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Nutrient and Arbuscular Mycorrhizal Amendments in Prairie Restoration

A THESIS
SUBMITED TO THE FACULTY OF THE GRAUDATE SCHOOL
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Joel Eric Tallaksen

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Iris Charvat, Advisor

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This is to certify that I have examined
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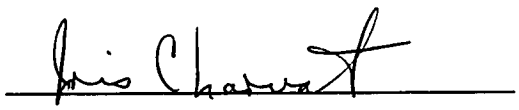

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Abstract

Joel Eric Tallaksen
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Prairie restoration emerged recently as a vegetation management technique and restoration protocols are quickly evolving to incorporate new, more ecologically sound and efficient plant propagation methods. The ability of fertilizers and arbuscular mycorrhizal fungi (AMF) to enhance native plant growth was tested at a wet prairie site in central Minnesota that had previously had a restoration failure.

The Beltsville hydroponic AMF inoculum production method was modified and studied as a means to produce prairie AMF inoculum. Inoculum produced by this method had fewer colonizing fungal spores than desired. This system has potential for AMF inoculum production, should culturing conditions be improved.

A one-time fertilizer and AM inocula application was tested at a restoration site in central Minnesota. Three fertilizer regimes (inorganic fertilizer, slow-release fertilizer [SRF], and an unfertilized control) were combined with four AMF inocula regimes (commercial inoculum/furrows, local inoculum/broadcast, local inoculum/furrows and uninoculated/furrows). Three measures were used to assess potential amendment benefits: biomass, species composition changes and diversity.

Changes in plant biomass were significant for the slow-release fertilizer (SRF) treatment, while the inorganic fertilizer treatment had little affect on biomass. SRF treatment corresponded to increased undesired forb and grass biomass for two years following application. AMF inoculation did not significantly affect biomass.

Species abundance and diversity were significantly altered by SRF. Inorganic fertilizer did not change plant community composition. Plants that increased in abundance were mostly weedy annual grasses, whereas those decreasing in abundance were typically perennial native grasses. AM inoculation treatments did not affect species abundance, however functional group abundance was slightly altered in a few instances. The changes in abundances may or may not have been due to the AMF propagules applied in the AM treatment regimes.

Overall, the restoration site studied did not significantly benefit from fertilizer or AM fungal amendment. Nutrients added in the inorganic treatment were likely leached; while the SRF treatment reduced native plant abundance and biomass. Other similar studies indicate that fertilization is likely not helpful, but some AMF inoculation studies suggest some benefits. Restoration site conditions appear to be an important factor determining whether fertilization or fungal amendments are helpful.

Chapter 1: Literature review- Soil amendments, possible new approaches to prairie restoration

Joel E. Tallaksen

Abstract

Prairie restoration has recently emerged as a vegetation management technique. Consequently, restoration protocols are advancing with the incorporation of new, more ecologically sound, and more efficient plant propagation methods. Soil amendments have been suggested to further improve restoration protocols by promoting plant establishment and growth at newly sown restorations. Fertilization and arbuscular mycorrhizal fungal (AMF) inoculum are two soil amendments thought by some to increase plant success at restoration sites.

Fertilization can increase growth of several native plants, yet many studies have found negative consequences to native plant communities when nutrients are added. The detrimental community responses to nutrient enhancement result from improved growth of more ruderal species under increased nutrient conditions. While direct nutrient addition may not be helpful in restoration, other means of increasing soil fertility, such as composting, may be beneficial.

Arbuscular mycorrhizal (AM) inoculation has been found to be greatly beneficial to potted plants and plant communities in microcosms. Additionally, preliminary field studies indicate possible restoration benefits from AM inoculation. However, at present the practicality of large-scale AM inoculation is limited by the small supply and high cost of AM inocula. Also, many questions remain about large-scale movement of AM fungal species and the potential for movement of invasive AMF.

Introduction

Prairie restoration has become an important tool in landscape management. Historically, restorations were conducted to maintain prairie habitat in the face of increasing agricultural and suburban land use, or to improve pastures that had been overgrazed (Weaver 1954). However, the aesthetic aspects of prairies have gained public recognition and consequently, prairie restorations have become more popular (Morgan et al. 1995). Equally important in increasing the prevalence of prairie restoration is the realization that established native prairies require less maintenance than similar stands planted with non-native species (Brakeman 1975). Facing limited budgets, many public and private organizations are looking towards restoration to reduce land management costs (Morgan et al. 1995). Together, public enthusiasm and cost savings have significantly increased the use of prairie restoration throughout the central United States.

As prairie restoration has become more common, so has the search for techniques of propagating native plant communities, especially techniques based on sound ecological principles. Initially, restoration involved planting native seed via traditional agricultural methods (Weaver 1954), however as their experience increased, restoration practitioners began to understand the planting and growth requirements of native plants. At the same time, a greater volume of prairie and grassland ecosystems research was being carried out (Greene & Curtis 1950; Penfound 1964; Good & Good 1971). Using this experience driven knowledge and a greater understanding of grassland community ecology, restoration protocols incorporating intensive site preparation, burning, and native seed collection were developed (Shirley 1994; SER 1997). The shift to these more holistic

techniques has increased our ability to transform large tracts of highly disturbed land into healthy, self-sustaining prairies in a far shorter period of time (SER 1997).

While current prairie restoration techniques are often successful, problems can and do occur in restoration (Anderson 1999). These difficulties can vary considerably, from unsuccessful establishment of all planted or seeded native species to low abundance of a particular species. In addition, native plant establishment can take years longer than anticipated. Correcting these restoration problems can require management changes and even further attempts at restoration. However, before additional effort is put into further maintenance or restoration work, the specific biological or ecological causes of the initial restoration difficulties should be understood.

I am interested in prairie plant ecology and working with the native plant community to avoid restoration failures. Questions I think are important to ask are: 'How do management practices affect native plant establishment and restoration community ecology?' and further, 'How can management practices be altered to improve restoration community health?' By continuing the trend of developing more ecologically conscious restoration methods, healthy plant communities may be created that are more resistant to environmental pressures and invasion by ruderal species.

This review focuses on the use of fertilizer and arbuscular mycorrhizal (AM) fungal amendments to enhance plant establishment and growth during prairie restoration. Both amendments have been proposed to alleviate plant stress, enhance plant growth, and improve the plant community at restoration sites. However, neither fertilization nor AM fungi have been extensively tested in field situations. Consequently, both their effects on restoration communities and their potential benefits are poorly understood. Also, the

practicality of using these amendments on a landscape scale has not been fully examined. This brief review provides readers a summary of these and other issues surrounding fertilizer and AM amendment use in restoration and also provides background information for later chapters of this thesis.

Fertilization during prairie restoration

A one-time application of fertilizer is currently a part of some of prairie restoration and native planting protocols (MN/DOT 2000; Henze 2001), though it is neither common nor universally accepted (Morgan et al. 1995). Vegetation managers who apply fertilizers during restoration feel that it increases plant establishment and improves plant growth, two important goals in restoration. Fertilization is also used to quickly increase plant biomass on a site, thereby producing plant cover on restoration sites that are often barren prior to restoration (Brakeman 1975). However, many restoration practitioners, restoration handbooks, and restoration protocols specifically avoid any mention of fertilization and usually suggest techniques to lower soil nutrient levels (Morgan et al. 1995; Delany et al. 2000).

In many cases, fertilization can have positive effects on individual native prairie species. Fertilizer applied to *Sorghastrum nutans* occurring in coastal prairie increased biomass several fold (Van Aucken et al. 1992) and cultivated stands of *Andropogon gerardii*, *Schizachyrium scoparium*, *Bouteloua curtipendula* and *Panicum virgatum* had a two fold increase in seed production with added nutrients (Cornelius 1950). In addition, other factors such as tillering of native plants appears to be enhanced with fertilization

(Valvarde & Pisanty 1999). Yet, examples of improved native plant growth with fertilization appear to be limited to situations where native plants were grown in monoculture or in a community heavily dominated by the species exhibiting enhanced growth.

Experiments in remnant prairies and other grasslands have found that regular fertilization negatively affects the native plant community as a whole by diminishing diversity, reducing richness and shifting species composition (Houston & Hyder 1975, Lauenroth 1979, Seastedt et al. 1991). Similarly, nutrients added to mixed-grass prairie (Power 1979), rangeland prairie pastures (Owensby & Smith 1975), and old-field succession sites (Tilman 1987) resulted in reduced diversity and fewer native grasses. Analogous species composition changes due to repeated or heavy fertilizer application have been seen in salt marsh (Boyer & Zedler 1999), serpentine grassland (Hueneke et al. 1990), and sand dune communities (Valvarde & Pisanty 1999).

Plant community changes induced by fertilization are thought to result from variations in the competitive interactions and life histories of plant species at different nutrient levels (Tilman 1986; Tilman 1987). Plants can be loosely assembled into two groups (early-successional and late-successional species), which are adapted to different soil nutrient concentrations (Wilson 1994). The first group (the early-successional species) tend to be annual or biennial, grow very quickly, and produce an abundance of seed (Rejmanek & Richardson 1996). These early-successional species are an indicator of disturbed sites. They grow vigorously in high nutrient soils, yet are less competitive in low nutrient soils. Many are considered undesirable species and are common colonizers during the first few years of restoration. The second group (late-successional species) are

often perennial, tend to grow slowly (often over several years), and have fewer seeds per growing season. These plants are adapted to growing in low nutrient soils and have less pronounced growth increases in response to added nutrients (Wedin & Tilman 1990; Tilman & Wedin 1991). Many of the dominant species common in native prairies are late-successional species.

Competition studies in several grassland habitats and soils clearly show that in low nutrient soils, late-successional prairie plants are more competitive than the ruderal early-succession species (Tilman & Wedin 1991; Wilson & Tilman 1993; Wilson 1994). Since the bulk of species that prairie restoration attempts to re-establish or re-invigorate are late-succession species, the desired restoration species would probably be more abundant in low nutrient soils. Therefore, fertilization does not seem an appropriate treatment for restoration, as it would likely increase the number of ruderal species and reduce the number of native plants.

One study examining fertilizer use during prairie restoration was conducted by Wilson and Gerry (1995), who examine artificially increased and decreased soil nitrogen levels at mixed-grass prairie restoration plots. They found that native seedling density in the restoration prairie was significantly lower in high nutrient plots and was highest in low nutrient plots. This evidence also suggests that a fertilization treatment would not be a useful addition to existing restoration protocols. However, it should be noted that the level of nitrogen added to the high nutrient plots in this study was substantial and that the fertilizer amendments were applied for two seasons.

So why is fertilization used? One reason for fertilizing restoration sites is the need to re-vegetate erosion prone areas. Though prairie restoration can take several years,

vegetation managers are often required to promptly provide plant cover to any denuded site. Fertilization can increase the growth of cover crops and ruderal species, helping the vegetation managers reach their goal of re-vegetation. Some vegetation managers also use fertilizer during restoration because they feel that agronomic principles can be directly applied in restoration (i.e. fertilization greatly increases the biomass of crops; therefore, fertilization will help promote native plant growth). While fertilization does increase total plant biomass in grasslands, biomass of the dominant native grasses can actually decrease under fertilization (Tilman 1987). Another potential use for fertilization in restoration is to enhance plant growth at sites so nutrient poor that plant growth is thought to be limited. In truth, sites that entirely lack nutrients may need more extensive soil enhancement and may be better served by other soil amendments, such as compost, combined with fertilization (Noyd et al. 1996). However, limited nutrient availability is typical of remnant prairies and does prevent growth of native plants. (Wilson & Tilman 1993). Overall, fertilization may be useful in filling some of the goals of restoration, but in general, fertilization is not needed and may slow the overall quality and pace of restoration.

Several alternatives to direct nutrient application via fertilization have been suggested that may enhance the fertility at restoration sites and limit the growth of ruderal species. One alternative is the use of timed or slow-release fertilizers, which add nutrients to the soil over a controlled length of time (Hummel & Wadington 1986). This regulated nutrient release provides some additional nutrients to the soil, but it minimizes sudden large increases in soil nutrient concentrations (Owens et al. 1999). Another alternative to fertilization is the use of compost as a soil amendment. Composted yard

waste has been shown to increase phosphorus levels and enhance water-holding capacity of soils (Noyd et al. 1996). Similarly, some researchers have also suggested the use of organic carbon amendments or so-called "reverse fertilization" (Reever-Morghan & Seastedt 1999). Organic carbon is thought to increase activity of the microbial community, increase water-holding capacity of the soil, and sequester nitrogen away from weedy plants. While reverse-fertilization techniques are only now being studied, some restoration protocols already suggest using this technique to reduce invading weeds (Delany et al. 2000).

Feasibility of fertilization

In general, fertilization of restoration sites with traditional inorganic (mineral) fertilizers is practical on a large scale. The use of fertilizers in agriculture, horticulture, and turf management has produced a wide variety of fertilizer formulations and many tools for their application. These fertilizers are inexpensive and simple to incorporate into the soil. In fact, compared with the cost of native prairie seed, the expense of using common agricultural fertilizers is minimal. However, using other forms of fertilization such as slow-release fertilizers, compost, or organic carbon would, at present, be more costly. In addition, compost and organic carbon are bulky materials that are more difficult to transport and require additional effort to apply.

Arbuscular mycorrhizae

The use of arbuscular mycorrhizal soil amendments has also been proposed to enhance establishment and growth of native plants at prairie sites. Arbusuclar mycorrhizae (AM)

are associations between arbuscular mycorrhizal fungi (AMF) and plant hosts, which occur in more than 80% of plant species (Smith & Read 1997). AM fungal hyphae colonize the roots of host plants and form specialized organelles inside the plant cell walls. These fungal associations often supply host plants with added nutrients, increased drought tolerance, and enhanced disease resistance (Smith & Read 1997) in return for a portion of the carbohydrates produced in photosynthesis. Thus, AMF colonization can enhance host plant fitness, and in fact, numerous plants species are dependent on AM fungi in poor growing conditions (Hetrick et al. 1988; Hetrick et al. 1992; Wilson & Hartnett 1998).

AM associations of prairie grasses and forbs have been extensively explored by Hetrick et al. (1988;1992), who found a wide variety of plant responses to mycorrhizal fungi in low nutrient soils. In general, the dominant prairie C4 grasses, such as big bluestem and little bluestem, are highly dependent on mycorrhizae, whereas C3 mycorrhizal relationships range from parasitic, in prairie junegrass (*Koeleria cristata*), to moderately dependent, in western wheatgrass (*Agropyron smithii*). AM dependency in forbs also varied widely between AMF dependent species and species that showed little benefit from AMF associations. Yet, overall the majority of plants common to native prairies do form associations with AMF.

The ability of AM fungi to ameliorate nutrient and water limitations affecting plants (Miller 1987; Auge 2001) is a likely reason for the prevalence of AM associations in prairies. Fungal hyphae extend the volume of soil from which plants can acquire nutrients, giving them an advantage in low nutrient soils (Smith & Read 1997). Some researchers have also suggested that fungal hyphae have more efficient hyphal nutrient

transporters than plants (Smith & Read 1997). In addition, AMF hyphae have been implicated in directly taking in phosphorus from decomposing organic materials. Increased drought tolerance is also seen in many plants with mycorrhizal associations (Auge 2001). Mycorrhizal plants may be more tolerant to water stress as a result of their enhanced nutrient status or because of the added soil mass that their associated hyphae inhabit; however, the mechanisms behind the added drought tolerance are not clear. Both limited nutrient availability (DeLuca & Keeney 1993) and periodic water shortages (Weaver 1954) are common features of prairies and grasslands.

Although prairie plant species are individually influenced by AM associations, the plant community as a whole is also greatly altered by interactions with AMF (Allen & Allen 1984; Miller 1987). The varying reliance of each plant species on AM symbiosis results in enhanced growth and abundance in some species in a mycorrhizal environment, while other species have little change in growth or abundance as result of mycorrhizal colonization (Francis & Read 1995). Therefore, the ability of AM associations to regulate species growth and abundance helps determine the structure of the plant community (Allen 1991). Prairie communities and prairie microcosms treated with fungicides to reduce AM symbiosis exhibit a decreased presence of the dominant prairie grasses, which are highly mycorrhizal. The niche left vacant by the missing dominant prairie grasses are filled be less mycorrhizal dependent species and, consequently, diversity and species richness sometime increased in the absence of AMF (Wilson et al. 2001).

AM symbioses are thought to play a significant role in plant community succession, which could be important in restoration communities. Plant species

commonly found in early successional habitats, such as those in the brassicaceae and chenopodaceae, are often non- or weakly mycorrhizal (Trappe 1987). In fact, AM fungal colonization of *Salsoli kali* (chenopodaceae) can damage the root system and kill newly emergent seedlings (Allen et al. 1988). In contrast, most late-successional species are mycorrhizal. The correlation between successional age and mycorrhizal dependence, plus the role of mycorrhizae in altering plant abundance in late successional communities, has led many mycorrhizal ecologists to suggest that mycorrhizae are one of the factors regulating succession (Allen 1991). Restoration ecologists have taken this one step further and proposed that AM associations may be necessary to quickly reproduce native vegetation at disturbed habitats (St. John 1998).

Disturbance of prairie mycorrhizae

Unfortunately, disturbance considerably reduces mycorrhizal populations in many historic prairie habitats, most often by elimination of host plants or destruction of fungal hyphae by soil movement. (Reeves et al. 1979; Call & McKell 1982; McGonicle & Miller 1996). In the central United States, the most widespread disturbances affecting prairie mycorrhizae have been agriculture (Johnson et al.1992) and mining (Allen & Allen 1980; Noyd et al.1996). In terms of sheer area, agricultural activity results in the largest reduction of prairie AM associations. While native prairie plant communities support a large and diverse AM fungal community, agricultural crops tend to form fewer mycorrhizal associations with a limited number of fungal species (Hetrick & Bloom 1983). By converting many prairies to cropland, native mycorrhizal host plants have been eliminated, and as a result, AM fungi numbers are lowered. In addition, tillage and

phosphorus fertilizers further reduce the AM fungal numbers on agricultural land (McGonigle et al. 1990a; McGonigle & Miller 1993; McGonigle & Miller 1996). Mining also damages AM populations by removing host plants and disturbing the soil. Although the area affected by mining is much less widespread, the level of damage to the ecosystem is far greater. While most mine site reclamation procedures replace vegetation and topsoil after removal of the ore, AMF propagules often have poor survival in the anaerobic conditions of soil storage piles (Allen & Allen 1980; Gould & Liberta 1981; Harris 1993). Most mine sites are therefore left without a viable population of AMF.

Following disturbance, the presence of the two mycorrhizal components, AM plants and fungi. is the most important factor governing natural recovery of native prairie AM communities. At less disturbed sites such as agricultural and pasture land, remnant AMF propagules and a local seed bank can re-establish mycorrhizal populations within a relatively short time (Johnson et al. 1991). In addition, fungal propagules and seeds can be transported onto the site by wind, animal or insect vectors. Although levels of AM colonization immediately after disturbance are low on moderately perturbed sites, AMF and plants can quickly reproduce and re-colonize sites. More heavily disturbed ecosystems often show little mycorrhizal recruitment over time (Reeves et al. 1979); this is especially true in mine sites (Wali & Freeman 1971; Call & McKell 1982). Between extreme changes to soil properties (pH, nutrients, texture) and the lack of remnant AMF and plants, natural recovery of prairie AM communities is often greatly limited. As an example, AM prairie plants required 40 years for re-establishment at an abandoned and unrestored North Dakota prairie mine site (Wali & Freeman 1971). In restored mine

sites, plant and AMF recovery does occur more quickly, but still often requires several years (Noyd et al. 1995).

AM amendments in prairie restoration

Some restoration ecologists have suggested amending soil with AMF inocula during prairie restoration to jump-start the microbial community (St. John 1998). The role AM associations play in enhancing succession and increasing resistance to environmental stresses indicates that AMF amendments could promote the growth of natives destined for re-establishment and hasten the succession of the restoration community. While typical restoration protocols replace the native vegetation, they do not commonly address other ecosystem components such as AMF (SER 1997). However, new restoration protocols are beginning to incorporate techniques to enhance the microbial community including AMF (Morgan et al.1995).

Arbuscular mycorrhizal fungal inoculation has been shown to improve some measures of native plant community development in prairie restoration studies. Noyd et al. (1995;1996) examined AM inoculation of a taconite mine reclamation site in northern Minnesota that was planted with native grasses. Plots treated with AMF amendments had increased colonization during the first and second seasons and greater plant cover during the third growth season. AM inoculum of an upland mesic prairie restoration site was studied by Smith et al. (1998), who applied AM inoculum during restoration seeding. At the end of the second year, inoculated plots had higher AM colonization levels and greater cover of planted prairie species than did non-inoculated plots. Both studies indicate that AMF inoculation can provide a source of propagules for re-establishing the

mycorrhizal community. However, both studies also suggest that colonization and plant cover differences fade by the third year. This implies that AM inoculation may assist native plants early in the restoration, but remnant and nearby AMF propagules numbers rebound by the third year and AMF symbiosis can again significantly affect the plant community.

While this early research indicates that AM inoculation may be a beneficial technique, it leaves many questions unanswered. An obvious question is the extent to which these amendments actually affect the overall restoration effort. And what amount of AM fungal propagules are required to generate a desired effect? Also, the temporary increase in colonization and cover demonstrated in these studies are impressive, but are these ephemeral benefits worth the amount of effort and expense to add these amendments? In addition, the narrow range of habitats in these studies limits their ability to predict what types of prairie habitats could be enhanced by AM inoculation.

