis measurements. While values in the control treatment were significantly higher than in treatment IR at the first sample period, they showed a declining trend throughout the entire experiment. Meanwhile, chestnuts in treatment IR increased from the beginning to the end of the experiment. Photosynthesis was linearly correlated with leaf N content in chestnuts from treatment IR but not in any of the other treatments. Moreover, the steady accrual of ^{15}N within poplar and chestnut leaves indicates the importance of transfer to foliar nitrogen. It is possible that leaf nutrient ratios could be partially responsible for this difference in photosynthesis activity. Ultimately, however, the importance of these values to this research is that nutrient competition did not seem to affect photosynthetic activity over the period of this short study.

In terms of growth measurements, no significant differences found between controls in chestnut pots indicates another variable that was seemingly unaffected by nutrient competition. In poplars, however, controls showed greater height and diameter growth. Considering that poplars were started from cuttings and chestnuts were bought as one-year saplings with variable heights and diameters, poplar growth data may be more reliable. However, the short period of time over which the experiment took place diminishes the importance of the growth parameters in general.

The purpose of establishing the controls was to get an idea of how the presence of clover affected the overall nutrition, growth, and metabolism of the tree species when grown together. The circumstances under which these were grown in this study are very different from what would occur in a field setting. Limited space for root growth in pots as well as limited potential for nutrient cycling capability and finite nutrient availability in the potting soil that wasn't regularly fertilized could explain why trees in controls did so much better. Thus, the results of the nutrient analysis probably do not accurately represent competition dynamics where roots are not inhibited by the space limits of a pot and where resources are provided by natural soils. Trees in the control treatment experienced almost no limitations in nutrients during the 63-day experiment, which would likely not be experienced in the field over time without external inputs. Since nitrate levels were the same between interaction and controls by the end of the experiment, trees in controls would likely begin to suffer from nitrogen limitations. At this point trees in the interaction treatment would have an advantage over controls based on the availability of nitrogenous exudate from clover.

4.6 Conclusion

The goal of this study was to establish and quantify the existence of a direct transfer pathway for nitrogen in a controlled environment. The values of percent nitrogen derived from transfer in both poplar (12.1%) and chestnut (33.3%) over nine weeks shows the likely importance of this pathway. Due to the age of the white clover, the generally higher $\boldsymbol{\delta}^{15}$N values in exudate compared to clover roots, and the use of potting soil, it is extremely likely that the majority of transfer occurred through root exudates and not through mycelial connections or root decay. The higher values of transfer, combined with the lack of difference in photosynthetic activity and morphological features between control and interaction treatments, indicate that chestnut root structure may be better suited to direct nitrogen transfer compared to poplar in the short-term. This difference falls in line with the influence of species selection on transfer stressed by Roggy et al (2004).

While nutrient analysis of controls revealed significant signs of competition between clover and trees, these circumstances are not representative of what would occur in the field. Additionally, the negative correlation between soil nitrate and transfer in interaction treatments indicates the ability of trees to persist in environments with low nutrient availability with the help of direct N transfer. This is reinforced by the fact that exudation is positively influenced by higher competition for N, typically as a result of increased biological nitrogen fixation by associated legumes [116,141,146]. The additions of N exudates to the soil would also likely benefit the long-term soil nutrient availability in such systems. It is therefore reasonable to believe that in a field setting, alley cropping chestnut and poplar plantations with white clover would benefit nutrient cycling and growth of trees over time. This has been shown to be true in similar studies [20–22,146,150]

Ultimately, this study, combined with previous research, shows a general trend of an increased importance of direct N transfer in systems. This implies that alley cropping systems such as these could be beneficial to landowners seeking low-input production, particularly on marginal lands with low fertility. They could also prove to be very effective at restoring soils, due to consistent N transfer and additions to the soil. The exudate transfer mechanism for nitrogen provide an important shortcut to the remineralization process that indirect N transfer requires, and could therefore be very useful to landowners. Similar tracing studies on a longer timeframe and in the field, preferably over the length of a full production cycle, are required to more fully understand how exudate-based transfer can benefit tree plantations.