Fungal diversity and its role in re-establishing an AM community is an especially important scientific and practical concern of any potential AMF inoculation protocol. Currently, around 160 species of AM Fungi are cataloged, but analysis of diversity in undisturbed systems reveals that a high number of AM fungal species are often present (Morton et al. 1995). Prairie habitats are no exception; roughly 20 AM fungal species were found in each of two well-analyzed prairies (Hetrick & Bloom 1983; Johnson et al. 1991). Unfortunately, most available AM inocula contain only a handful of AMF species. Therefore, understanding the link between AMF diversity and AMF function in a plant community is important. AMF species exhibit a wide range of functional differences, including spore production (Daniels [Hetrick] et al. 1981), plant colonization

rates (Sylvia & Hubbell 1986) and ability to influence plant growth (Bever 1994; Sanders et al. 1996). The diversity of mycorrhizal species in undisturbed habitats and studies of individual AMF species suggest a functional or ecological niche for each fungal species. Restoring the functionality of an AM community may be achievable using AM inocula, if only a few keystone AMF species are required. However, to fully restore the diversity of prairie AM communities with AM inocula would be difficult, as some species are challenging to culture, and many grow slowly and form few reproductive propagules.

Many questions have yet to be answered about AM amendments including, 'How AM amendments affect the native plant and AMF communities?' When enhancing the growth of native plants, do these AM amendments promote the growth of plant species originally present on the site? Do they maintain the level of plant diversity seen in the original prairie? Are plant biomass increases seen only in plant species targeted for the restoration, or are some undesired species also benefited? How does adding AM fungi to the soil affect the local population of AMF?

Answering these questions will require further restoration studies examining AM amendments in a variety of habitats. Field trials of AM inoculation in soils with ranges of nutrients, organic matter, soil textures, moisture levels and pHs would vastly improve our knowledge of the responses in AMF in different soil types. Plant community responses such as differences in abundance and composition could be tested in experiments similar to those of Smith et al. (1998) and Noyd et al. (1995). Perhaps the most difficult studies to conduct would be those analyzing how AMF inocula diversity affects the AMF diversity of the resulting soil AMF community. The effort and expense required to collect and analyze this data are great, and as a result few field studies have

examined AM inoculation in restoration. However, as more emphasis is being placed on ecological restoration, there is more interest in examining new techniques such as AMF inoculation.

AM Inoculum

Potentially the largest limiting factor for use of AM amendments in restoration is the supply of AM inocula. Research scientists have in the past produced their own AM inoculum out of necessity and therefore, were the primary source of AM inocula and inoculum production methods (Ferguson & Woodhead 1982; Douds and Schenck 1990). As their time and resources have often been limited, what little inocula has been produced was destined for use in their research. The supplies of AM inoculum that they produced could not be used on a large-scale and were cost prohibitive in restoration. However, research methods have been modified to allow commercial inoculum production. Continued advances in inoculum production research are likely to lead to more concentrated inoculum produced in larger quantities. In addition, further collection of AM fugal cultures will allow for more diverse and likely better performing AM inocula. Therefore, the cost, availability, and performance of AMF should continue to improve over time.

Many competing commercial inoculum products are more widely available than in the past. At present most of these products are marketed for high management horticultural applications such as flower, turf, and tree establishment, although some companies advertise their use in restoration. To keep costs low, most commercial inoculum products contain a few commonly occurring easily cultured AMF species.

These products often include other growth enhancing compounds such as fertilizers, hormones, or water-absorbent polymers. Unfortunately, companies marketing inocula are reluctant to provide detailed information on how the inocula are cultured, what concentrations of fungal propagules are present, and what other ingredients are included in these products. So determining which AM inocula products would be most useful in prairie restoration is difficult. While anecdotal evidence of their usefulness in several branches of restorations sounds very promising (St. John 1998), scientific analysis of commercial AM inocula and their effects on plant communities has not been widely reported.

Feasibility of AM inoculation in restoration

While AM inoculation greatly benefits the growth of many plants in pot culture and microcosms (Hetrick et al. 1994; Wilson & Hartnett 1997; Wilson & Hartnett 1998), the degree to which these amendments could aid native plants in restorations is less certain. On the practical side, AMF inoculation would not be a great deal of added effort during restoration. Application could often be done using modified restoration seeding equipment. Pelletized inoculum has also been tested, which would allow additional flexibility in seeding and planting (Hall 1979). Therefore, inexpensive supplies of AM inocula are the major issue in AMF inoculation feasibility. Should AM inoculation cause only a slight increase in native plant cover, then the high cost of inoculum may not be justified. However, should inoculation result in dramatically improved native plant establishment and growth, then inoculation may protect against restoration failure and

may be warranted. So the extent of AMF inoculation benefits must be known before the feasibility of AM inoculation can be judged.

Conclusions

On a limited basis, fertilizer or AMF amendments are already being used to enhance growth and aid establishment at restoration sites. While incomplete evidence suggests that these amendments may be useful in some circumstances, many questions remain about their effectiveness. To determine the value of these amendments in restoration will require an organized scientific examination of their influence on restoration communities. Especially important in assessing these amendments for restoration applications is analyzing whether the plant community they foster accurately reflects a native community in species composition and diversity. In addition, practical concerns, such as the degree of amendment effectiveness and cost, must be addressed. In the absence of such information, widespread use of fertilizer or AM fungal inoculations in restoration is not warranted.

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Chapter 2: Native prairie arbuscular mycorrhizal fungal inoculum production using the Beltsville soilless production method

Joel Tallaksen

Abstract

Recent interest among restoration practitioners in applying arbuscular mycorrhizal fungal (AMF) inocula to restoration seedings and plantings has the potential to create a demand for AMF inocula. Research production methods are not sufficient to supply large amounts of AMF inocula and, therefore alternative production methods are needed to meet demand. The Beltsville hydroponic system, a research method of producing arbuscular mycorrhizal (AM) inoculum, has the potential to be scaled up to produce large amounts of AMF inoculum for prairie restoration. This system was modified and tested in the production of a mixed species AMF inoculum. Alterations to the system included the use of the prairie grass *Andropogon gerardii* as a host for the AM fungi and a reduction of the nutrient concentration in the hydroponic solution. The study also examined whether a liquid spore suspension could be used as a starter culture to inoculate host plants.

Inoculum produced in this study had lower AMF spore numbers than desired; however, AMF root colonization was modest. Factors that may have reduced spore number in the produced inoculum were nematode contamination, poor environmental conditions, and/or an overly rich nutrient solution. With further testing and refinement, AM spore production and root colonization could likely be increased. These improvements would likely allow the system to produce a significant amount of inoculum with a relatively small input of effort and expense.

Introduction

The application of arbuscular mycorrhizal (AM) inoculum at restoration and reclamation sites has created an interest in production of large quantities of AM inocula (St. John 1998). Arbuscular mycorrhizal fungal (AMF) inoculants are cultured for several months with live host plants in a well-maintained, nutrient-controlled environment (Ferguson & Woodhead 1982). In addition, most production methods require significant inputs of labor and supplies that increase the cost of inoculum beyond what is affordable for restoration use (St. John 1998). While most common methods of inoculum production function well in supplying small quantities of inocula for research purposes, typically they do not generate sufficient inocula for restoration and reclamation.

My interest in large-scale production of native prairie AM inoculum led me to examine the Beltsville soilless inoculum production method (Ojala & Jarrell 1980; Millner & Kitt 1992), a promising AMF inocula culturing technique that reportedly required less labor and expense than traditional inocula production systems. The Beltsville method is a hydroponic culturing system that uses silica sand in flow-through hydroponic pots (Millner & Kitt 1992). Components for the Beltsville method are readily available plumbing and electrical hardware that can be assemble with only a basic mechanical and electrical knowledge (Fig. 1). Daily operation of the system is computer controlled, reducing the labor required to generate inoculum. The Beltsville method has been used previously for generating single species AMF inocula for research studies (Miller & Kitt 1992), but it has the potential to be scaled up to produce large quantities of mixed species inoculum.

The primary objective of this pilot study was to determine whether the Beltsville method, with slight modifications, could efficiently supply large amounts of inocula for restoration. To be successful, the Beltsville method must accomplish this goal with limited inputs of labor and expense, so that it can supply an inexpensive inocula for prairie restoration. In its initial tests (Millner & Kitt 1992), inocula produced by the Beltsville system had high concentrations of AMF spores and colonized roots. In addition to examining the feasibility of increased inoculum production, this study focused on whether adaptations can be made to produce mixed AMF species (general) inoculum specifically tailored for prairie restoration.

A second objective of this study was to assess the use of a native plant as the host for AM fungi in the Beltsville culturing system. While the original Beltsville study used maize as a host plant, maize may not be as effective in facilitating the growth of prairie AM fungal species. *Andropogon gerardii* (big bluestem) was selected as the host plant because it is AM dependent in low nutrient soils (Hetrick et al. 1988, Schultz et al. 2001), is a large component of many native prairies (Weaver 1954), and has been used with success in conventional inoculum production methods (Charvat et al. 1996). However, growth of *A. gerardii* has not been examined as part of a sand hydroponic system and its shoot and root growth characteristics are not known.

The study's third objective was to examine liquid AMF inoculation of culturing pots. Traditionally, soil based AM inoculum has been used as a starter culture for field and potted research studies (Hayman et al. 1981; Noyd et al. 1995; Smith et al. 1998). However, AMF colonized roots and hyphae do not persist in soil inoculum after extended storage, leaving only spores to begin new colonization (Smith & Read 1997). By

extracting spores from the soil, a concentrated liquid inoculum could be obtained that would be less bulky, easy to store, and simple to apply. Yet, since only limited data exists on the use of liquid AMF inocula to initiate AM associations (Hayman et al. 1981), determination of whether a spore suspension has the same inoculum potential as soil is necessary.

Materials and Methods

Inoculum production setup

The AM fungal starter cultures used to begin inoculum production in this study were derived from previous experiments (Charvat et al. 1996). The fungal species were originally collected at Crosstown prairie, a remnant prairie located in Minneapolis, MN (44' 53" N, 93' 12" W). AMF inoculum was used in three treatments with sterile controls for each. AMF Starter culture treatments included a soil based inoculum, a liquid inoculum, and a soil based inoculum combined with a liquid inoculum. The soil starter culture was a mixture of fungal hyphae, spores, and colonized root pieces in a soil media. The liquid starter culture contained AMF spores isolated from the soil starter culture suspended in water. Inocula controls were prepared by autoclaving the soil and spore suspensions (121°C @ 20 psi) for 20 min.

Fifteen pots were established for each of three inoculation treatments, with five additional pots prepared as sterile controls for each inoculation treatment. The black plastic pots, 14.5 cm in diameter, were filled 11 cm deep with approximately 1.5 kg moist silica-sand microspheres (Unimen Corp., Le Sueur, MN). Pots inoculated with the soil starter culture had 51.7 grams of AMF colonized soil added with the seeds. The liquid

starter culture was applied by evenly spreading 5 ml of the suspension directly on the seeds. Approximately 348 imbibed and sterilized *A. gerardii* seeds were placed in each pot. Seeds were covered with a layer (1.5 cm) of silica-sand and drip irrigation watering rings were placed on top of the sand. Pots were covered for 4 days with plastic to prevent drying and contamination.

Cultivation

Pots were placed in the University of Minnesota College of Biological Sciences Greenhouse (44°59' N 93°11' W). For the first 13 weeks, potted plants were irrigated 5 times daily with 65 ml of a modified Hoagland's nutrient solution (Table 1) (Hoagland & Arnon 1938). The modifications included lowering macronutrient levels to half-strength and adding buffers to maintain a constant pH. Phosphorus levels were also altered, with phosphorus given in excess (100 μM) during the first 2 weeks of growth and then limited to 10 μM for the remainder of the study. Beginning at week 14, an additional 30 mls of solution was supplied to the plants during each of the 5 waterings. Plants were maintained at ambient greenhouse temperatures from August 5th, 1996 until Jan 16th, 1997 (16 wks.). In addition to naturally occurring light, artificial sodium vapor lighting was continuously applied. A "controlled" burn of the weeds growing under greenhouse benches slowed growth of some plants for several days, but all plants recovered with the exception of plants in one pot. Temperature was sporadically measured by a thermocouple buried inside one pot during the first two months of the study, but for the final two months was logged on two digital thermometers (one air temperature and one soil temperature). During cultivation, pot soil temperature typically measured between

16° and 32° C; however, a maxiumum of 41° C was reached during the beginning of the study (late summer) and minimum of 13°C was observed later in the study (mid-winter).

Data Collection

Root analysis was conducted on three pots from each treatment and all control pots. The root mass was cleaned and divided, with half dried, then weighed and the other half stored in ethanol for colonization analysis. Roots stained for colonization analysis were cleared for 7.5 min. in 10% KOH at 90° C, acidified with 1% HCl for 1 hr at 90°C and, finally, stained with trypan blue (McGonicle et al. 1990). Destaining was performed in acid glycerol. Roots were then cut into 1-cm pieces and random subsamples were permanently mounted on microscope slides. Percent colonization was evaluated using the magnified intersection method at 100-400x magnification (McGonigle et al. 1990).

Pots not used for root examination were sampled for fungal spore density after being dried in the greenhouse to ambient moisture levels (approximately 6 weeks). For spore counts, 15 grams of dry soil from each pot was wet sieved to collect material between 250 and 38 μm in size. Sieved material (containing mycorrhizal spores) was further purified by sucrose density centrifugation, which separates spores from unwanted debris (Tommerup & Kidby 1979). Spores were placed on gridded filters and counted under a dissecting scope at approximately 60X.

Plant stem height and phosphorus deficiency were measured prior to harvest. Height of plant stems was measured from the soil surface to the tip of the tallest stem of the mostly uniformly sized plants. Phosphorus deficiency was visually assessed on a 1 to 5 scale based on anthocyanin pigmentation, a dark red-purple discoloration symptomatic

of phosphorus stress (Marschner 1995). During harvest, stems were removed from plants, placed in separate paper bags, dried at 65° C, and later weighed.

Statistical Analysis

Statistical analyses were conducted using the StatView statistical software package. Control treatments were combined after no significant differences were noted among all three controls and their means observed to be nearly identical. One-way ANOVAs were conducted on all data, with Fischer's LSD used to calculate significant differences between treatments at $p < 0.05$.

Results

AM colonization was found in roots of plants inoculated with the soil or combination (soil + liquid suspension) inocula treatments (Fig. 2). In contrast, plants treated with the liquid suspension or sterilized inocula had few colonized roots and were not significantly different. Soil in all pots inoculated with unsterilized AMF inoculum contained a limited numbers of spores (Fig. 3). However, in two treatments, the soil and combination treated pots, there were significantly more spores than in pots treated with the liquid suspension. Identification of spores from soil in treatment pots revealed *Glomus mossea*, *Gl. occultum*, and *Gl. etunicatum*, but immature spores of other species may have also been present (H. Agwa, personal communication). Nematode contamination was also noted during spore identification and quantification. Each pot produced approximately 1.5 kg of inoculum (soil containing AMF spores, hyphae, and colonized root pieces), and the

system handled 60 culture pots (45 treatment +15 controls) with no noticeable nutrient delivery problems.

A. gerardii began germinating within 5 days of seeding, and by 21 days plants formed a dense foliage layer, which functioned as a barrier to limit soil surface contaminants. Plants receiving the liquid spore suspension or control treatments were slightly taller (Fig. 4) and had more above ground biomass (Fig. 5) than plants treated with soil inoculum or the combination treatments. Soil and combination treatment plants had no floral development by the conclusion of the study, while control and liquid spore inoculation pots had floral development and often fully mature seeds. Plants inoculated with soil or the combination treatment exhibited only minor phosphorus deficiency symptoms. However, plants in pots treated with either the liquid spore suspension or control (sterilized inocula) began exhibiting anthocyanin pigmentation (phosphorus deficiency) within weeks of lowering phosphorus levels and appeared distinctly pigmented at the conclusion of the study (Fig. 6). Pot root mass averaged 11.96 ± 3.85 g per pot with no significant differences in root mass among treatments. Dissection of the root mass revealed both course and fine roots present throughout the pots of all treatments.

Discussion

The Beltsville system

This study provided baseline data as to the quantity and quality of prairie AMF inoculum produced with the Beltsville method (Millner & Kitt 1992). While my pilot study was limited to 90 kg of inoculum (60 pots), the Beltsville system can, in principle, produce large amounts of native prairie AMF inoculum. In addition, with added culturing pots a

greater volume of inoculum could be produced. Daily operation and maintenance of the system, along with time spent setting up inoculum production pots, required relatively little time and in terms of effort, was comparable to other inoculum production schemes.

Although the inoculum generated had modest AMF colonization and some spores, the relatively minimal spore number indicated a low inoculum potential (ability of spores, hyphae and colonized root pieces to initiate new colonization). Therefore, the inoculum produced in this pilot study was not deemed suitable for field use. The colonization percentage for the soil inoculum treatments (approximately 45%), while modest, was in the range of those found for a wide variety of AM fungi and plant hosts (Thompson 1986; Volkmar & Woodbury 1989; Doud & Schenck 1990). However, the spore counts for the soil inoculated pots (3.5 spores/gram dry soil) were far below those of the original Beltsville trials, which had a combined average of 116 spores/gram. The spore count was also much lower than those of other inoculum production systems, which typically top 40 spores/gram (Struble & Skipper 1988; Douds & Schenck 1990).

One of several factors that may have reduced spore numbers was the nematode contamination found in culture pots. Nematodes, microscopic thread worms that consume the contents of mycorrhizal spores, were found to be common during spore identification, though nematode numbers were not quantified. Nematodes are likely common in the greenhouse and may have entered the greenhouse through the ventilation system, which is next to heavily tilled agricultural fields. Another source of nematode contamination may have been the soil and liquid spore inocula used to inoculate the pots, which was originally collected at a field site. The presence of nematodes in this study

indicated that the Beltsville system is susceptible to nematode infection. More rigorous methods are needed to assure that a pathogen free inoculum is produced.

High greenhouse temperatures during the study were another factor that may have hampered inoculum production. For a two week period during the middle of August, pot soil (sand) temperatures commonly reached 38°C and a peak temperature of 41 °C was recorded. Though temperatures between 30 and 35°C have been shown to be beneficial for germination and colonization of AM fungal spore isolates from tropical and subtropical regions (Schenck et al. 1975), isolates from temperate climates have optimum germination and colonization maxima at between 21 and 22°C (Zhang et al. 1995). Schenck & Schroder's data (1974) also suggests that while sup-optimum temperatures have a minor effect on AM fungal activity, high temperatures greatly reduce germination and colonization. Therefore, for maximum inoculum potential in greenhouse cultures, the temperature should be regulated to match the needs of the fungal symbiont. Work subsequent to this study showed that the Beltsville method could easily be established in an environmental growth chamber or other temperature-controlled facility. (J. Tallaksen, unpublished data)

The nutrient concentration of the hydroponic solution may also have discouraged production of AM fungi and fungal propagules. In the original Beltsville study, the nutrient solution was designed to produce inoculum with maize as the plant host (Millner & Kitt 1992). Since *A. gerardii* is a native plant adapted to soils with relatively low nutrient availability, I reasoned that a significantly lower concentration of nutrients would produce healthy *A. gerardii.* The key element is phosphorus, which needs to be kept at low concentration to encourage mycorrhizal colonization and spore production (Mosse &

Thompson 1984; Douds & Schenck 1990; Millner & Kitt 1992). However, plants must receive sufficient phosphorus to remain healthy and stimulate root growth and photosynthesis, or the symbiotic relationship can suffer (Smith & Read 1997). In this study, I lowered the phosphorus concentration to one-half that used by Millner and Kitt (1992) for maize. However, plant growth data suggest that an even less concentrated nutrient solution should have been used. *A. gerardii* is AMF dependent in low nutrient soils and will not grow without AM fungal partners under such conditions (Hetrick et al. 1988). Yet in more rich soils, *A. gerardii* can function effectively without mycorrhizal associations (Anderson et al. 1994). In control treatments (those without AMF propagules), *A. gerardii* had relatively normal growth and aside from some symptoms of phosphorus deficiency was able to complete its life cycle. Therefore, nutrient levels were probably high enough that the plants did not need AM fungi for growth and, as a result, colonization and spore numbers were low. Optimal nutrient levels for plants appear both to be plant specific and to vary with the inoculum production method (Elmes & Mosse 1984, Thompson 1986, Douds & Schenck 1990).

Another nutrient related concern is use of high phosphorus concentrations in the first two weeks of the study. While additional phosphorus was applied to enhance growth of the tiny seedlings, it may have discouraged early mycorrhizal symbiosis. By delaying symbiosis, fungal propagule viability may have been lowered, thus reducing mycorrhizal colonization. Again, further examination of nutrient conditions may reveal the optimal nutrient regime for promoting AMF reproduction.

Andropogon gerardii in inocula production

The excellent above and below-ground growth of *A. gerardii* indicates that this plant should function well as a host plant for the Beltsville hydroponic system. The plant grew normally in the variable environment of the greenhouse. The ability of the plant to tolerate some phosphorus deficiency also allows more leeway in tailoring the phosphorus level to enhance spore production without risking severe plant damage. Since spore production peaks during plant senescence, the 16 week maturation time of *A. gerardii* in in the Beltsville system is also conducive to AM inoculum production. Based upon the success of big bluestem, other native hosts should be considered for use in the Beltsville method as a replacement for common agronomic host plants such as maize or sorghum. Use of native plants as AMF hosts in inoculum production is particularly appealing in production of native prairie inoculum. Several studies have demonstrated that host plant feedback helps determine AM fungal species in the rhizosphere (Bever et al. 1996, Schultz et al. 2001), and therefore using a native host plant may be more beneficial in fostering growth of native prairie AM fungal species.

Method of Inoculation

As a starter inoculum, the soil inoculum treatment was more effective than the liquid spore suspension treatment in initiating AM colonization or spore production. While the liquid and soil starter inocula treatments were designed to each have approximately 500 spores, propagule concentrations were very likely higher in the soil starter compared with the liquid inoculum due to an undercounting of spores in the soil inoculum. In addition, colonized roots and fungal hyphae may have enhanced the propagule number in the soil. Therefore, the differences in the produced inoculum were likely due to higher propagule

numbers in the soil starter culture. Alternately, the liquid spore suspension could have been washed through the large textured sand microspheres, which held larger soil and root pieces in place near the roots. Further work using various spore suspension concentrations and soil particle sizes may resolve the difficulties found using the liquid spore suspension.

Conclusion

This pilot project demonstrated that native prairie AM inocula can be produced with the Beltsville hydroponic system and that large volumes of inoculum could be generated by increasing the scale of the inoculum production equipment. The successful use of *A. gerardii* as a host indicates that it can be used hydroponically for native prairie inoculum production and suggests other native plants may work as well. Due to experimental problems, I was not able to determine the effectiveness of a liquid inoculum solution, and thus additional studies are needed to determine its efficacy. Unfortunately, in this study, inoculum produced by the Beltsville method did not have the desired level of AMF spores, which indicates further refinements to the method are needed to enhance inoculum quality. Overall, the Beltsville system was simple to set up, inexpensive to operate, and with refinement, holds potential for large-scale inoculum production.

Acknowledgements

The author would like to thank Professor Hamdy Agwa for assistance in identifying fungal spores present in AMF inoculum generated during this study.

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Table 1 Modified Hoagland's solution
(Based on Hoagland & Arnon 1938)

Compound	Concentration	mg/liter
$Ca(NO_3)_2*4H_2O$	2.5 mM	590
KNO_3	2.5 mM	253
$MgSO_4*7H_2O$	1.0 mM	246
NaFe EDTA (H_20)	0.05 mM	18.4
KH_2PO_4	10 µM	0.174
$CuSO_4*5H_2O$	0.5 µM	0.062
$CoCl_2*6H_2O$	0.2 µM	0.024
$NiSO_4*6H_2O$	0.2 µM	0.026
H_3BO_3	10.0 µM	0.307
$MnCl_2*4H_2O$	2.0 µM	0.198
$ZnSO_4*7H_2O$	1.0 µM	0.144
$NaMoO_4*2H_2O$	0.2 µM	0.024
Buffers/pH adjustments		
HCL 3N	As needed	
KOH 10%	As needed	
MES buffer	0.5 mM	0.117

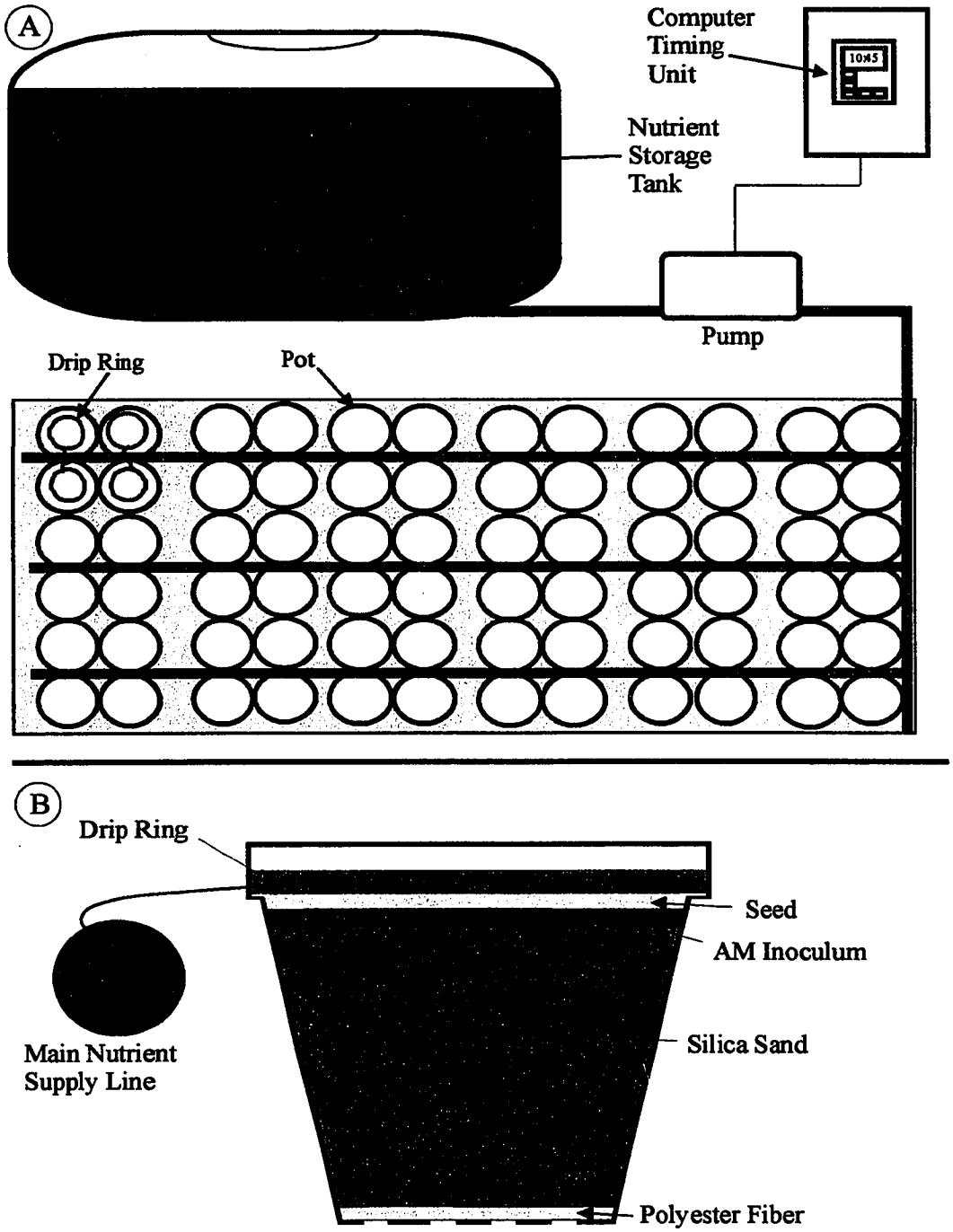

Figure 1. Beltsville AM inoculum production system layout. Schematic of nutrient distribution network (A). Cross-section of typical inoculum production pot after application of seed, inoculum, and a drip ring (B).

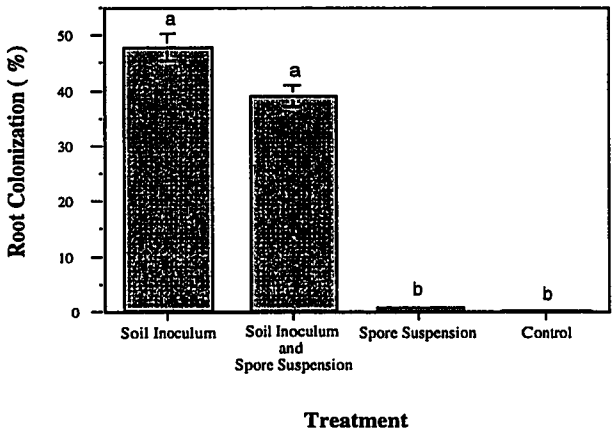

Fig. 2 Percentage of *Andropogon gerardii* roots colonized by arbuscular mycorrhizal fungi after receiving one of four inoculation treatments. Bars represent colonization ± SE. Different letters above bars indicate significant differences between treatments at $p < 0.05$.

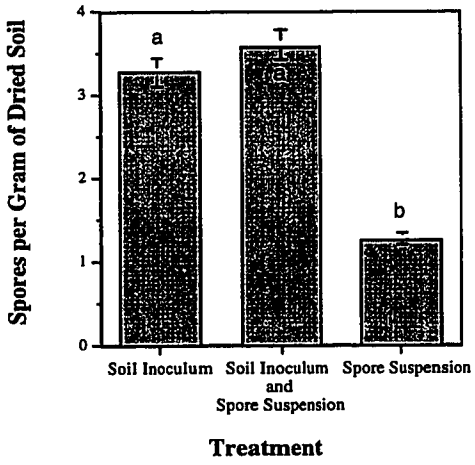

Fig. 3 Number of arbuscular mycorrhizal fungal spores per gram of soil in pots receiving one of three inoculation treatments. Bars represent spores per gram of soil ± SE. Different letters near the top of bars indicate significant differences between treatments at $p < 0.05$.

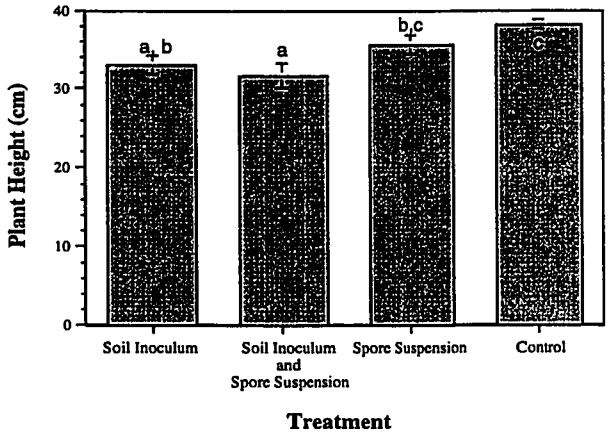

Fig. 4 Final height of inoculated *A. gerardii*

Final height of *Andropogon gerardii* stems 16 weeks after plants received one of four inoculation treatments. Bars represent height ± SE. Different letters next to bars indicate significant differences between treatments at $p < 0.05$.

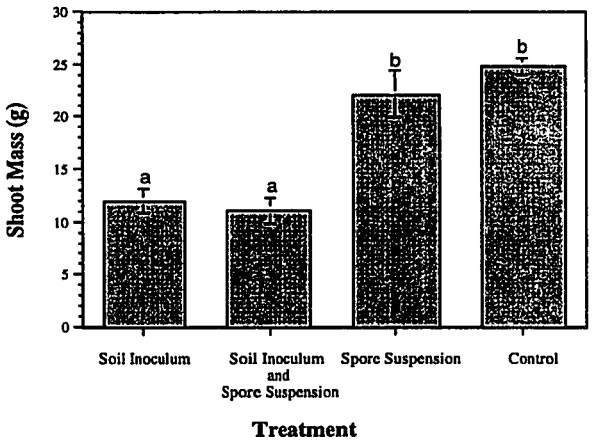

Fig. 5 Shoot mass of *Andropogon gerardii* 16 weeks after plants received one of four inoculation treatments. Bars represent dry weight ± SE. Different letters above bars indicate significant differences between treatments at $p < 0.05$.

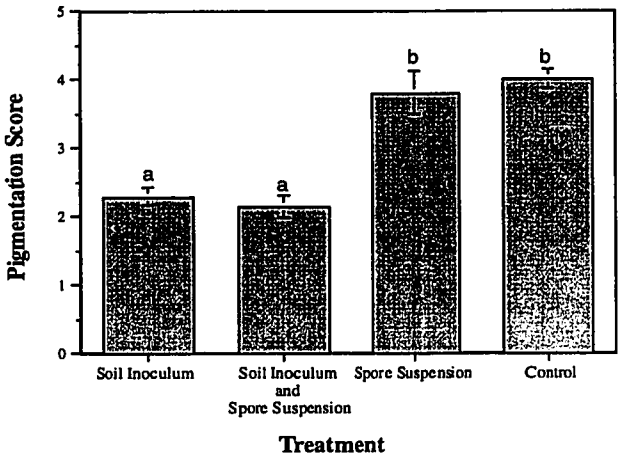

Fig. 6 Visually scored anthocyanin pigmentation of *Andropogon gerardii* at 16 weeks after germination in plants that received one of four inoculation treatments. Bars represent mean pigmentation score ± SE. Different letters above bars indicate significant differences between treatments at $p < 0.05$.

Chapter 3: The effects of fertilization and arbuscular mycorrhizal inoculation on a wet prairie restoration plant community

Joel E. Tallaksen

PLEASE NOTE: Reference to specific commercial formulations of fertilizer and/or mycorrhizal inoculum does not constitute a recommendation for or against such products by the author, the University of Minnesota, or any group/agency providing funding.

Abstract:

Applying soil amendments to highly disturbed restoration sites has been proposed to improve restoration of plant communities. The ability of fertilizers and arbuscular mycorrhizal (AM) fungi to enhance native plant growth was tested at a wet prairie site in central Minnesota that had previously had a restoration effort fail. Plant biomass and community diversity were monitored for three years at restoration plots treated with combinations of an inorganic or slow-release fertilizer and one of three AM fungal/seeding regimes.

Significant changes in biomass were observed under the slow-release fertilization regime throughout the study. Specifically, undesired grass and forb biomass were higher in slow-release fertilized plots during the first two seasons, leading to a higher total biomass in those seasons. Biomass in inorganic nutrient fertilized plots was not significantly different from controls. Diversity was also altered by slow-release fertilization, which lowered both total diversity and diversity of grasses. Inorganic fertilization did not affect total or grass diversity. AM fungal inoculation had little effect on biomass or diversity.

Overall, seeded native vegetation grew effectively in all plots regardless of treatment. Inorganic fertilizer was likely leached from the site by heavy rain and, therefore there was no effect from its application. The one-time application of slow release fertilizer negatively influenced the plant community for the three seasons that were monitored. However, fertilization could be useful in satisfying other goals of restoration projects such as providing rapid cover for erosion control. In contrast to the results of other studies, AM inoculants were not found to benefit vegetation at this site.

Introduction

Establishment is a critical phase of prairie restorations as it is the time when competition for resources between desirable native plants and unwanted species begins. In addition to competition, a host of harsh environmental conditions can reduce native seedling establishment and increase the time required to develop the desired prairie. Though many former prairie areas may revert to prairie given sufficient time (Weaver 1954; Inouye et al. 1987), the goal of restoration is to reproduce the historic vegetation without an extended successional period. The inhospitable environment of many restoration sites and resulting poor plant establishment have led vegetation managers to suggest several alternative approaches to more successfully and rapidly restore sites (Reever Morghan & Seastedt 1994; Wilson & Gerry 1995; St. John 1998). I was interested in examining these proposed alternatives to restore native prairies in Minnesota, specifically wet prairie communities.

Found along the margins of wet meadows, wet prairies are one of many prairie types being restored in Minnesota. The dominant vegetation in wet prairies is a mixture of hydric wetland species and mesic upland prairie species, which are protected from minor droughts by a persistent, shallow water table (MNHP 1993). The Minnesota Department of Transportation (Mn/DOT) is one organization interested in restoring wet prairies and has extensive right-of-ways including many which offer excellent restoration opportunities. While Mn/DOT has restored prairies for several years as part of their integrated roadside management plan, they strive to improve practices for establishing native prairies and thus promote the study of prairie restoration methods.

I designed a study to examine current and proposed wet prairie restoration protocols used by Mn/DOT (1996). I focused on how fertilizers and arbuscular mycorrhizal fungal amendments enhance or inhibit the restoration plant community. Fertilization is used in the current protocol both to aid in early native growth and to quickly produce standing vegetation that resists erosion. However, an increased understanding of the ecological role nutrients play in plant competition and succession indicates that added nutrients may not be beneficial to prairie restorations (Tilman 1997). Additionally, newer alternative soil amendments, such as arbuscular mycorrhizal fungi, may further enhance restoration outcomes.

Plant community responses to nutrients are mediated by variations in the competitive ability of individual plant species at different nutrient concentrations (Tilman 1987). Hence, alterations in soil nutrient concentration will change the competitive interaction between species, and thus alter the plant community. Nitrogen, in particular, has been shown to influence plant communities via plant competition (Tilman 1997). Invasive species tend to be more competitive at higher levels of available nutrients, whereas late successional native plants are more competitive at low concentrations of available nutrients (Inouye & Tilman 1995; Huenneke et al. 1990). Nutrient availability is an important component of these competition responses; while prairies can contain significant quantities of total nutrients locked in root systems and detritus (DeLuca & Kenney 1993), only those nutrients readily available to plants can influence competition. Therefore, maintaining low levels of available nutrients in restoration prairies may allow native plants a competitive advantage over non-natives.

Indeed, Wilson and Gerry (1995) suggest that native prairie restoration is favored on soils with low nutrient availability.

Although inorganic fertilizers have been shown to increase the biomass of native plants in several settings (Van Auken & Bush 1992; Valvarde & Pisanty 1999), plant communities as a whole exhibit reduced diversity (Wilson & Shay 1990), a reduction of the dominant native grasses (Lorenz & Rogler 1972), a loss of species richness (Tilman 1987), or a combination of these responses following inorganic fertilizer application. Detrimental effects of inorganic nutrient addition have been observed in remnant tallgrass prairie (Seastedt et al. 1991), mixed-grass prairie (Power 1979), rangeland prairie pastures (Owensby & Smith 1979), and old-field succession sites (Tilman 1987). Few studies have examined fertilization of prairie restoration sites; however, Wilson and Gerry (1995) found that nutrient addition significantly decreased native seedling density in a mixed-grass prairie restoration. Therefore, inorganic fertilization would probably not benefit prairie restoration plant communities.

A better alternative to inorganic fertilization may be slow-release fertilizers such as sulfur-coated urea or fertilizer encapsulated in a semi-permeable membrane (Hummel & Waddington 1986). Slow-release fertilizers deliver smaller quantities of nutrients over a given amount of time, which varies depending on the coating, membrane or decomposition process regulating nutrient dissolution (Hummel & Waddington 1986). This is in sharp contrast to inorganic fertilizers, whose full quantity of inorganic nutrients are released at the rate their elements dissolve in solution. As plant community competition is nutrient mediated, a slight long-term increase in available nutrients by

slow-release fertilizer may be sufficient to promote the growth of native plants, while at the same time not significantly enhancing undesirable plant growth.

Another method that may increase native plant establishment and aid their long-term survival is inoculation of the soil with arbuscular mycorrhizal (AM) fungi. AM fungi are symbiotic soil-borne fungi that aid plants in nutrient uptake, alleviate water-stress and enhance disease resistance (Smith & Read 1997). Most native prairie plants form strong mutualistic relationships with AM fungi, while many invasive species do not (Hetrick et al. 1990; Hetrick et al. 1992). The mycorrhizal relationships of native and invasive species suggest that the presence or absence of mycorrhizal fungi is pivotal in the balance between native prairie species and invasive weedy vegetation. Indeed, a great deal of evidence indicates that mycorrhizal fungi play an important role in plant community succession and maintenance of climax communities (Francis & Read 1996; Hartnett & Wilson 1999). Unfortunately, heavily disturbed sites often either lack an AM population or have had the number of AM propagules drastically reduced (Reeves et al. 1979; Warner 1983;Smith et al. 1998). The low numbers of mycorrhizal fungi at some restoration sites has prompted a number of restorationists to propose the application of AM fungal inocula during restoration to augment existing AM propagules (St. John 1998; Anderson 1999). I was interested in determining whether AM fungal inoculation would aid in restoration of wet prairies and how AM fungal inoculation alters the plant community.

I established an experimental wet prairie restoration in order to study plant community responses to fertilizer and AM fungal amendments during the first three

years of restoration establishment. In our data collection, specific emphasis was placed on species considered desirable for restoration, which were defined as native prairie species not considered by federal, state, or local law as noxious weeds. I also tracked the occurrence of undesirable species, which we did not want in our restoration; these undesired species included exotics and plants regulated as weeds. My objective was to identify how these amendments affected the emergent plant community structure in terms of the diversity and productivity of desired versus undesired plants.

Materials and Methods

Study Site

The restoration site (Fig 1) is within the city limits of Shakopee, Minnesota (44°46' N 93°24' W). Annual mean temperature and precipitation are 5.9 °C and 70 cm respectively. Historic data indicate that prior to European settlement, vegetation at the site consisted of wet meadow species, and soils were a Hubbard fine sand series (Harms 1959).

Following construction of a new highway in 1995, the Mn/DOT engineered a prairie floodplain adjacent to a roadside water-storage pond. The origins of the post-construction topsoil and sub-soils at the site are unknown, but the soils are very sandy and reminiscent of the locally occurring sandy soil types. Originally seeded with regionally occurring native prairie plants in 1995, the prairie vegetation was sparse and patchy by 1997. Research was conducted on a patch presenting no visible evidence of desired native vegetation from the 1995 restoration.

In the two days before planting and amending the site in June of 1997, it was tilled twice to a depth of approximately 15 cm with a commercial roto-tiller. Light rain between tillage treatments moistened the dry sandy soil and provided an excellent seeding bed. As pre-existing vegetation was sparse, application of glyphosate herbicide was not deemed beneficial.

Seeding and amendment application

Research plots were fertilized, inoculated and seeded on June 26-27, 1997. Three fertilization treatments and four mycorrhizal treatments were used in a factorial design for 12 different treatments (see Appendix IIa). Treatments were applied in a completely randomized block design with five blocks. Each treatment plot was 2-m by 2-m with a restored buffer zone of 1-m surrounding each plot (2-m between treatment plots). A further buffer zone extending 4-m around the entire research site was seeded with native grasses.

Twenty forbs and seven grass species were selected for seeding based on the pre-settlement range of each species (Ownbey & Morley 1991) (see appendix IIb). Native seeds were purchased from Prairie Restorations Inc. (Princeton, MN), Prairie Moon Nursery (Winona, MN), or Peterson Seed Company (Shakopee, MN).

Two fertilizer treatments (inorganic and slow-release) and a non-fertilized control treatment were investigated. Inorganic (mineral) fertilizer 6-24-24 (N-P-K) was applied at a rate of 225 kg/ha (United Horticultural Supply, Brooklyn Center, MN). Encapsulated slow-release fertilizer (22-5-10 + micronutrients) with a three-month

release time was applied at the manufacture's suggested rate of 305 kg/ha (United Horticultural Supply, Brooklyn Center, MN). Fertilizers were evenly broadcast over the study plots, then raked into the top 4-cm of soil. Control treatments were similarly raked.