5 Discussion and Research Implications

Previous research has suggested that decaying biomass is the transfer pathway that has the most potential for N transfer. The field study in this research did not discern between modes of transfer, and is therefore not applicable to the argument of relative pathway importance. The greenhouse study, however, revealed an important belowground relationship between trees and herbaceous legumes. White clover (*T. repens*) directly supplied 12.1% of poplar N and 33.3% of chestnut N. Due to the age of the white clover and the high exudate potential during early root growth, it is extremely likely that the majority of transfer occurred via root exudates. Since these values were derived under controlled conditions, they could differ in a field setting. However, research has suggested that direct transfer pathways increase with time in the field, as exudates accrue in the soil [16,98]. While these findings do not disprove the relative importance of indirect transfer, they may suggest that the role of direct transfer has been historically underestimated. Nevertheless, the role of indirect transfer via green manure in improving soil health and poplar nutrition in the field study was obvious. Treatments involving red clover (*T. pratense*) regularly returned more green biomass and nutrients to the soil during mowing, which elevated soil nitrate and nitrogen in poplar leaves over the course of just two years. Despite, this there was a lack of apparent benefits to poplar growth. However, similar studies have shown that this amount of biomass return benefits tree growth in the long-term [19–22]. The results of the field study suggest a similar relationship is likely between poplar and red clover over the next several growing seasons.

Ultimately, this research affirms the importance of nitrogen transfer in agroforestry systems and the need to gain a better understanding of it. It falls in line with several other studies that stress the underrated importance of exudate-based N transfer and the benefits of cover crops to tree plantations in the field. It also reinforces the importance of species selection to N transfer dynamics. Nitrogen transfer can differ widely depending on nitrogen-fixing capacity of the donor, differing root patterns, and differing growth phases [14,136]. The notable difference in N transfer between poplars and chestnuts seen in the greenhouse study supports this theory.

Much more research is needed to understand the mechanisms of N transfer, particularly in the long-term. As previously mentioned, most longer studies have shown that species relations of these kind are often competitive in the short term but ultimately end up being beneficial. However, no long-term field studies have discerned between N transfer mechanisms that result in beneficial relationships. Additionally, few studies have attempted to analyze the effects of management methods on direct transfer. Regular cutting of cover crops, for instance, is shown to increase N transfer through root turnover [149]. The effects of cutting on exudate transfer, however, have not been measured. Similar isotope studies in the field are required to understand how direct mechanisms change over time and to determine their actual importance at the end of a production cycle. This research adds to a small, but powerful block of research that has proven the important role of N transfer dynamics in alley cropping systems. Hopefully, it will assist future studies that seek to address remaining research gaps regarding the effects of time scales, physical and biological factors, and species selection on transfer.

Locally, this research could directly benefit the biofuel and tree nut orchard industries in temperate climates. Clearly, clover-based cover crops have the potential to benefit both poplar and chestnut plantations either through indirect or direct means of N transfer. In the eastern part of the United States, hybrid chestnut trees have provided a potential comeback for a tree that has been nearly wiped out by disease. Michigan, for instance, has more chestnut plantation area than another state in the U.S and a growing chestnut market [151]. The chestnut-clover alley cropping system described in this research has potential to offer a low-input form of production for landowners. At the small scale, this provides an opportunity for landowners to diversify their production and become more resilient to socio-economic stresses. At the large scale, this could aid the growth of a burgeoning industry while providing multiple ecosystem services.

Additionally, biofuels derived from hybrid poplars represent a burgeoning market for low-input production systems in temperate and tropical climates. Despite the ongoing debate over their carbon neutrality, the importance of short-rotation woody crops (SRWC's) intended for biofuels is growing. The Kyoto Protocol of the UNFCCC stressed the importance of agroforestry and short-rotation woody crops to providing ecosystems services such as carbon sequestration while remaining productive [132]. Maintaining these systems without further artificial nitrogen inputs is critical considering the high carbon cost of producing them and the GHG emissions and runoff risks involved during application [2]. This research, as well as others, suggests the potential for utilizing nitrogen-fixers as a replacement for such fertilizers [20]. Moreover, the utilization of direct N transfer could encourage “no-till” agricultural practices which could reduce emissions of nitrous oxide and carbon dioxide when applied at a wide scale. Lastly, the relatively higher biodiversity, soil carbon additions, soil microbial activity, and efficient water use from these systems can be highly beneficial to production and ecosystem protection.

What is perhaps the most intriguing implication of this research is that both of these species, when incorporated with clover into agroforestry systems could be effectively grown on infertile and marginal lands. In fact, since N transfer tends to increase under competitive conditions, this could be the best use for herbaceous legume-tree systems such as these. Since 38% of global land area is currently considered degraded and that number is likely to grow, this could have potentially massive implications for production systems. Inputs to the soil from clover both below and above ground can help to restore soil nutrients on a site while remaining productive. This could ultimately result in healthier soils by the end of one or several production cycles. At a wide scale this could mean increased carbon sequestration and soil protection per unit of land area. Alley cropping systems on marginal lands also provide opportunities for producing other products, such as livestock. Gamble et al. (2014) studied short-rotation willow and poplar plantations alley cropped with forage grasses in Minnesota to determine carbon sequestration and biomass production potential. They found that the poplar hybrid 'NM6' was capable of providing substantial biomass potential while maintaining opportunities for livestock forage [134]. However, a problem posed by SRWC's is that frequent harvest removes considerable nutrients from an ecosystem, making them unsustainable without inputs. Here the potential for alley cropping systems that focus on herbaceous species that could benefit woody species becomes very apparent.