Four AM inocula treatments were used: 1) locally produced inoculum applied in rows (2.54 kg/m^2, 23,000 spores/m^2), 2) locally produced AM inoculum broadcast (2.54 kg/m^2, 23,000 spores/m^2), 3) a control treatment of sterilized local inoculum in rows (2.54 kg/m^2, 23,000 spores/m^2), and 4) non-local commercially produced inoculum in rows (0.585 kg/m^2, 4,800 spores/m^2).

The local prairie inoculum used in this study was generated in three batches using the pot culturing techniques (Ferguson & Woodhead 1982; Millner & Kitt 1992) and a fungal inoculum originally collected at Crosstown Prairie (44° 54' N, 93° 12' W), a nearby remnant prairie. The three batches were cultured for 16 weeks with *Andropogon gerardii* or *Bouteloua curtipendula* as a host plant. Soil, spores and chopped root pieces from the different batches of inocula were thoroughly mixed prior to use. This combined inoculum had a spore density of 36.2 spores/g (Charvat et al. 1998; Charvat et al. 2000). The commercial inoculum, Mycor™ VAM Cocktail™ Flowerbed Inoculant, used in the study was purchased from Plant Health Care Inc. (Pittsburgh, Pennsylvania). According to product labeling, the commercial inoculum was formulated to provide a suitable AM inocula for a wide range herbaceous species. It also contained yucca, seaweed, humic extracts, and nitrogen fixing/phosphorus solubilizing bacteria.

Seed and inocula were simultaneously added to plots by one of two methods, broadcast or row application. Broadcast application involved evenly hand-spreading seed and inoculum over the 4-m^2 treatment area and raking them into the soil to an estimated average depth of 1 cm. Row application of seeds and inoculum was used to simulate seeding and inoculation via a native seed drill. Inocula and seed were evenly dispensed into rows 3 cm deep by 2-m long (16 cm apart) and covered to a depth of 1 cm with soil. The buffer zone between the plots was broadcast seeded using the same seed mix and rate as treatment plots.

Soil sampling

In the third week of September during each of the three growing seasons, ten soil cores per plot were collected to a depth of 15 cm with a 2.5-cm diameter soil corer. Each season, the five replicate samples from the 12 treatment combination (fertilizer x inoculum) were combined for nutrient analysis. Samples were stored at -20° C until analyzed by the University of Minnesota Research and Analytical Laboratories. Nitrate (Henriksen & Selmer-Olsen 1970) and phosphorus (Olsen method) (Frank et al. 1998) were quantified by colormetric assay, while potassium was quantified by ICP analysis (Fassel & Kniseley 1974). Nitrate, phosphorus, and potassium concentrations were statistically tested using ANOVA ($\alpha = 0.05$), with the mycorrhizal/fertilizer interactive effects used as the error factor.

Vegetation sampling

Plant material was also collected during the third week of September each fall. A different 10-cm by 100-cm strip of vegetation at the same location in all plots was harvested from each plot each year. All aboveground material was taken down to bare soil. Samples from individual plots were frozen for preservation within 10 hours of harvesting. Later, plants from each plot were thawed in water, counted, and measured over a 4-month period. Following counting, plants were sorted by species, grouped into related types, packaged in paper bags and placed into a 65° C drying oven. Five plant characterizations were used to categorize plants for biomass analysis: desired grasses, desired forbs, undesired grasses, undesired forbs, and litter. Unidentified plants were placed into the appropriate categories based on morphology and similarity to known species. Dry weight measurements were taken after the samples had dried a minimum of 3 weeks. For statistical analysis, a blocked two-way ANOVA was performed on transformed (log+1) data using Systat 9.0 (SPSS, Chicago, IL). Significant differences were determined at $\alpha = 0.05$ using Tukey's pair-wise comparison.

Diversity analysis

Plant species diversity was calculated each year on the major components of that year's plant community. Major components of the plant community were defined as plants whose numbers made up more than 0.5 percent of the total number of plants in a given season; using this criteria, I removed plants from consideration which had too few representatives for satisfactory species identification. Less than 2 percent of all plants

were in the trimmed data. Calculation of diversity used the Shannon Index (H') (Kent & Coker 1992):

$$H' = - \Sigma p_i \ln p_i$$

where p_i is the proportion of species i in the vegetation sample. ANOVA of diversity data used a blocked, two-way design at $\alpha = 0.05$ (Systat 9.0), followed by Tukey's pair-wise comparison test to detect treatment differences.

Results

Plant biomass

Plant aboveground biomass measurements revealed significant differences in productivity between fertilized plots. Total biomass was highest for the first two seasons in plots treated with slow-release fertilizer (Fig. 2), but by the third season, total biomass was similar to unfertilized control plots. Biomass in inorganic nutrient fertilized plots did not significantly differ from that of control plots, nor did it appear that mycorrhizal inoculation affected total biomass (Fig. 3).

To further analyze biomass, plants were divided into 4 categories: 1) desired grasses, 2) undesired grasses, 3) desired forbs, and 4) undesired forbs. While fertilization had significant effects on biomass in the breakdown categories (Fig. 4A-D), mycorrhizal inoculation had little effect on biomass (not shown).

Desired grass biomass, consisting mostly of the native perennials *Andropogon gerardii*, *Sorghastrum nutans* (Indian grass), and *Schizachyrium scoparium* (little bluestem), became an increasing component of total biomass over the three seasons that I

analyzed. Though significant fertilization effects were observed in desired grass biomass only in the second season, slow release fertilized plots tended to have a lower biomass of desired grasses (Fig. 4A).

Overall, undesired grass biomass decreased each season of the experiment (Fig. 4B) and corresponded to decreases in the size and number of the major grasses in this category (*Setaria spp.* [foxtails], *Echinochloa crusgalli* [barnyard grass], and *Digitaria spp.*[crab grasses]), which are all annuals. However, in each season undesired biomass was highest in slow-release fertilized plots.

As the restoration progressed, desired forb biomass (Fig. 4C) increased mainly due to the larger size and increasing recruitment of the predominant species *Monarda fistulosa* (bee balm), *Astragalus canadensis* (Canada milk-vetch), and *Rudbeckia hirta* (blackeyed-Susan). In the second and third years, control plots had significantly more desired forb biomass than those treated with slow-release fertilizer.

Undesired forbs (Fig. 4D) were most prominent in the second season of the study. The main species recorded were the annuals *Ambrosia artimsiifolia* (common ragweed), *Conyza canadensis* (horsetail weed), and *Amaranthus ssp.* (pigweeds), which all decreased in abundance during the third season. Slow-release fertilized plots tended to have greater undesired forb biomass throughout the experiment; however, the increase was only significant during the second season.

Plant diversity

Plant community diversity, as measured by the Shannon index (H'), was significantly affected by slow-release fertilizer treatments (Fig. 5A). Plots treated with slow-release fertilizer consistently had the lowest diversity, which likely reflected the reduced grass diversity seen in slow-release fertilized plots (Fig. 5B). Inorganic nutrient addition did not significantly affect diversity (Fig. 5A), nor did mycorrhizal treatments (Fig. 6).

Nutrient levels following fertilization

Soil nutrient levels were significantly affected by fertilization for the first year of the study (Table 1). Nitrate increased in slow-release fertilized plots, but not in inorganic fertilized plots. Potassium was elevated in inorganic fertilizer plots, though not in slow-release fertilized plots. Although phosphorus concentrations increased in both slow-release and inorganic fertilized plots, they were not significantly different from control plots. Nutrient patterns established during the first season followed the same trend during subsequent seasons; however, with the exception of potassium, no significant differences in nutrient concentrations were seen past the first year.

Discussion

Fertilization

The results demonstrate that an early one-time addition of nutrients can extensively affect the restoration plant community. Applied concurrent with seeding, slow-release fertilizer significantly altered total biomass and decreased diversity in the restoration plots. However, a similarly applied inorganic fertilizer had little affect on the plant

community and did not resulted in a significant biomass increase. These results indicate that soil nutrients at restoration onset are important in determining plant community composition and that the plant restoration community is sensitive to additions of fertilizer based on the type and/or amount of nutrients added.

Inorganic fertilizer responses

I reasoned, based on data from other prairie nutrient addition studies, that inorganic fertilization would have a significant effect on the restoration community. However, under my inorganic fertilization regime, fertilized plots were comparable to control plots in terms of both diversity and biomass. One explanation of the similarities of inorganic fertilized and control plots is that the nutrient application rate I examined was not sufficient to elicit a response. Nitrogen, thought to be limiting, was applied at 13.5 kg/ha in my experiment versus 100 kg/ha in other experiments (Lorenz & Rogler 1972; Power 1979). In addition to a low nutrient application rate, I also suspect that leaching of inorganic fertilizer reduced nutrient levels and could have eliminated plant community responses. Leaching likely occurred in heavy rains immediately following planting, which temporarily produced standing water on a portion of the study site. Since few seedlings emerged until 7-14 days following planting, fertilizer granules would have had time to dissolve into soil solution and leach into the sandy soil before plants could begin to acquire the added nutrients. Leaching is also suggested by soil tests, which found that nitrate and phosphorus concentrations, which are susceptible to leaching in sandy soils, were comparable in inorganic fertilized and unfertilized control treatments (Table 1).

Yet potassium, which is less subject to leaching, increased in inorganic fertilized plots. Based on these facts, I believe that nutrients were not effectively added to inorganic fertilized plots and as a result no differences were seen between the inorganic fertilized and control treatments.

Slow-release fertilizer responses

Slow-release fertilization was intended to improve biomass and occurrence of desired natives. However, both desired grasses and forbs had reduced biomass in plots treated with slow-release fertilizer. Furthermore, slow-release fertilizer increased the biomass of undesired grasses and undesired forbs. Slow-release fertilization also reduced plant diversity, primarily by increasing the occurrence of undesired grasses. Therefore, slow-release fertilization does not appear to enhance native establishment or provide any benefit to desired native plants. While I can not say that this is true in all prairie habitats at all application rates, the significant changes observed in my restoration study and other grassland fertilization studies (Power 1979; Tilman 1987) suggest that slow-release fertilization should not be used in prairie restoration.

The magnitude and time-course of the changes in the plant community were much greater than I anticipated based on previous nutrient addition studies (Tilman & Wedin 1991). I felt that using only a single application of slow-release fertilizer would limit changes in biomass to the first season and would not significantly change diversity. However, both biomass and diversity were affected during the three seasons of the experiment. Even in the third year, differences in biomass and diversity were highly

significant (often with p values less than 0.01) and suggest that the plant communities in slow-release fertilized plots would remain quite different from control plots for the next few seasons.

Why the plant community in slow-release plots changed considerably with a relatively small amount of fertilization is unclear; however, a major factor may have been the lack of pre-existing vegetation. My predictions were based on nutrient studies in which mature plants in established communities experienced competition with other established plants; yet in my restoration, competition was exclusively between newly germinated seed from the applied native seed mix and remnant seed bank on the site. It would be logical that seedlings are more sensitive to nutrients than established vegetation, as their relative growth rate is higher and their root system much smaller. In fertilized plots, seedlings that rapidly extract nutrients from the soil would have a competitive advantage over those that obtain nutrients more slowly. Therefore, seedling competition and growth may be more affected by increased nutrients than by competition and growth in established vegetation. However, seedling competition has not been fully explored, and to conclude that plant growth stage is important in nutrient regulated plant competition is premature.

Implications of fertilization

Though my experiment did not elucidate the effects of inorganic fertilization at wet prairie restorations, they did demonstrate the potential problem of leaching associated with inorganic fertilizer application. Given the emphasis on conducting environmentally

sound prairie restorations, inorganic fertilization of restoration areas near sensitive sites, such as those prone to flooding, seems risky due to the negative consequences associated with leaching (Lowery 1998). In addition, regulation by local, state and federal agencies may prohibit the use of fertilizers, especially inorganic based fertilizers, in some locations. While slow-release fertilizer is less likely to be leached, my results demonstrate that it can lower diversity and promote the growth of non-native species.

Fertilization results also indicate the sensitivity of prairie restorations to conditions present during restoration initiation. A small fertilizer application at the time of restoration influenced vegetation for at least the three years studied and likely longer. This sensitivity to initial nutrient conditions should serve as an example to restoration practitioners as to the importance of soil parameters, such as nutrients, in a restoration's outcome.

A potential benefit of slow release fertilization was the rapid above ground growth and fullness of vegetation, which were observed during the first season of the experiment. At some restoration sites, the benefits of rapid aboveground growth of plants (even undesirable species) may outweigh the desire for the best possible restoration. For example, sites prone to erosion may be better served by stimulating rapid growth via slow-release fertilization. Yet, my results show that by the third season, the transient effects of slow-release fertilization on aboveground biomass had subsided and that fertilized plots had the same biomass as unfertilized controls. In addition, the deep-rooted native prairie vegetation favored by lower nutrients may best serve sites prone to erosion in the long-term. Another reason that may compel vegetation managers

to use fertilizer in prairie restoration is the need to rapidly produce a full, green, and 'healthy' looking restoration in order to meet expectations of clients, governmental bodies or the public.

Arbuscular mycorrhizal amendments

The results indicate that mycorrhizal amendments had a limited impact on the vegetation at the wet prairie site. A possible explanation is that AM fungi were not beneficial to plants under the conditions present at this site. The main nutrient AM fungi provide plants is phosphorus, which is found in adequate concentrations for native plant growth in the moist soils of my study site based on data from other prairies (Hetrick & Bloom 1983; Benjamin et al. 1989) and on my soil data (Table 1). Nitrogen limitations, however, may not have been mitigated by AM fungi, which are thought to provide plants only limited amounts of additional nitrogen in moist environments (Smith & Read 1997). Therefore, the beneficial effects of our AM inoculation may not be sufficient to influence the plant community at this site.

These findings are different than those of Noyd et al. (1995; 1996) and Smith et al. (1998), who examined AM fungal inoculation as an aid to growth and establishment of native prairie plants. Noyd et al., reclaiming a mine site, and Smith et al., restoring an upland prairie, found increases in plant cover and native grass cover, respectively, with the application of inocula. An important difference between my inoculation study and these prior studies is the long 'fallow' period between disturbance and restoration in my study. Noyd et al. (1995) established plots on a berm of iron ore tailings that had little if

any vegetation before their work. Similarly, Smith et al. (1998) had only a sterile grass cover crop present on their site in the year prior to inoculation. My experiment was conducted at a site that had had a restoration planting approximately 2 years before the current restoration planting. Though the original restoration did not result in successful establishment of natives, weedy mycorrhizal plants on the site provided a refugia for AM fungal colonization (Charvat, unpublished data). Thus, though the pre-existing presence of AM fungi was low, my site likely had more fungal propagules present at restoration onset than those of Noyd et al. (1995) or Smith et al. (1996). These remnant propagules in my study may have reduced colonization differences between inoculated and un-inoculated plots and lowered the corresponding plant community differences.

Another important difference between previous prairie AM fungal inoculation studies and this study is the soil moisture level at the restoration sites. AM fungal recovery from stored topsoil is thought to occur more slowly in drier soils (Miller 1987). Both Noyd et al.(1995, 1996) and Smith et al.(1998) inoculated well drained sites, whereas my site had a moist, yet aerated, topsoil maintained by a shallow water-table. Therefore, in my moist soil, applied and remnant inocula may have colonized plants and reproduced faster, resulting in high colonization rates in all inoculation treatments and little difference in vegetation between treatments. Low soil moisture levels on the sites used by Smith et al. (1998) and Noyd et al. (1995,1996) may have limited growth of any remnant AM fungal propagules; therefore, added fungal inoculum may have had a stronger influence on the plant community at their drier sites.

Restoration implications

My results and the contrasting findings of Smith et al. (1998) and Noyd et al. (1995, 1996) indicate that AM fungal inoculation varies in its ability to influence prairie restoration plant communities. Habitat differences (especially soil properties, nutrients, and moisture) are likely to be important factors in determining whether inoculation will enhance restoration efforts. In less disturbed areas, remnant mycorrhizal populations are more likely to be present and can serve as a source of inoculum. Additionally, AM fungal re-colonization fostered by wind and animal vectors will occur over time if host plants are present. Therefore, even heavily disturbed areas idle for many years prior to restoration should have some AM colonization, provided suitable host plants (including weedy hosts) have colonized the site. Unfortunately, the paucity of AM fungal inoculation research in prairie restoration limits our ability to predict which sites could benefit from inoculation.

Conclusions

Although fertilization does not appear to be a sound technique for ecological restorations, it may be of use in situations where the long-term goal of restoration is secondary to the short-term goal of revegetation. Slow-release fertilizer increased the total biomass in the first few seasons, but lowered overall diversity and reduced the presence of the desirable natives. Inorganic fertilizer did not have a noticeable effect on vegetation and was likely leached into the nearby waterways. Careful consideration must be given to fertilization

during prairie restoration, and if applied, the amount and type of fertilizer used should be tailored for that site and its soil properties. The contrasting AM inoculation results between my study and those of others makes generalizations about the effectiveness of AM fungal inoculation difficult. Restoration site differences are likely important in determining whether AM fungal amendments will aid in prairie restorations.

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Table 1. Soil nutrient levels in fertilized plots at the Shakopee restoration site. Numbers are mean nutrient levels (ppm) ± SE. Soil was sampled in the third week of September each Fall. Letters following numbers indicate significant differences between treatments during the same growing season ($p < 0.05$). Treatments without letters had no significant differences.

	Nitrate (ppm)		
	1997	1998	1999
No fertilizer control	2.68 ±0.31 a	2.38 ±0.18	3.35 ±1.09
Inorganic	2.50 ±0.09 a	2.48 ±0.21	3.90 ±0.34
Slow release	4.65 ±0.72 b	3.50 ±0.56	4.93 ±0.69
	Phosphorus (ppm)		
	1997	1998	1999
No fertilizer control	22.63 ±0.99	23.00 ±1.00	21.50 ±0.96
Inorganic	26.50 ±1.66	29.50 ±2.06	29.75 ±2.47
Slow release	24.50 ±1.44	27.00 ±2.61	27.00 ±3.24
	Potassium (ppm)		
	1997	1998	1999
No fertilizer control	31.88 ±1.66 a	34.50 ±1.85 a	36.50 ±0.65
Inorganic	39.50 ±0.96 b	42.25 ±2.50 b	42.75 ±3.37
Slow release	28.00 ±1.08 a	34.13 ±0.43 a	38.00 ±3.19

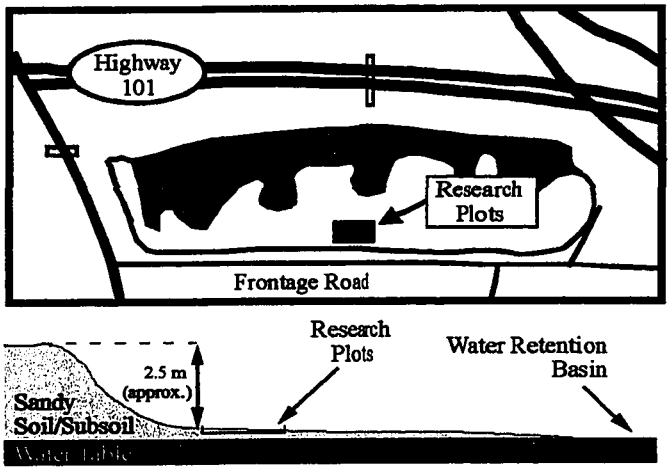

Figure 1. Shakopee research site. Top panel- location of the Shakopee research site three miles SE of downtown Shakopee, Mn at the Highway 101/169 interchange. The research plots are approximately 50 meters from the water retention pond and lie on a gently sloping flood plane. Bottom panel- cross section showing the location of the plots and relative elevation at the roadside site.

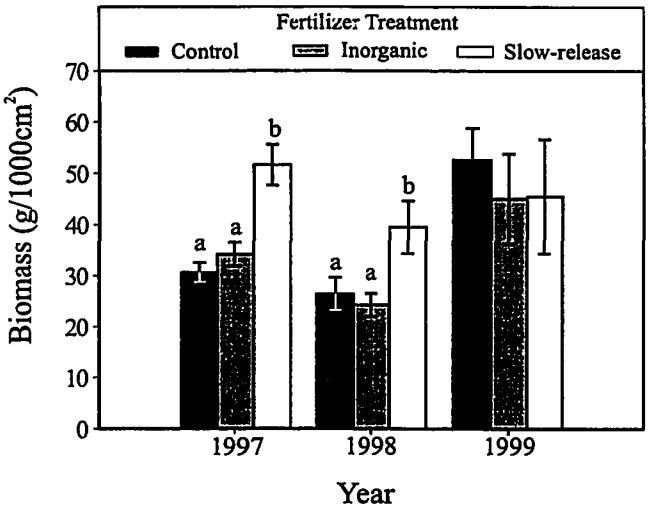

Figure 2. Total biomass in fertilized plots. Collected biomass in restoration plots fertilized with no fertilizer control (black bars), inorganic fertilizer (gray bars), or slow-release fertilizer (white bars). Bars represent mean biomass (g) ± SE. Different letters above bars indicate significant differences between treatments during the same growing season ($p = 0.05$).

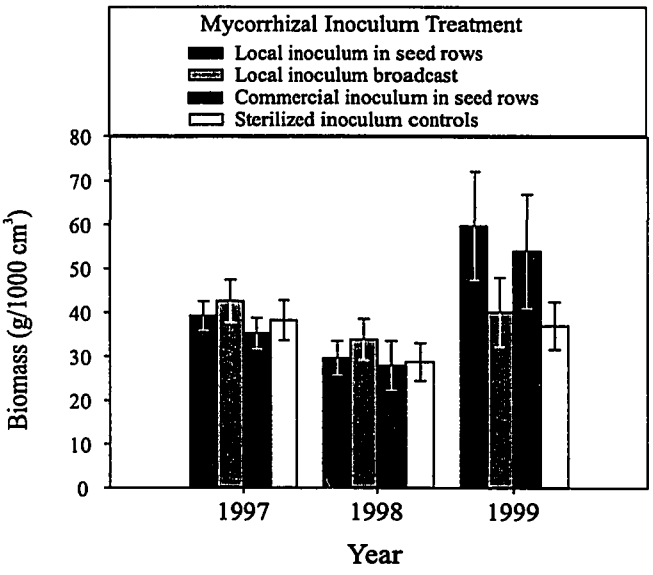

Figure 3. Effects of arbuscular mycorrhizal inoculum on total biomass and plant diversity. Total aboveground biomass in plots amended with lab produced inoculum broadcast (black bars), lab produced inoculum in seed rows (light gray bars), commercial mycorrhizal inoculum in seed rows (dark gray bars), and a sterilized control inoculum in seed rows (white bars). Bars are mean value ± SE.

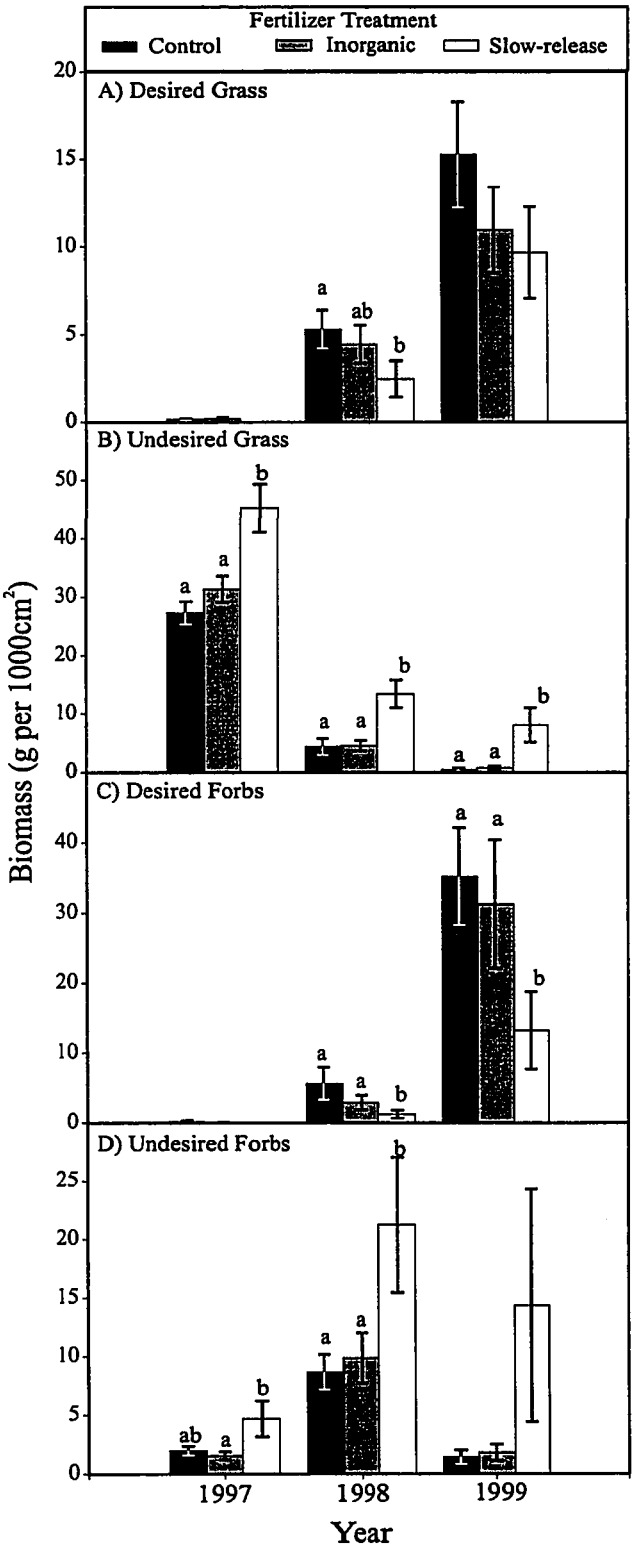

Figure 4. Biomass of vegetation groups in fertilized plots. Biomass of desirable grasses (A), undesirable grasses (B), desirable forbs (C), and undesirable forbs (D) measured in plots treated with no fertilizer control (black bars), inorganic fertilizer (gray bars), and slow-release fertilizer (white bars). Bars are biomass(g) ± SE. Different letters above bars indicate significant differences between treatments during the same growing season ($p = 0.05$).

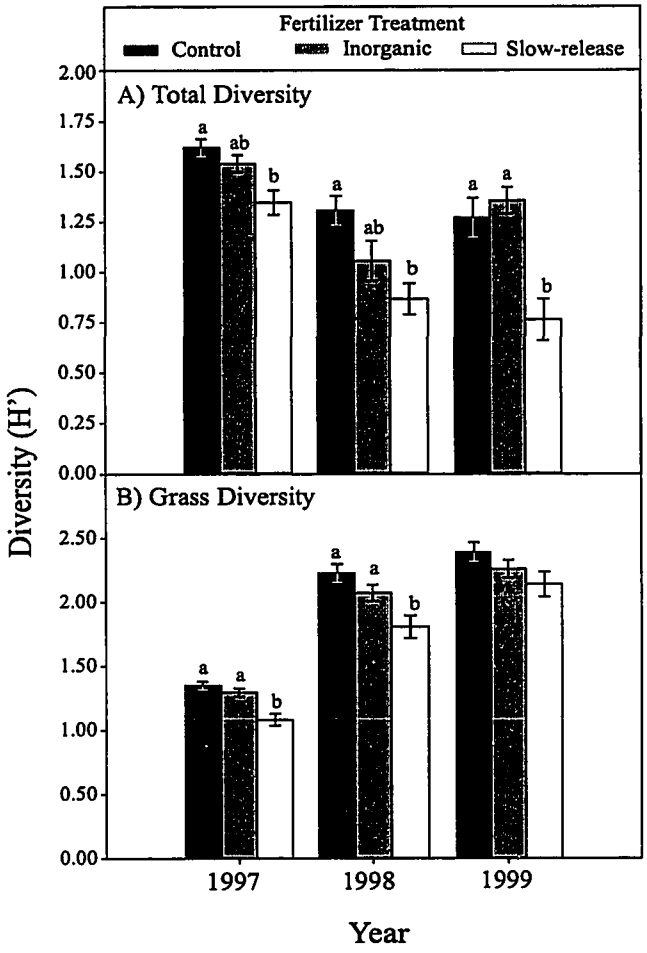

Figure 5. Plant diversity in fertilized plots. Diversity of major components of the entire plant community (A) and major grasses (B) found in restoration plots treated with no fertilizer (black bars), inorganic fertilizer (gray bars), and slow-release fertilizer (white bars). Bars are plant diversity (H') ± SE. Different letters above bars indicate significant differences between treatments during the same growing season ($p = 0.05$).

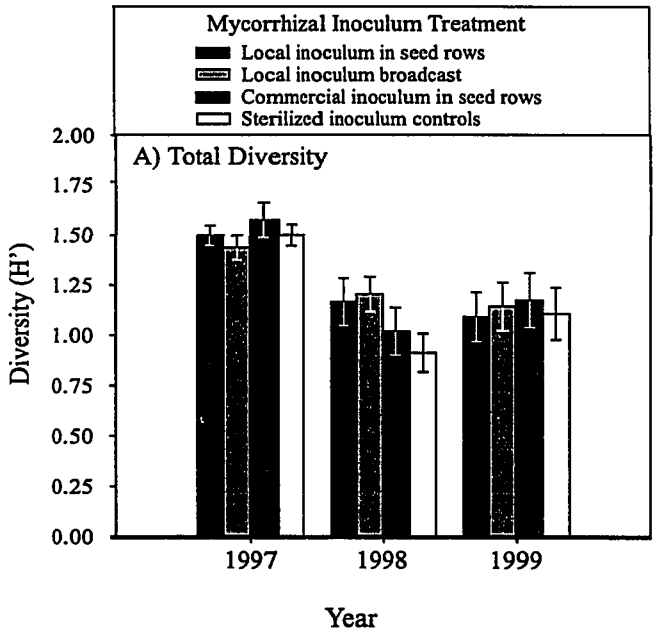

Figure 6. Effects of arbuscular mycorrhizal inoculum on plant diversity. Plant diversity in plots amended with lab produced inoculum broadcast (black bars), lab produced inoculum in seed rows (light gray bars), commercial mycorrhizal inoculum in seed rows (dark gray bars), and a sterilized control inoculum in seed rows (white bars) amended plots (B). Bars are mean value ± SE.

Chapter 4: Response of prairie restoration plant community members to nutrient and arbuscular mycorrhizal amendments

Joel E. Tallaksen

PLEASE NOTE: Reference to specific commercial formulations of fertilizer and/or mycorrhizal inoculum does not constitute a recommendation for or against such products by the author, the University of Minnesota, or any group/agency providing funding.

ABSTRACT

To improve prairie restoration efforts, many vegetation managers desire new techniques and treatments that maximize use of their resources. This study examined the restoration potential of two soil amendments (fertilization and arbuscular mycorrhizal fungal [AMF] inoculation) applied once during restoration to improve native prairie plant growth. An experimental prairie restoration was established at the site of a previously failed restoration effort. The restoration was designed to study whether these amendments could increase prairie plant abundance and reduce weedy species abundance. Three fertilizer regimes (inorganic fertilizer, slow-release fertilizer [SRF], and an unfertilized control) were combined with four AMF inocula regimes (commercial inocula/furrows, local inocula/broadcast, local inocula/furrows and uninoculated/furrows) for a total of twelve treatments. Portions of the treated plots were harvested and plants counted each September for three years. Analysis of plant data from inorganic nutrient fertilized plots found few significant differences in species abundance. The lack of major inorganic fertilizer effects can be explained by the leaching of inorganic nutrients during rain. Plots treated with SRF had significant increases in the abundance of the annual weedy species (*Echinocloa* spp. and *Seteria* spp.) and decreases in perennial native grass abundance (*Schyzacurium scoparium*, *Sorgashtrum nutans*, *Elymus* spp.). Thus, SRF appeared to limit native establishment at the study site. AM inoculation treatments did not affect species abundance, however functional group abundance was slightly altered in a few instances. The changes in abundances may or may not have been due to the AM propagules applied in the AM treatment regimes. Therefore, neither the fertilizer nor AMF treatments were helpful at this restoration site.

INTRODUCTION

Many organizations conducting prairie restorations have resource limitations that constrain their ability to perform high-maintenance restorations and must often reduce resources dedicated to site preparation and periodic maintenance. Instead, they rely on restoration protocols that require little pre- or post-restoration management. One approach used to increase the success of these low input restoration protocols is amending the soil with biotic and abiotic substances purported to enhance native plant establishment and/or growth (Anderson 1999; Henze 2001). Many soil amendments are relatively inexpensive and easy to apply during a restoration planting or seeding, and thus from a resource allocation standpoint they fit well into these low input restorations. However, most soil amendments have not been thoroughly studied in prairie restoration and from a restoration management standpoint are unproven.

Fertilization is an amendment used by some prairie restoration practitioners to improve plant health in low input restoration protocols (Henze 2001; Mn/DOT 2000). Fertilizers, especially with high nitrogen content, have been shown to be beneficial for some native plants. For example, fertilization increases native plant biomass several fold (Van Auken & Bush 1992), enhances seed production (Cornelius 1950) and induces other favorable responses such as increased tillering (Valvarde & Pisanty 1999). However, these beneficial growth responses are mainly seen in native C4 grasses grown in monoculture or in the dominant species of well-established native communities. In old-field succession and mixed native/non-native prairie sites, native species are often selected against with fertilization treatments (Houston & Hyder 1975; Wedin & Tilman 1990; Heuneke et al. 1990; Tilman & Wedin 1991). Native plants appear to be adapted to

lower nutrient soils and can survive in nutrient poor soils (Wedin & Tilman 1990), while more ruderal species rapidly acquire easily available nutrients and incorporate them into new growth. Yet, few studies have examined fertilization of prairie restoration sites. Wilson and Gerry (1995) found that fertilization reduces native seedling density and increases cover of other species. Restorations of other habitats, such as tidal wetlands (Boyer & Zedler 1999) and eastern woodlands (Carson & Barrett 1988), using nutrient amendments have also resulted in unfavorable changes in plant community composition. Therefore, restoration practitioners need to know definitively whether fertilization is a beneficial addition to restoration protocols and whether it enhances native plant establishment and growth.

Inoculation of the soil with arbuscular mycorrhizal (AM) fungal amendments during restoration seeding is another alternative for increasing the nutrients available to plants, which might fit well in a low input restoration protocol. AM fungi (AMF) are soil-borne fungi that form symbiotic associations with most native prairie plants (Hetrick et al. 1988; Wilson & Hartnett 1998; Hetrick & Todd 1992). These associations often provide plant hosts with increased access to nutrients and other benefits in return for a small portion of the host's carbohydrate production (Smith & Read 1997). AM associations are typically found to increase fitness of the plant host (Allen 1991). Field and microcosm studies of prairie plant communities both indicate that the dominant native grass species are more competitive when colonized by AMF (Wilson & Hartnett 1997; Wilson et al. 2001). These findings have led some in the restoration community to suggest the use of AMF inocula in habitat restorations involving highly disturbed sites

(Smith et al. 1998) and, indeed, some restoration practitioners are regularly applying AM amendments at such locations (St. John 1998).

This study focuses on how nutrient and AMF amendments affect the species composition of the prairie restoration plant community at a site known to be difficult to restore. Because both amendments affect the plant community at the species level, examination of how particular species react when these amendments are used is important. While fertilization studies have examined plant species response in old-field succession (Inouye & Tilman 1995) and AMF inoculation studies have looked at plant cover in inoculated restoration prairies (Smith et al. 1998), species responses to these amendments have not been fully examined in prairie restoration communities. These species responses are especially important in restoration as the perceived success or failure of restoration can depend on the abundance of a handful of species.

Two fertilizer types were tested as alternative approaches of directly adding nutrients to the newly seeded restoration community: inorganic and slow-release. Each was selected to represent an amendment specified in existing restoration protocols (Henze 2001; MN/DOT 2001). My hypothesis was that the rapid release of nutrients by inorganic fertilizer would increase the abundance of ruderal species. I further hypothesized the slow-release fertilizer (SRF) would provide plants with nutrients at a rate more close to that of natural litter decomposition and thus avoid possible negative effects from a surge of nutrients.

To examine AMF inoculation, two AM inocula and two application methods were tested. Both the inocula sources and the inoculation methods used were among those available to restoration practitioners. One AMF treatment used a locally derived prairie

inoculum and the other used inoculum from a commercial company located in the eastern United States. I hypothesized that AMF inoculation would improve plant establishment and growth, but that local prairie inoculum would be better adapted to local soil conditions, seed sources and environmental conditions. Therefore, enhancement of native plant growth than would more likely occur with local vs. the non-local inoculum. Broadcast and seed furrow application methods were tested with local inoculum, while the commercial inoculum was tested only with the seed furrow method, due to experimental size limitations. My hypothesis was that if all inoculum was placed in the immediate vicinity of the native seed (furrow method), native plants would have a higher likelihood of being colonized by the inoculum and would be more likely to demonstrate increased abundance, whereas the dispersed seed and inocula would be less likely to form mycorrhizal associations and, hence, native plant abundance would be lower.

MATERIALS AND METHODS

Study Site

The restoration site is in Shakopee, Minnesota (44°46' N 93°24' W) and is on right-of-way land managed by the Minnesota Department of Transportation (Mn/DOT). Prior to Mn/DOT purchase of the land, it was used as a residential trailer park. In 1995, Mn/DOT transformed the site into a prairie floodplain to contain excess water from an adjacent water-storage pond. Annual mean temperature and rainfall at the site are 5.9 °C and 70 cm respectively. Historic data indicates that pre-settlement vegetation consisted of wet meadow species and that soils were a Hubbard fine sand series (Harms 1959). The

origins of the post-construction topsoil and sub-soils at the site are unknown, but they are very sandy and reminiscent of the locally occurring sandy soil types.

The roughly 13 acre site was originally seeded by Mn/DOT with prairie natives in 1995, but by 1997 the seeded prairie vegetation was extremely sparse and patchy. A small section of the site that had no remaining natives from the 1995 restoration was chosen to be restored a second time. Although the seed bank could have retained some native seed from the 1995 restoration and a few native species may have been present in nearby refugia, native plants in the new restoration plot should almost entirely be progeny of seed from this study.

In the two days before seeding and amending the site in June of 1997, it was tilled twice to a depth of approximately 15 cm with a commercial walk behind roto-tiller. Light rain between tillage treatments moistened the dry sandy soil and provided an excellent seeding bed. Existing vegetation was lacking due to unseasonably cool and dry weather, so pre-treatment with glyphosate herbicide was not deemed beneficial.

Seeding and amendment application

Research plots were fertilized, inoculated and seeded on June 26-27, 1997. Three fertilization regimes and four mycorrhizal regimes were used in a factorial design for 12 different treatments. Treatments were applied in a complete random block design with five blocks (Appendix IIa). Each treatment plot was 2 m by 2 m with a restored buffer zone of 1 m surrounding each plot (2 m between treatment plots). A further buffer zone extending 4 m around the entire research site was planted with native grasses.

The seed mix applied (Appendix IIb) to the restoration site was selected based on herbarium collections of locally occurring species (Ownbey & Morley 1991), local habitat, and regional specification of the Minnesota Department of Transportation. The mix consisted of twenty-seven native species and a sterile cover-crop each individually purchased from Prairie Restorations Inc. (Princeton, MN), Prairie Moon Nursery (Winona, MN), or Peterson Seed Company (Shakopee, MN).

Broadcast application involved evenly hand-spreading seed and inocula over the 4 m^2 treatment area and raking them into the soil to an estimated average depth of 1 cm. For in furrow application, inocula and seed were evenly dispensed into rows 3 cm deep by 2 m long (16 cm apart) and covered to a depth of 1 cm with soil. The buffer zone between the plots was broadcast seeded using the same seed mix and rate as treatment plots.

Fertilizer formulations were selected based on then current restoration protocols (MN/DoT 1997). Inorganic fertilizer 6-24-24 (N-P-K) was applied at a rate of 225 kg/ha (Howe Fertilizer, Minneapolis, MN). Encapsulated slow-release fertilizer (22-5-10 + micronutrients) with a three-month release time was applied at the manufacture's suggested rate of 305 kg/ha (Howe Fertilizer, Minneapolis, MN). Fertilizers were evenly broadcast over the study plots, then raked into the top 4-cm of soil. Control treatments had nothing added, but were similarly raked.

The local prairie inoculum used in this study was generated in three batches using the sand-soil pot culturing technique (Ferguson & Woodhead 1982) and a small amount of starter inoculum collected at Crosstown Prairie (44° 54' N, 93° 12' W), a nearby remnant prairie. The three batches were cultured for 16 weeks with *Andropogon gerardii*

or *Bouteloua curtipendula* as a host plant. Soil, spores and chopped root pieces from the different batches of inocula were thoroughly mixed prior to use. This combined inoculum had a spore density of 36.2 spores/g (Tallaksen 2002). The commercial inoculum, Mycor™ VAM Cocktail™ Flowerbed Inoculant used in the study was purchased from Plant Health Care Inc. (Pittsburgh, Pennsylvania). According to product labeling, the commercial inoculum was formulated to provide a suitable AM inoculum for a wide range of herbaceous species. While product literature did not list specific organisms or quantities, the commercial inoculum also included yucca, seaweed, humic extracts, and nitrogen fixing/phosphorus solubilizing bacteria.

The AM inocula treatments were applied at the following rates: 1) local AMF inoculum broadcast (2.54 kg/m^2, 23000 spores/m^2), 2) local AMF inoculum applied in-furrow (2.54 kg/m^2, 23000 spores/m^2), 3) commercial inoculant in-furrow(0.585 kg/m^2, 4800 spores/m^2), and 4) a sterilized local AMF control inoculum in-furrow (2.54 kg/m^2, 23000 spores/m^2).

Vegetation sampling

Plant matter was collected during the third week of September each fall. A different 10-cm by 100-cm strip of vegetation was harvested from each plot each year. All aboveground material was removed, exposing the bare soil. Plants from individual plots were frozen for preservation within 10 hours of harvesting and thawed over the next 2-5 months for quantification. Plants from each plot were identified to genus and species when possible; however, during the first two seasons, many newly germinating species

lacked reproductive structures and/or were too small to identify with certainty. In these cases, unknown plants of very similar appearance were grouped as a species and placed into functional groups based on similarity of morphological characters to known locally occurring species. In some cases, native grass species were not able to be identified and were given identifying letters (e.g. unknown native grass A).

Soil sampling

In the third week of September during each of the three growing seasons, ten soil cores per plot were collected to a depth of 15 cm with a 2.5-cm diameter soil corer. Each season, the five replicate samples from the 12 treatment combination (fertilizer x inoculum) were combined for nutrient analysis. Samples were stored at -20° C until analyzed by the University of Minnesota Research and Analytical Laboratories. Nitrate (Henriksen & Selmer-Olsen 1970) and phosphorus (Olsen method) (Frank et al. 1998) were quantified by colorimetric assay, while potassium was quantified by ICP analysis (Fassel & Kniseley 1974). Nitrate, phosphorus, and potassium concentrations were statistically tested using ANOVA ($\alpha = 0.05$), with the mycorrhizal/fertilizer interactive effects used as the error factor.