These systems could also be critical to protecting water sources when serving as riparian buffers while also improving landowner income at the small scale. A considerable focus of recent research has been on using SRWC species in riparian buffers, because of their dense root systems that can capture leaching nutrients and runoff [64]. Additionally, other potential ecosystem services provided by these systems are numerous and would likely be paired with an improved and diversified income for landowners. This would improve landowner resiliency to climate change as well as future economic and political shifts, which would be particularly important in tropical climates, where there is a considerable amount of degraded land and higher dependency on agriculture [2]. Many of these areas have already used agroforestry practices with great success, largely because of their ability to enhance nutrient cycling and reduce the need for inputs, which are expensive and often difficult to find. While the exact species combinations used in these experiments are not necessarily feasible in tropical climates, the basic principles regarding nitrogen transfer dynamics discovered in this research can be paralleled to these ecosystems with great benefit.

Global consumption of synthetic nitrogenous fertilizer was estimated at 113,100,000 tonnes in 2013 and is expected to grow at 1.4-1.5% into the next decade [49]. As mentioned before, there is a disproportionate global access and use of fertilizer. Many of the landowners in developing countries cannot get the fertilizers they need to improve production while industrial agriculture systems in developed countries have greater access to fertilizer and over-use it to the point of degrading the surrounding ecosystems. A critical element of this research is outlining the potential for cover crops to replace the need for synthetic nitrogenous fertilizer. This can reduce costs to the landowner and allow them to engage in more diverse production systems (such as silvopasture) that would additionally improve landowner income.

Ultimately, effectively utilizing the nitrogen transfer mechanism can be beneficial to both temperate and tropical landowners and help begin to address many of the issues mentioned in

Chapter 2 through the enhancement of agroecological feedback cycles. In both regions, barriers to the adoption of agroforestry systems most often have to do with fear over direct and incurred costs. The systems described by this research require minimal inputs and labor, have a high ability to restore soil health, and are extremely adaptable to marginal and low-fertility lands. These characteristics could make them more enticing to landowners that are hesitant to change their agricultural practices.

6 Conclusion

The experiments conducted in this research provide further evidence for the existence of a direct transfer mechanism for nitrogen between white clover (*T. repens*) and the two chosen tree species (*Castanea sativa x C. crenata* ("Colossal") and *Populus nigra* L.×*P. maximowiczii* A. Henry ('NM6')). Relative estimated nitrogen contributions by the donor to the trees were 15.9% of poplar N and 33.3% of chestnut N. The majority of this transfer occurred via root exudates. By the end of the experiment, the amount of available N exudate absorbed by the trees was ranged between 7-17.5%, depending on the species, indicating that exudates are a larger source of soil N even than estimated by transfer. Disparity in transfer values between tree species was likely due to varying rooting structures, but the chestnut trees appeared to be more adept to direct N transfer. Ultimately the estimates under controlled greenhouse condition may not accurately reflect transfer over the same time period in the field and could be overestimated. However, since direct N transfer typically increases in the field over time, these estimates may actually represent long-term N transfer in field settings. Furthermore, the field study proved that the presence of red clover (*T. pretense*) in hybrid poplar plantations (*Populus nigra* L.×*P. maximowiczii* A. Henry ('NM6')) significantly improved poplar leaf and soil nitrogen concentrations after just two years at wider spacings. The benefits seen in clover treatments were not reflected in poplar growth. However, previous research suggests that positive effects on growth would likely be seen in ongoing seasons. Spacing treatments in this study showed that narrow row gaps incurred greater signs of competition, while wider row spacing showed signs of beneficial nitrogen transfer.

The results of these studies reveal a high potential for clover species (*T. pretense* and *T. repens*) to benefit nitrogen cycling and relationships in tree plantations. Longer and more in-depth field studies are needed to determine how nitrogen transfer changes over the course of a full production cycle and the relative importance of indirect and direct transfer pathways. Ultimately these cover crops can present a cheap and low-labor solution to maintaining and restoring soil fertility in long-term productive agroforestry systems, the implications of which could be great for both the environment and landowners.

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