Statistical analysis

Relative abundance was determined by dividing the number of individual plants in a species or group by the total number of plants in the plots. Individual species abundance data was calculated only for species commonly found in the treatment plots, using the criterion that a given species or group had to be present in at least 30 of the 60 treatment

plots. Plant species that occurred infrequently (rare species) were combined into groups according to their functional role in the community (rare weedy forbs, rare weedy grasses, rare native grasses, and rare native forbs). In addition, functional groups were established that included both frequently and infrequently occurring species (weedy grasses, weedy forbs, native grasses, and native forbs). Analyses were performed using a blocked two-way ANOVA in the MacANOVA statistical analysis software package. A fertilizer by inoculum interactions term was used in the ANOVA, however no interactive effects were observed. Significant differences between treatments were calculated using Tukey's pairwise comparison at $p < 0.05$.

RESULTS

Fertilization

Concentrations of specific nutrients increased in fertilized plots according to the type of fertilizer applied (Table 1). Inorganic nutrient fertilized plots had increased levels of potassium during the first and second year, whereas plots treated with SRF had higher concentrations of nitrogen in the first year. Nitrogen levels in the sandy soils of this site were low (2.68 ppm nitrate in untreated plots) and indicate nitrogen limiting conditions. Phosphorus levels were not significantly different in either nutrient addition treatment.

Plant species abundance in inorganic nutrient fertilized plots did not significantly differ from control plots in most of the analyses (Figs. 1 thru 4). Exceptions to this were the weedy forb *Conyza canadensis* (Fig. 1a) and unknown native grass B (Fig. 1b), both of which responded to inorganic nutrient fertilization with decreases in abundance. However, in the majority of cases, plant abundance in inorganic nutrient fertilized plots

followed the trend of plants in the SRF fertilized plots. Hence, if a significant increase in plant species abundance occurred in SRF plots, inorganic nutrient fertilized plots usually had a slight increase in abundance.

Weedy grasses were the most abundant functional group seen at the site during the study and showed significantly altered abundance levels in SRF treated plots. Overall, the most common weedy grasses were *Seteria* spp., which were observed during all three years of the study (Fig. 2a). While SRF did not influence the abundance of *Seteria* spp. during the first season, plots treated with SRF had significantly higher *Seteria* spp. abundance than did control plots during the second and third seasons. Other weedy grasses found in the first two seasons were *Echinocloa* spp. (Fig. 2b), whose abundance was increased in SRF treated plots at the end of the first season. However, during the second season, no differences were detected in *Echinocloa* spp. abundance, and by the third season too few plants were available to analyze. Members of the genus *Digitatiria* (Fig. 2c), though very numerous, were only a minor plant component at the site due to their small stature compared to the larger plants of *Seteria* spp. and *Echinocloa* spp. The large increases in *Seteria* spp. and *Echinocloa* spp. abundance in SRF treated plots increased the relative abundance of weedy grasses as a functional group (Fig. 2d).

Several weedy forb species occurred sporadically during the study, although only *Amaranthus* spp., *Chenopodium* spp., and *Conyza canadensis* occurred in sufficient number for statistical analysis. *Amaranthus* spp. and *Chenopodium* spp., seen in the first season, were grouped for analysis due to the difficulty in distinguishing between the tiny, almost fully senesced seedlings. No significant difference was observed in *Amaranthus* spp. / *Chenopodium* spp. abundance in plots receiving SRF (Fig. 3a). Only during the

second year of the study was *C. canadensis* (Fig. 1a) present in sufficient quantity for analysis, at which time it was found to be less abundant in plots treated with SRF. The abundance of the infrequently occurring weedy forbs did not change in SRF treated plots (Fig. 3b). As a whole, weedy forbs (Fig. 3c) changed abundance in SRF plots only during the second season, when weedy forbs abundance was lower in SRF plots (Fig. 2d middle pane).

Native grasses were most prominent in the second and third seasons of the study. However, *Panicum capillare* (Fig. 4a) and unknown native grass A (Fig. 4b) were seen during the first seasons, but neither species appeared to respond to SRF. In the second season, SRF reduced the abundance of three native grasses: *Schyzacurium scoparium* (Fig. 4c) and unknown native grasses B (Fig. 1b) and C (Fig. 4d). Abundance data from the native genus, *Elymus*, also included a small number of individuals of the species *Agropyron repens*, which looked somewhat similar. The abundance of *Elymus* spp./*A. repens* (Fig. 4e) was not affected by SRF in the second season. However, during the third season *Elymus* spp./*Agropyron* spp. was very significantly reduced in SRF treated plots. Similarly, during the third season, *Sorghastrum nutans* (Fig. 4f) was found in much lower numbers in SRF treated plots. Changes in the abundance of the native grasses as a group corresponded to treatment with SRF during all three seasons; both the infrequently occurring native grasses (Fig. 4g) and the entire group of native grasses (Fig. 4h) showed significant reductions in SRF treated plots.

Rudbeckia hirta (Fig. 5a) was the only native forb that occurred in sufficient numbers for analysis, and only in the third season was the population large enough to be analyzed. However, no significant differences were observed in *R. hirta* abundance in

SRF treated plots, nor was the abundance of the rare native forb group (Fig. 5b) significantly different from that in unfertilized controls.

Mycorrhizal response

There were no individual species responses to the different mycorrhizal application and inoculation treatments; however, increased abundances of two functional groups corresponded to two AMF treatments. In plots treated with the commercial inoculum treatment, rare native grasses (Fig. 6a) were more abundant during the first season. Plots treated with the broadcast seeding/inoculum regime had increased abundance of native forbs (Fig. 6b) during the second year.

DISCUSSION

Fertilization

The absence of major plant responses to inorganic fertilizer treatment may be explained by leaching of the nutrients in the inorganic fertilizer. Inorganic fertilizers are composed of inorganic salts that readily dissolve in water and release nutrients into the soil solution (Hummel & Waddington 1986). Thus with moderate moisture, all the inorganic nutrients are available to plants immediately as they dissolve into solution. Shortly after fertilization (and seeding), heavy rains saturated the research plots. Most of the seeds had not yet germinated or had tiny root systems. The heavy rain probably dissolved the inorganic fertilizer and flushed the nutrients out of the soil and into the watershed before the seedlings were able to acquire them. Soil nutrient concentrations (Table 1) provide evidence for leaching of the inorganic fertilizer. Concentrations of

nitrogen, the most easily leached nutrient, are similar in control and inorganic nutrient fertilized plots. However, potassium, which is less subject to leaching, was found in significantly higher concentrations in inorganic nutrient fertilized plots. The lack of an increase in nitrogen concentrations in the nitrogen limiting soils likely eliminated the potential for a change in plant abundance. Since leaching apparently reduced this studies' ability to establish any positive or negative effects on the restoration plant community, I could not verify my hypothesis regarding inorganic fertilizer effects on the plant community.

Slow release fertilizer was included in this study because it potentially offered a way to avoid the negative effects of inorganic fertilizers on the plant community. SRF application was thought to somewhat mimic natural soil decomposition processes and provide only slight increases in soil nutrient levels, thereby yielding relatively minor changes in the plant community. Unfortunately, SRF addition significantly altered the abundance of several species and functional groups (Table 2).

The species that benefited the most from this one-time application of SRF were the undesirable weedy annual grasses common in the first year of restorations. Key native grass species, typically perennial, appeared to be at a disadvantage in SRF treated plots, exhibiting substantial reductions in abundance. My results fit well with studies of other restoration and old-field succession sites, which found that long term SRF application resulted in increased nutrient levels, decreased the abundance of native species and increased the presence of weedy species (Tilman 1987, Carson & Barrett 1988, Boyer & Zellder 1999). Similarly, I found that nutrient addition via SRF increases the time that early successional weedy species dominated the restoration plant community

(Inouye & Tilman 1995). However, unlike their studies, my study demonstrates a measurable response to nutrients after only one application of SRF (with a three month release time). The fact that the single application of nutrients was able to influence plant abundance for the three years also indicates the major role nutrients play in a developing plant community.

Overall, my data suggests that fertilizers are not helpful in promoting native species establishment and growth in a prairie restoration, especially at sites with a weedy seed bank. The nutrients added via SRF decreased the abundance of native species and increased the population of undesired species. Although I found that nutrient addition via SRF reduced native species abundance, total plant biomass was increased in plots treated with SRF (Chapter 3). Thus for erosion control of barren slopes on a restoration site, SRF might be a useful component of the restoration protocol. While inorganic fertilizer did not have the same unfavorable effects to native species abundance seen in other studies, the findings that nutrients likely leached into the nearby watershed are unfortunate as added nutrients can negatively impact waterways (Lowery 1998). Therefore, fertilization may be of little benefit in prairie restoration.

Arbuscular Mycorrhizal Inoculation

The increasing abundance of the rare native grasses and forbs in AM inoculated plots may support the idea that AM amendments can increase native plant presence in prairie restoration. However in the AM inoculated plots, treatment factors other than the actual AMF offer equally likely explanations for the observed increases in abundance.

Therefore, more detailed examinations of the AM inoculation treatments need to be conducted.

The commercial inoculum product included AMF inoculum, but also contained what the product literature referred to as biotic and abiotic growth stimulants, including bacteria, plant and seaweed extracts, and humic acid. Each of these could be responsible for plant growth changes, meaning that AMF inoculum is not necessarily inducing the altered abundance. The inability to separate the response to these individual commercial inoculum components limits what can be explicitly stated about AMF and indicates the need for further study of this product and its individual components.

The broadcast seed/inoculum application used the same seeding and inoculation rates as the furrow method; therefore, plant abundance changes in the broadcast treatment indicated that the treatment method was important in the response not simply the addition of the fungal inoculum. However, the increased abundance could result from the seed being broadcast, the inoculum being broadcast, or the interactions of both seed and inoculum being broadcast. One scenario for the change in plant abundance is that the planted native seed faced less competition from other planted native species when broadcast over a larger area. An alternative explanation is that in the broadcast treatment the inoculum came into contact with more weedy plants and may have suppressed establishment and growth of these weedy species (Allen & Allen 1988; Allen et al. 1988). The design of this experimental treatment does not allow either of these explanations to be ruled out, and consequently, the AMF component of the treatment can not be explicitly linked to the change in native forb abundance. However, results of these treatment do suggest the need to further examine which application method is best for native seeding

and inoculation, as one may hold an advantage over the other in promoting plant establishment.

Although the changes in abundance due to AM inocula sources and application methods are statistically measurable, the differences could not be considered major changes in the plant community. Plant responses to AMF inoculation may have been limited by natural recolonization of the AMF population in the two years between the initial construction-restoration (1995) disturbance and the current restoration (1997). AM spore counts in the year between the restoration efforts (1996) indicated recovery of some AM propagules (I. Charvat & N. Berg, personal communication), which were not likely destroyed by tillage during the second restoration. In a parallel study during the second restoration, it was found that plants in control plots had 50% of their root length colonized by AMF after only one season's growth (Charvat et al. 2000), which suggests that remnant AMF propagules sufficiently inoculated plants and likely reduced the importance of the added AMF inoculum.

Habitat differences may also have played a role in attenuating any AMF effects on the plant community. Previous studies of AMF inoculation demonstrating positive affects on restoration plantings were conducted on mesic soils (Noyd et al. 1995; Smith et al. 1998). Plants in more arid conditions may be more responsive to AM inoculation because AMF increases drought tolerance (Auge 2001). In addition, AMF amendments may be more critical in drier habitats as AM colonization and reproduction are thought to occur more slowly on mesic soils (Miller 1987). The Shakopee site was hydric which might have allowed more rapid recolonization and consequently limited the differences between AMF inoculation treatments. Unfortunately, a complete understanding of the

relationship between habitat and AM communities is lacking. More studies such as this one in a variety of habitats are needed to fully understand the dynamics of AMF inoculation.

From the vegetation management perspective, AMF inoculation was not proven beneficial in this restoration. Even if the noted plant abundance increases could be solely attributable to AMF inoculation, the responses were not sufficient to justify the expense of purchasing the inoculum or the effort needed to inoculate the restoration site. In other words, the statistical differences did not translate into an economically useful difference. Yet, the information from this restoration is only one piece of the larger AMF inoculation issue. Differences such as habitat type, planted/seeded species, fallow period, and inoculation method are all likely to affect mycorrhizal inoculation responses. Therefore, the results of this study should be considered in conjunction with those of other studies to determine when and where AMF inoculation may be beneficial.

CONCLUSIONS

Results of this study suggest that fertilizer is not a beneficial amendment for low input restoration protocols at all restoration sites. The inorganic fertilizer applied to the site was leached into the watershed, which can pollute waterways and is a waste of resources. Although SRF did not leach, SRF treated plots had reduced abundance of key native grasses. The negative fertilization results from my study are supported by a large body of fertilization studies that suggest fertilizers are not appropriate in a prairie restoration setting.

Arbuscular mycorrhizal amendments also may not be beneficial in restoration, though they did not appear to impair the restoration effort. The abundance increases in certain native plant groups in AMF treated plots were not necessarily caused by the AM fungal propagules applied in the AMF treatment regimes, nor were these increases reason to justify the effort and expense of AMF inoculation. While AMF inoculation does not appear to be beneficial under the restoration conditions examined in this study, this study represents only one particular situation in which AMF inoculation was tested. As few AM inoculation trials have been scientifically conducted, more research should be conducted to determine when and where AMF inoculation might help in restoration.

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Table 1. Soil nutrient levels in fertilized plots at the Shakopee restoration site. Numbers are mean nutrient levels (ppm) ± SE. Soil was sampled in the third week of September each Fall. Letters following numbers indicate significant differences between treatments during the same growing season ($p < 0.05$). Treatments without letters had no significant differences.

	Nitrate (ppm)		
	1997	1998	1999
No fertilizer control	2.68 ±0.31 a	2.38 ±0.18	3.35 ±1.09
Inorganic	2.50 ±0.09 a	2.48 ±0.21	3.90 ±0.34
Slow release	4.65 ±0.72 b	3.50 ±0.56	4.93 ±0.69
	Phosphorus (ppm)		
	1997	1998	1999
No fertilizer control	22.63 ±0.99	23.00 ±1.00	21.50 ±0.96
Inorganic	26.50 ±1.66	29.50 ±2.06	29.75 ±2.47
Slow release	24.50 ±1.44	27.00 ±2.61	27.00 ±3.24
	Potassium (ppm)		
	1997	1998	1999
No fertilizer control	31.88 ±1.66 a	34.50 ±1.85 a	36.50 ±0.65
Inorganic	39.50 ±0.96 b	42.25 ±2.50 b	42.75 ±3.37
Slow release	28.00 ±1.08 a	34.13 ±0.43 a	38.00 ±3.19

Table 2. Summary of plant occurrence in response to slow-release fertilization (SRF)

	Genus/Species		
	1997	1998	1999
Increased presence	*Echinocloa crusgali*	*Seteria* spp.	*Setaria* spp.
Differences not detected	*Digitaria* spp. *Amaranthus/chenopodium* *Setaria* spp. Grass A	*Echinocloa crusgali* Unknown Grass A *Lolium* spp.	*Rudbeckia hirta*
Decreased presence	*Panicum capillare*	*Conyza canadensis* *Digitaria spp.* *Schyzacurium scoparium* Unknown Grass B Unknown Grass C	*Lolium* spp *Sorgastum nutans*

	Functional Groups		
	1997	1998	1999
Increased presence	Weedy Grasses	Weedy Grasses	Weedy Grasses
Differences not detected	Weedy Forbs Rare Weedy Forbs	Rare Weedy Forbs Rare Native Forbs Native Forbs	Weedy Forbs Native Forbs
Decreased presence	Rare Native Grasses Native Grasses	Weedy Forbs Native Grasses Rare Native Grasses	Native Grasses Rare Native Forbs Rare Native Grasses

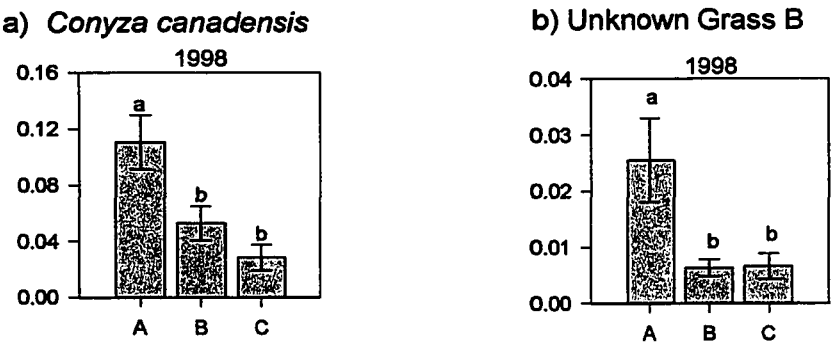
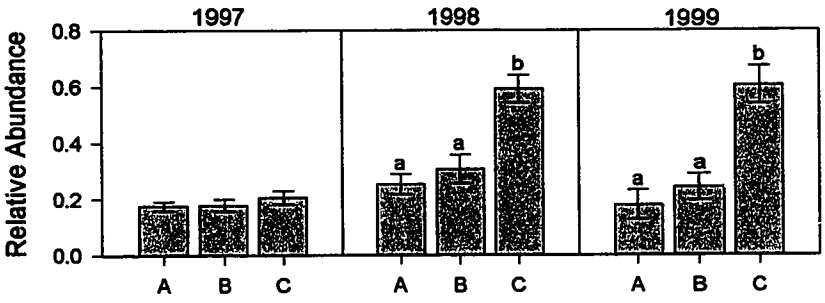
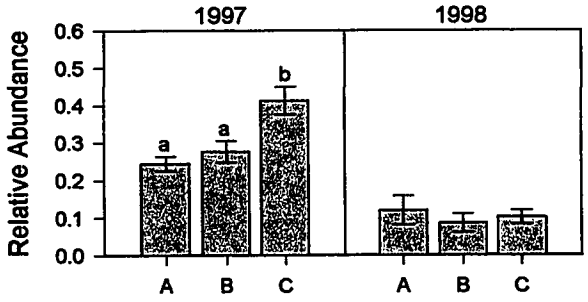


Figure 1. Relative abundance of a) *Conyza canadensis* and b) unknown grass B in plots treated with (A) no fertilizer, (B) inorganic fertilizer, or (C) slow-release fertilizer. Bars represent relative abundance of plant species (± SE). Different letters above bars indicated significant differences at $p < 0.05$

a) *Seteria* spp.



b) *Echinocloa* spp.



c) *Digitaria* spp.

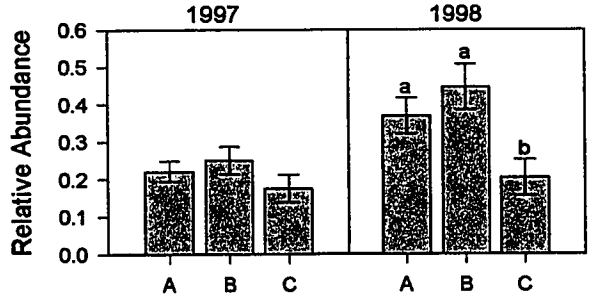
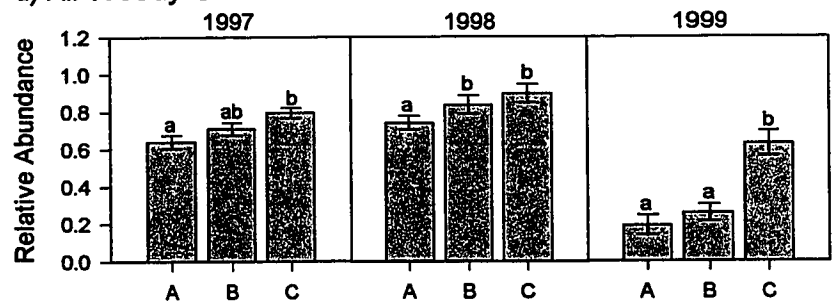


Figure 2. Relative abundance of weedy grasses in plots treated with (A) no fertilizer,(B) inorganic fertilizer, or (C) slow-release fertilizer.Bars represent relative abundance of plant species or functional groups (± SE). Different letters above bars indicated significant differences at $p < 0.05$.

d) All Weedy Grasses



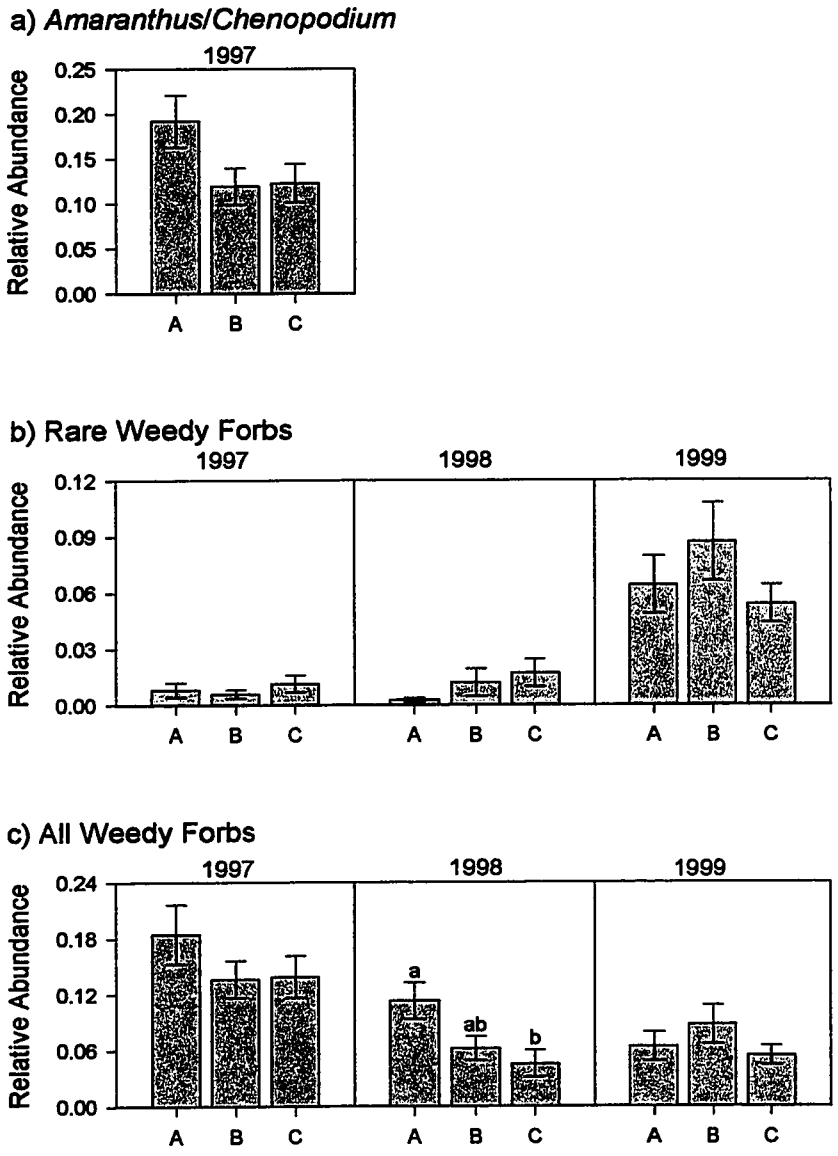


Figure 3. Relative abundance of weedy forbs in of plots treated with (A) no fertilizer, (B) inorganic fertilizer, or (C) slow-release fertilizer. Bars represent relative abundance of plant species or functional groups (± SE). Different letters above bars indicated significant differences at $p < 0.05$.

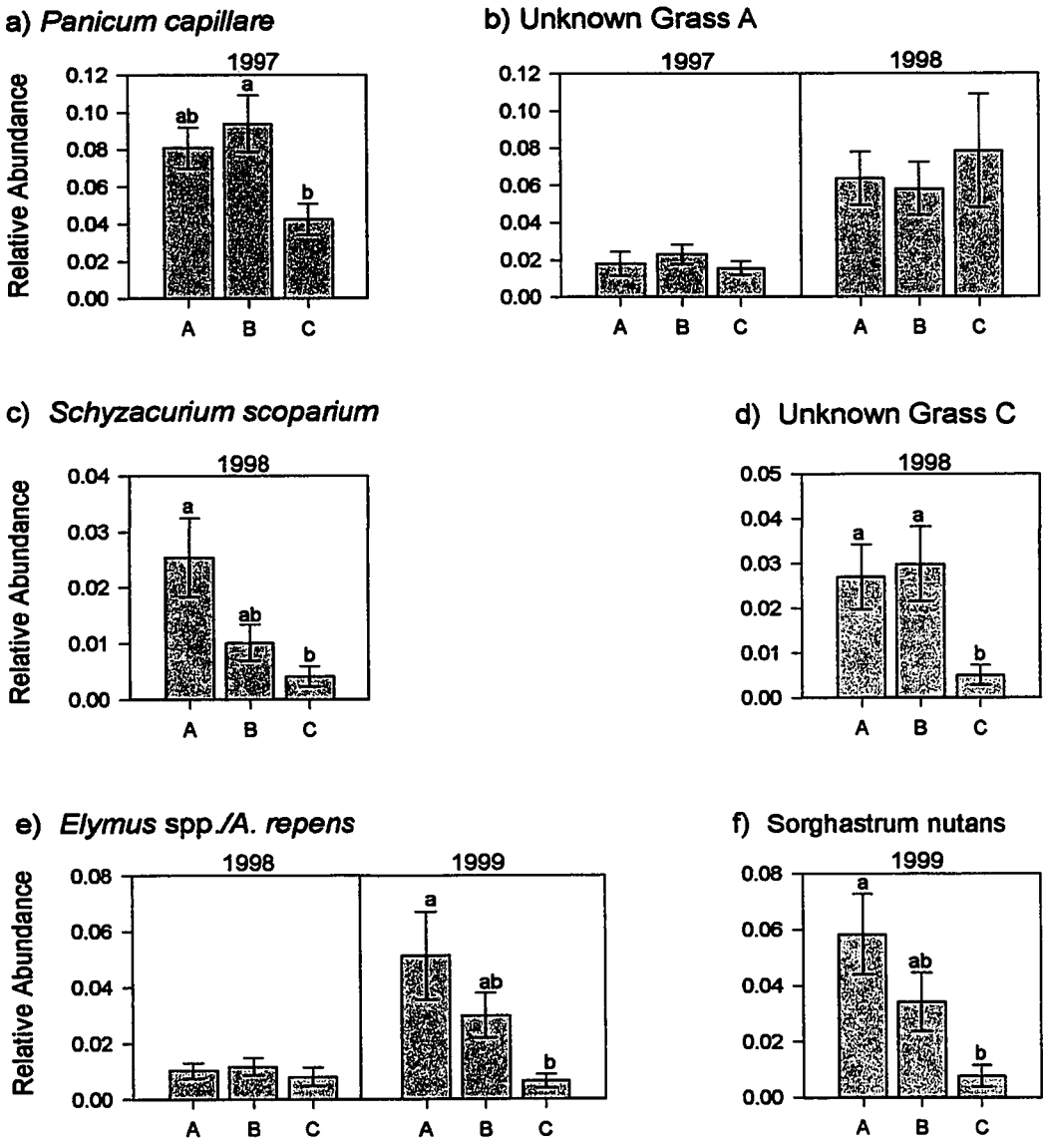


Figure 4. Relative abundance of native grass species in plots treated with (A) no fertilizer, (B) inorganic fertilizer, or (C) slow-release fertilizer. Bars represent relative abundance of plant species or functional groups (± SE) Different letters above bars indicated significant differences at $p < 0.05$

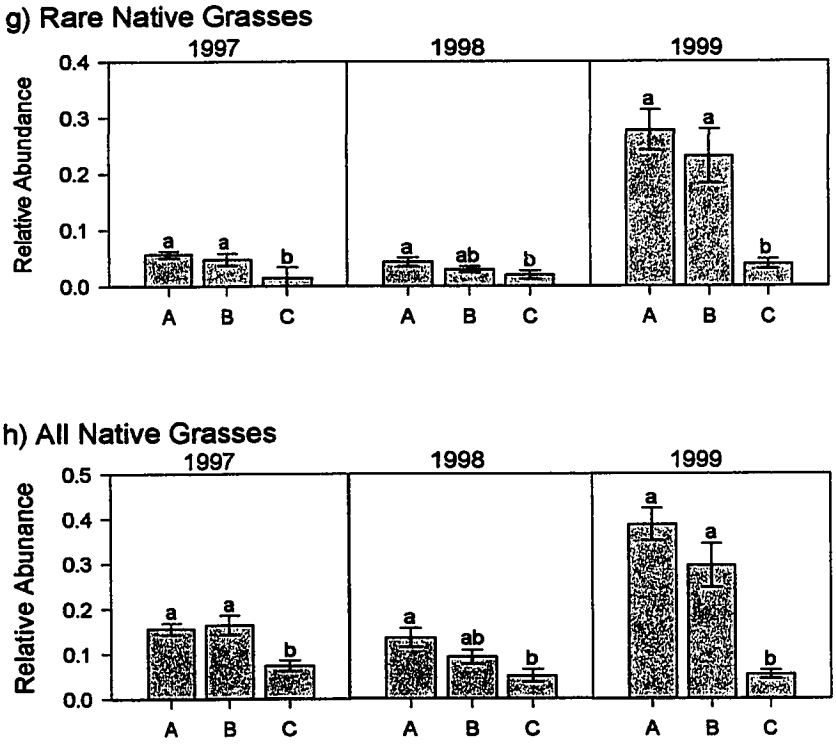


Figure 4. continued

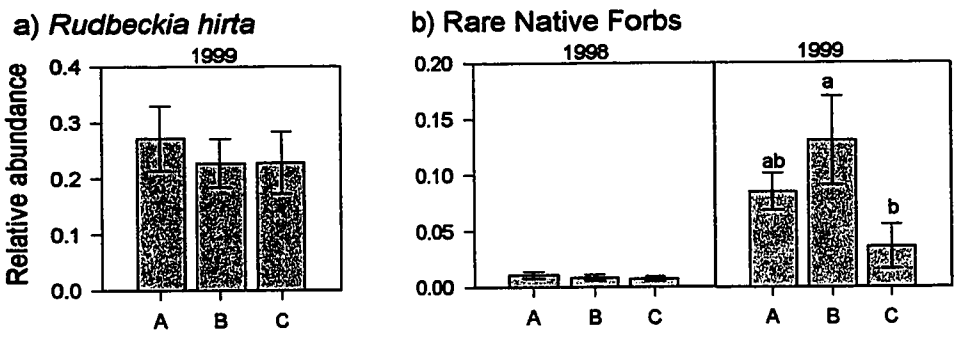


Figure 5. Relative abundance of native forbs in plots treated with (A) no fertilizer, (B) inorganic fertilizer, or (C) slow-release fertilizer. Bars represent relative abundance of plant species or functional groups (± SE). Different letters above bars indicated significant differences at $p < 0.05$.

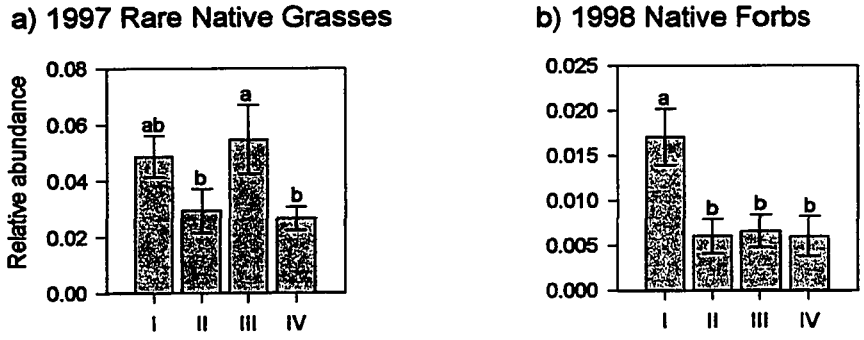


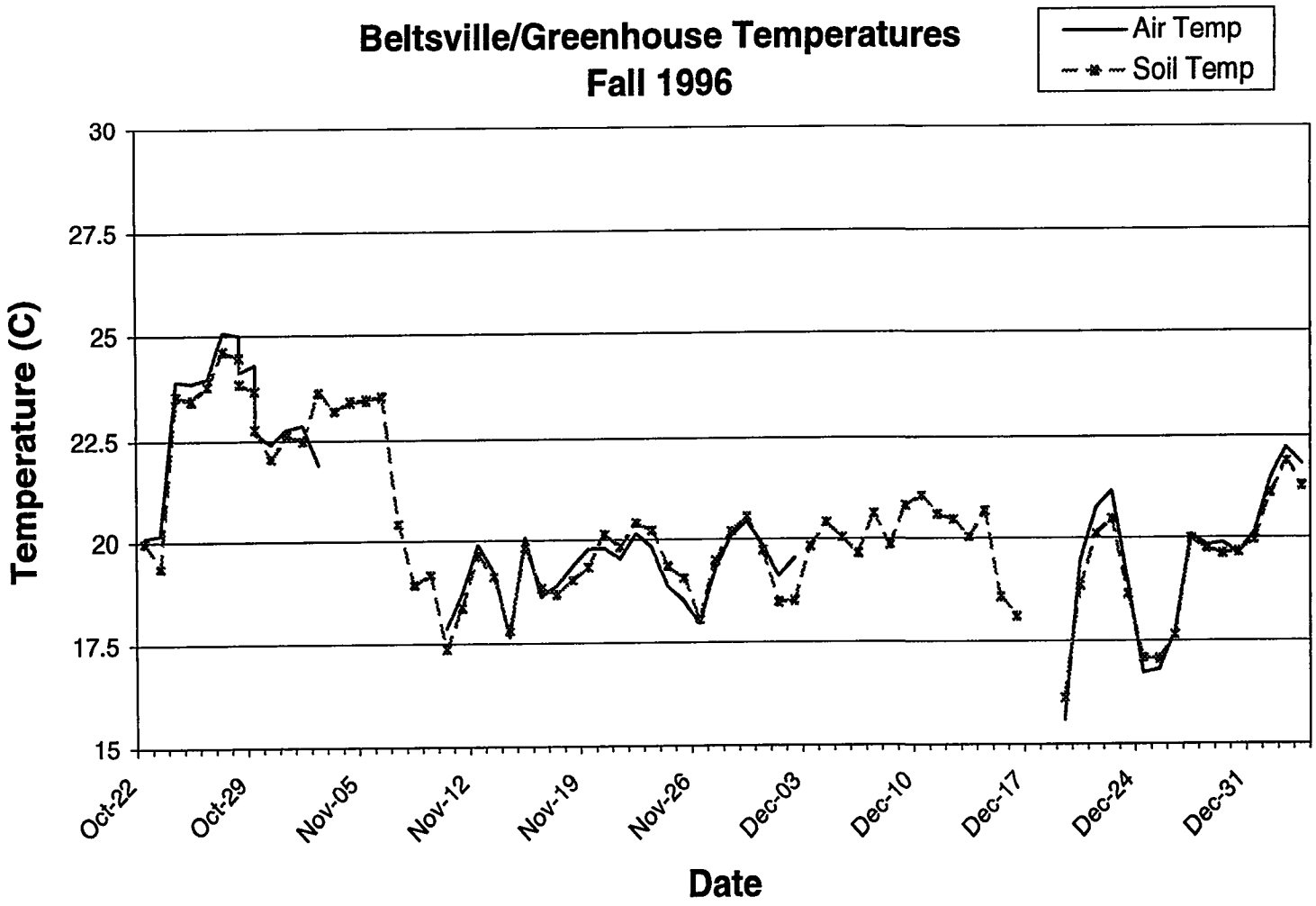
Figure 6. Relative abundance of weedy forbs in of plots treated with (I) lab produced inoculum broadcast with seed, (II) lab produced inoculum in seed furrows, (III) commercial inoculum in seed furrows, (IV) or with a sterile inoculum in seed furrows. Bars represent relative abundance of plant species or functional groups (± SE) Different letters above bars indicated significant differences at $p < 0.05$.

Appendix I: Soil Temperature Data

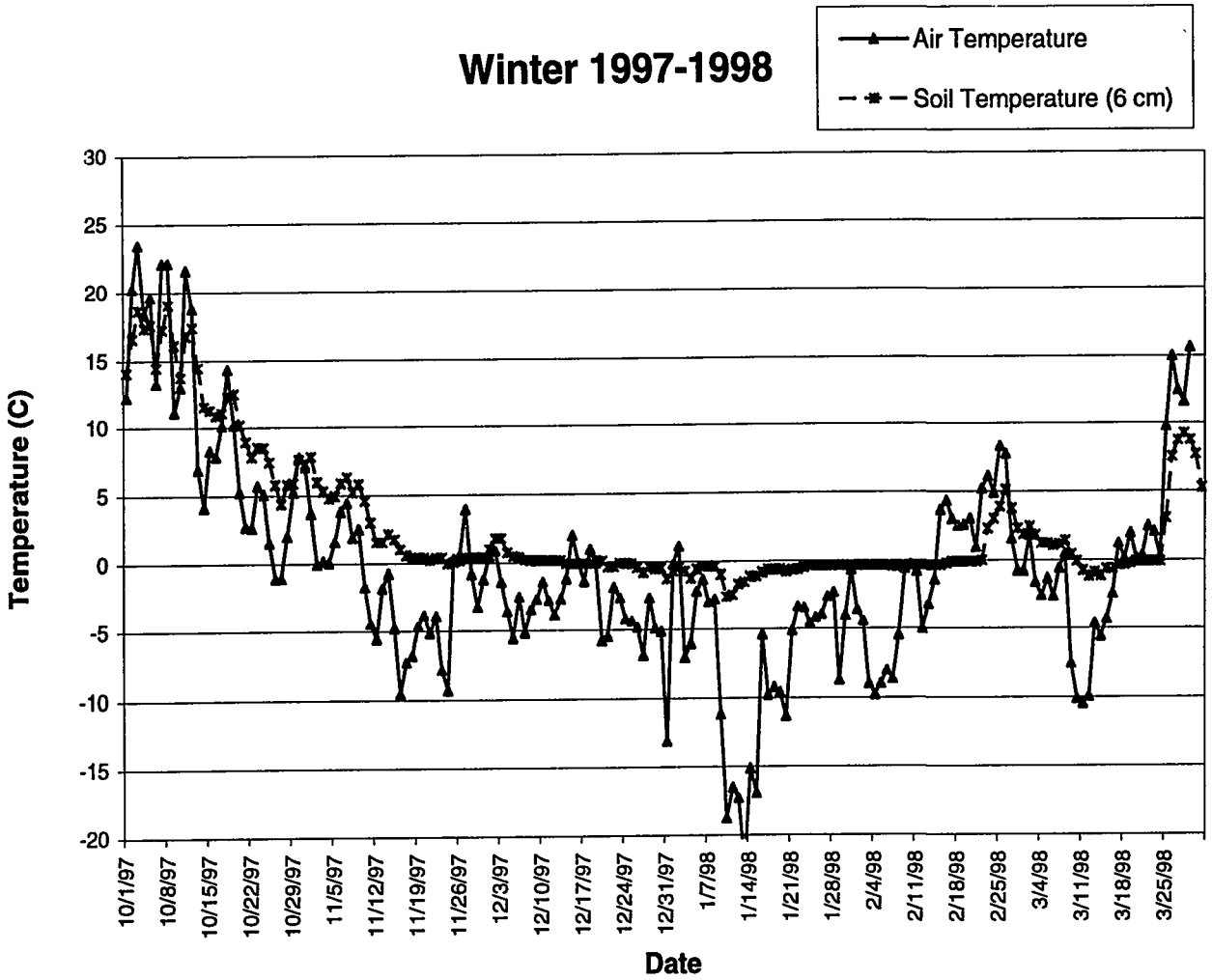
Appendix I: Soil temperature in field and greenhouse soils

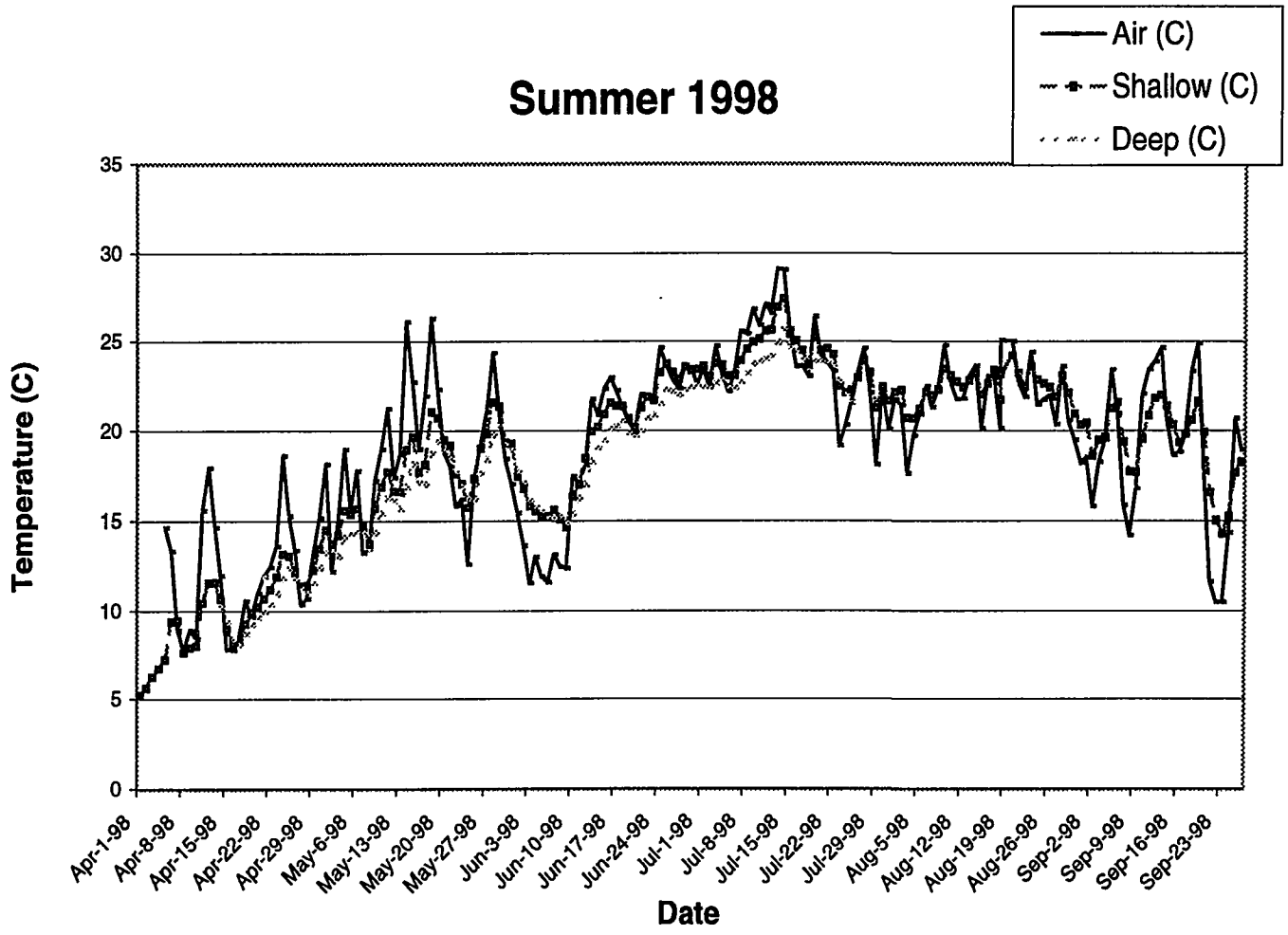
Early data collection during the inoculum production study indicated that greenhouse soil temperature may have been considerably higher than those found in field situations. To further examine this observation, more extensive testing was undertaken. Soil temperatures were monitored during the restoration inoculation experiment (Chapter 3 & Chapter 4) and during the greenhouse inoculum production (Chapter 2). Soil temperatures from the restoration plots were monitored for 30 months at two different soil depths. Greenhouse temperature data was recorded in the sand media of inoculum production pots and in the air.

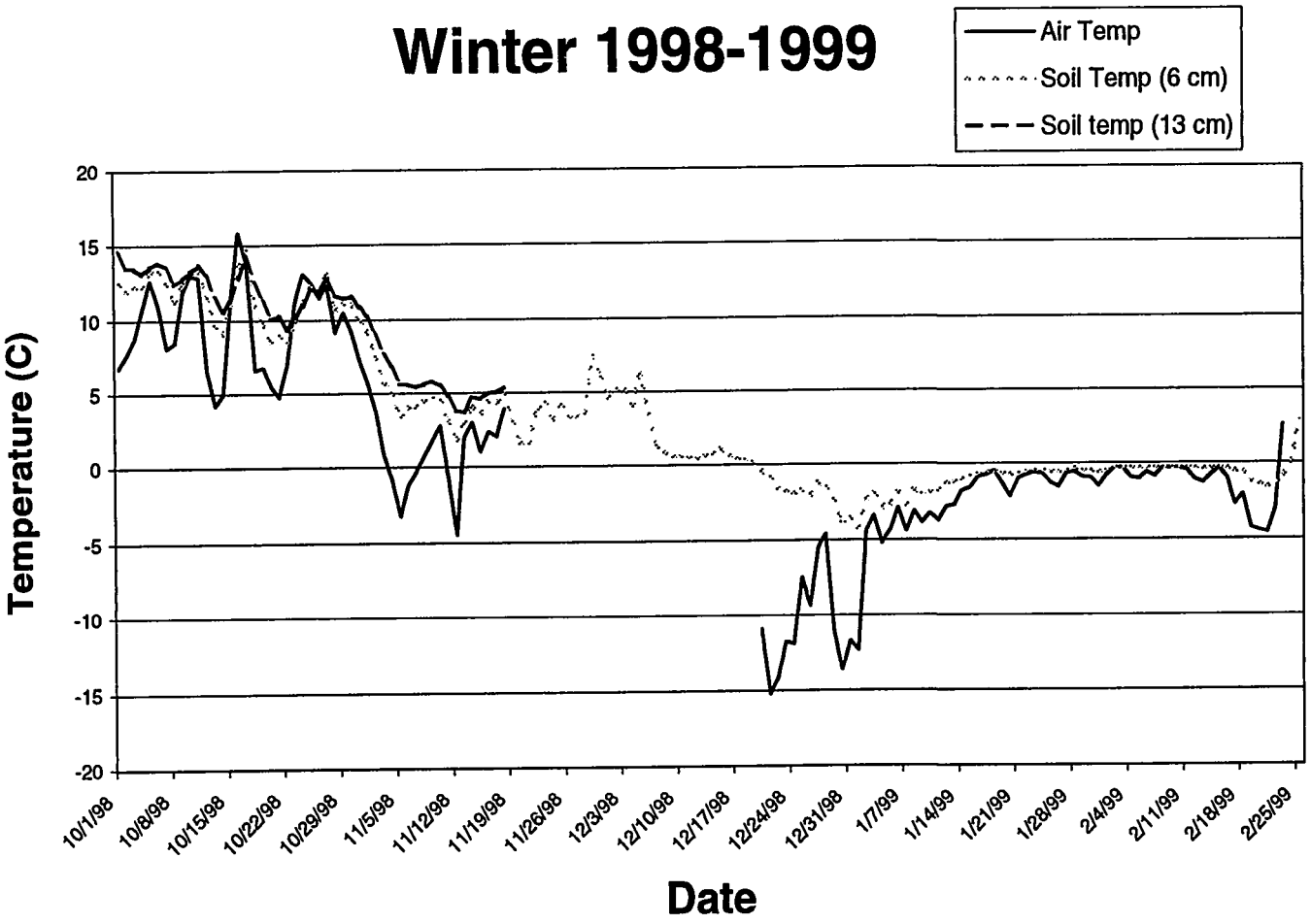
The following graphs represent the data collected. Unfortunately due to technical problems, readings were sometimes not collected by the probes or able to be downloaded from them. These are indicated as gaps in the data.


Beltsville/Greenhouse Temperatures
Fall 1996
Air Temp
Soil Temp
Temperature (C)
30
27.5
25
22.5
20
17.5
15
Oct-22
Oct-29
Nov-05
Nov-12
Nov-19
Nov-26
Dec-03
Dec-10
Dec-17
Dec-24
Dec-31
Date

Summer 1997
Air Temp
Soil Temp (6 cm)
Temperature (C)
30
25
20
15
10
5
0
Date
May-6
May-13
May-20
May-27
Jun-3
Jun-10
Jun-17
Jun-24
Jul-1
Jul-8
Jul-15
Jul-22
Jul-29
Aug-5
Aug-12
Aug-19
Aug-26
Sep-2
Sep-9
Sep-16
Sep-23
Sep-30

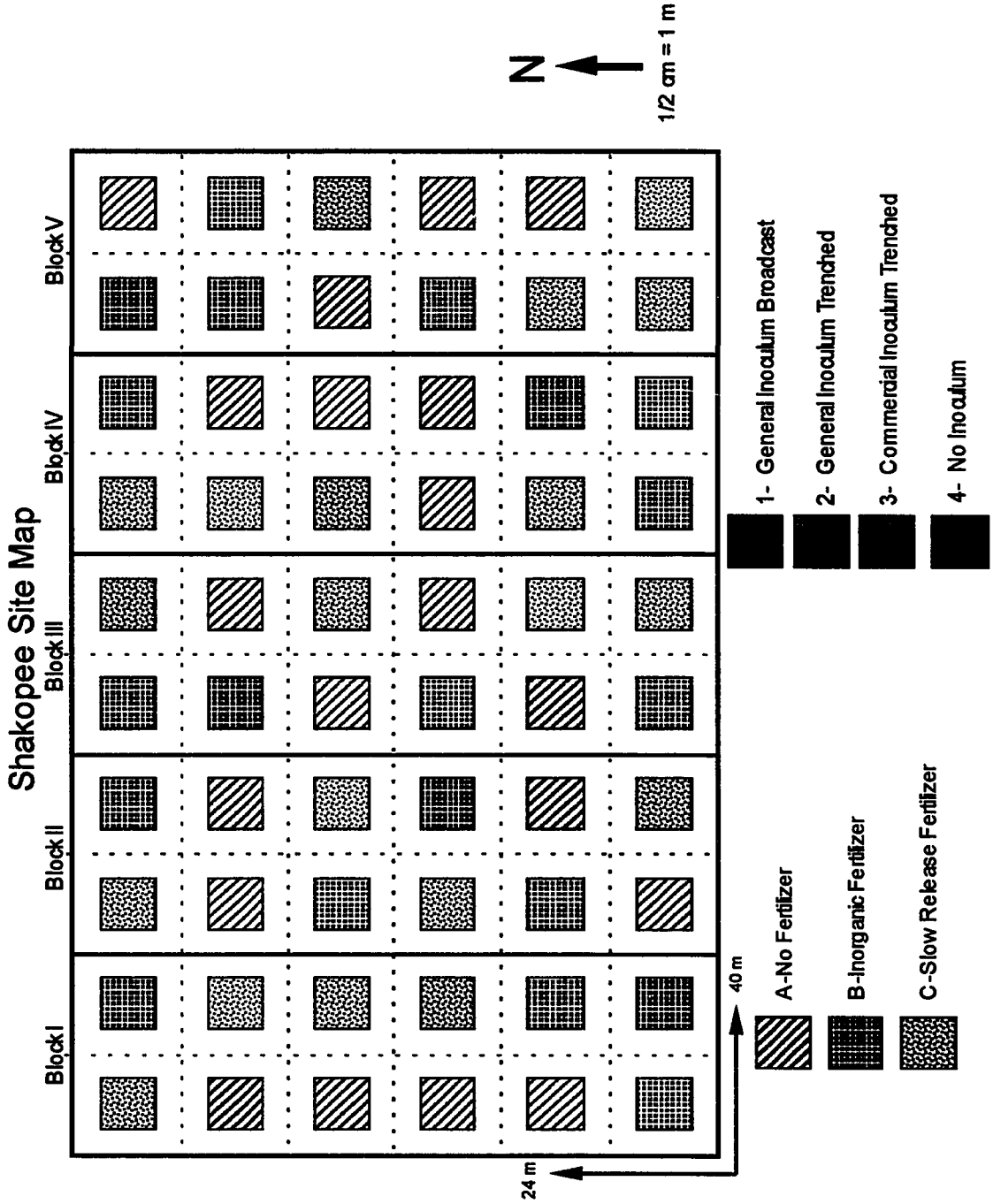

Winter 1997-1998
Air Temperature
Soil Temperature (6 cm)
Temperature (C)
30
25
20
15
10
5
0
-5
-10
-15
-20
10/1/97
10/8/97
10/15/97
10/22/97
10/29/97
11/5/97
11/12/97
11/19/97
11/26/97
12/3/97
12/10/97
12/17/97
12/24/97
12/31/97
1/7/98
1/14/98
1/21/98
1/28/98
2/4/98
2/11/98
2/18/98
2/25/98
3/4/98
3/11/98
3/18/98
3/25/98
Date


Summer 1998
Air (C)
Shallow (C)
Deep (C)
Temperature (C)
35
30
25
20
15
10
5
0
Apr-1-98
Apr-8-98
Apr-15-98
Apr-22-98
Apr-29-98
May-6-98
May-13-98
May-20-98
May-27-98
Jun-3-98
Jun-10-98
Jun-17-98
Jun-24-98
Jul-1-98
Jul-8-98
Jul-15-98
Jul-22-98
Jul-29-98
Aug-5-98
Aug-12-98
Aug-19-98
Aug-26-98
Sep-2-98
Sep-9-98
Sep-16-98
Sep-23-98
Date


Winter 1998-1999
Air Temp
Soil Temp (6 cm)
Soil temp (13 cm)
Temperature (C)
20
15
10
5
0
-5
-10
-15
-20
10/1/98
10/8/98
10/15/98
10/22/98
10/29/98
11/5/98
11/12/98
11/19/98
11/26/98
12/3/98
12/10/98
12/17/98
12/24/98
12/31/98
1/7/99
1/14/99
1/21/99
1/28/99
2/4/99
2/11/99
2/18/99
2/25/99
Date

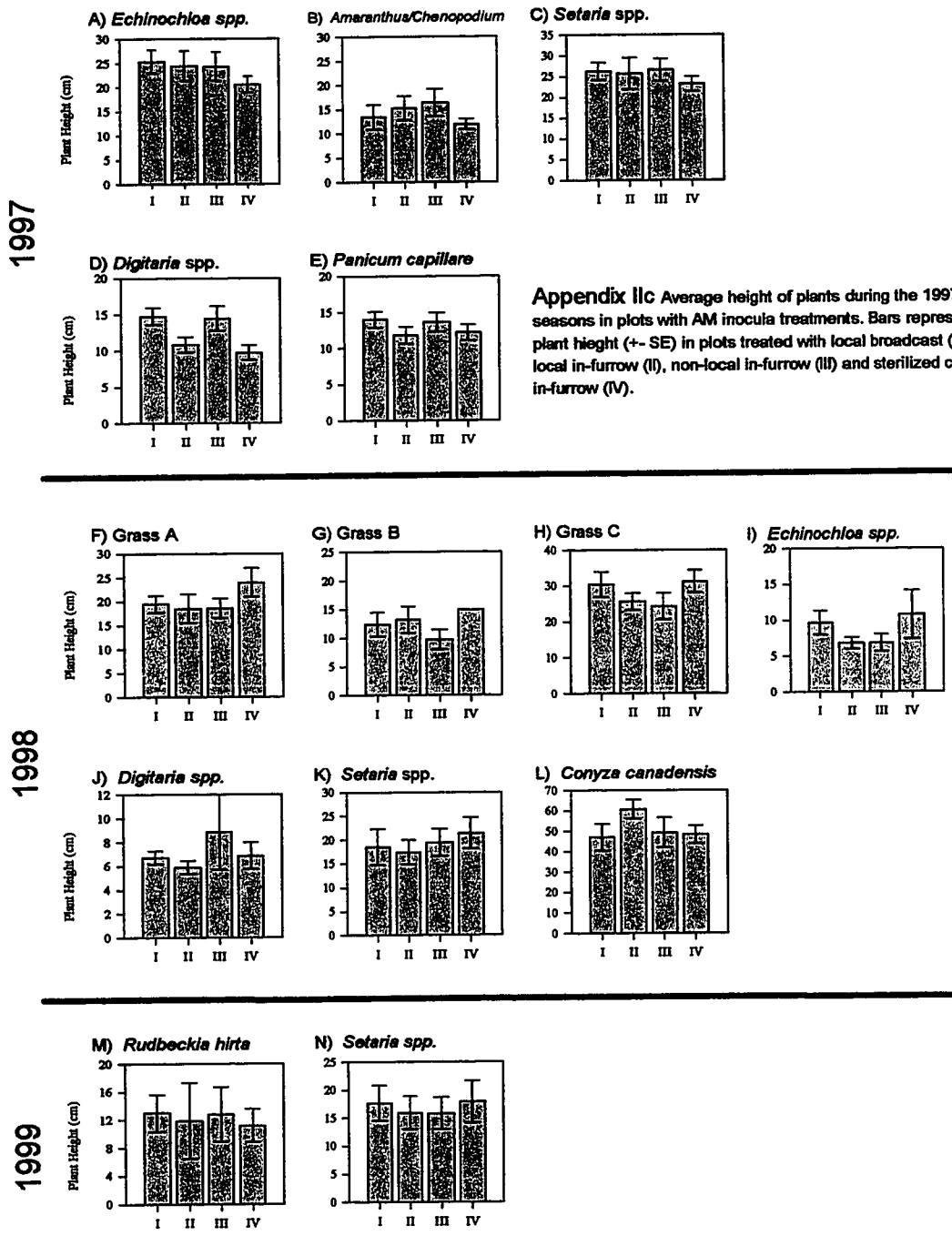
Appendix II: Additional Shakopee Information

Appendix IIa

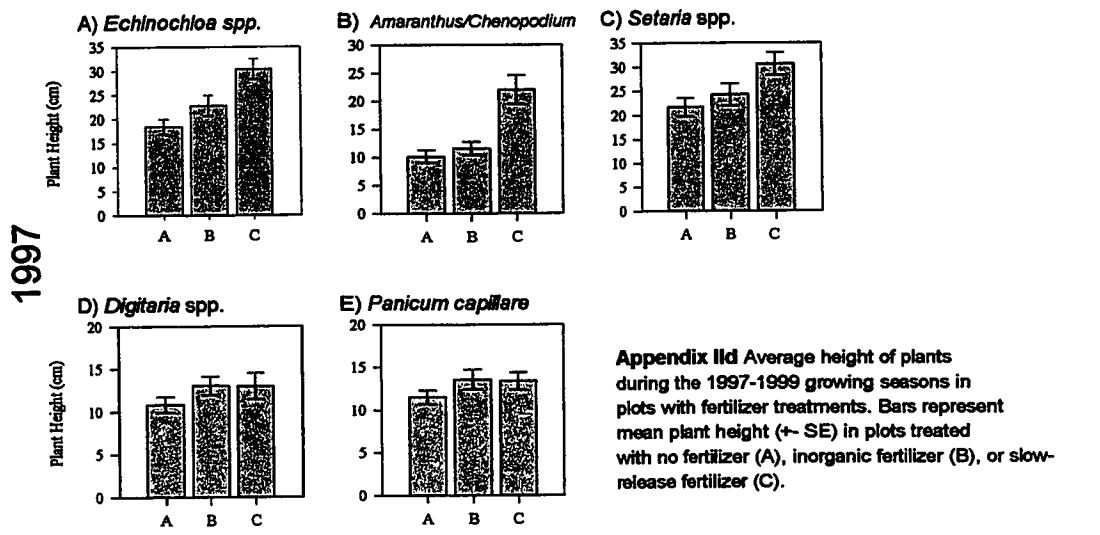


Appendix IIb Planted seed mixture at the Shakopee, MN restoration site

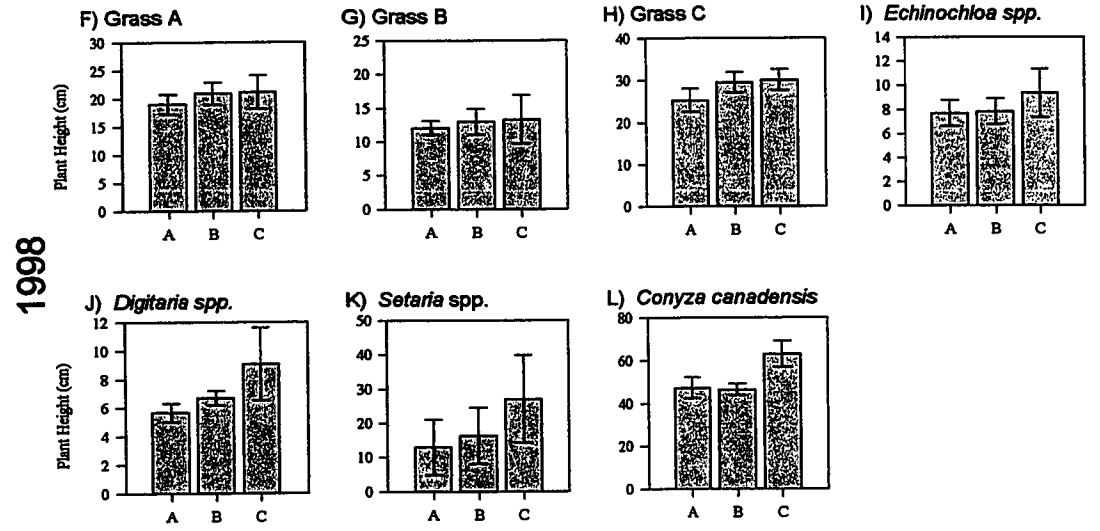
Common name	Scientific Name	Planting Rate kg/ha	Planting Rate seeds/m
Grasses			
Big bluestem	*Andropogon gerardii*	4.5	778
Canada wild-rye	*Elymus canadensis*	3.5	404
Indian grass	*Sorghastrum nutans*	3.5	933
Little bluestem	*Schizachyrium scoparium*	2.5	1167
Re-Green (hybrid cover crop)	*Triticum sp.x Agropyron sp.*	18	-
Sideoats grama	*Bouteloua curtipendula*	3.5	467
Slender wheat-grass	*Agropyron trachycaulum*	1	537
Switch grass	*Panicum virgatum*	1	1089
Forbs			
Black-eyed susan	*Rudbeckia hirta*	0.25	325
Blue vervain	*Verbena hastata*	0.25	353
Butterfly milkweed	*Asclepias tuberosa*	0.25	15
Canada milkvetch,	*Astragalus canadensis*	0.25	60
Common ox-eye	*Heliopsis helianthoides*	0.25	39
Grey-headed coneflower	*Ratibita pinnata*	0.25	95
Hoary vervain	*Verbena stricta*	0.25	21
New England aster	*Aster novae-angliae*	0.25	233
Ohio spiderwort	*Tradescantia ohiensis*	0.25	7
Partridge pea	*Chamaecrista fasciculata*	0.25	10
Purple prairie clover	*Dalea purpureum*	0.25	22
Roundheaded bushclover	*Lespedeza capitata*	0.25	282
Showy goldenrod	*Solidago speciosa*	0.25	85
Showy penstemon	*Penstemon grandiflorus*	0.25	63
Showy tick-trefoil	*Desmodium canadense*	0.25	39
Smooth-blue aster	*Aster lavies*	0.25	35
Stiff goldenrod	*Solidago rigida*	0.25	29
Tall blazingstar	*Liatris pycnostachya*	0.25	49
White prairie clover	*Dalea candidum*	0.25	4
Wild bergamot	*Monarda fistulosa*	0.25	67

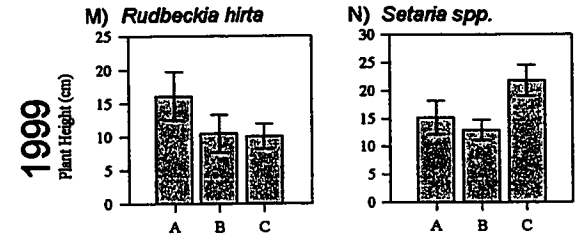


Appendix IIc Average height of plants during the 1997-1999 seasons in plots with AM inocula treatments. Bars represent mean plant hieght (+- SE) in plots treated with local broadcast (I), local in-furrow (II), non-local in-furrow (III) and sterilized control in-furrow (IV).



Appendix IId Average height of plants during the 1997-1999 growing seasons in plots with fertilizer treatments. Bars represent mean plant height (+- SE) in plots treated with no fertilizer (A), inorganic fertilizer (B), or slow-release fertilizer (C).





Appendix III: Plant Data Computer Program

Appendix III: Plant Count 1.0

Plant count 1.0 is a computer software application designed to summarize field data matrixes and perform summary computations on the data. It was developed to assist in compiling plant count data from spreadsheet programs such as Excel™. Once summarized, the data can be imported to many statistical analysis programs. Plant count 1.0 also calculates descriptive data for comparing study plots, including diversity indices, richness, and similarity.

The program was designed with expandability in mind, with both the size of data matrixes and functions upgradeable with needed. Currently, the data matrix file can accommodate 80 test plots with 80 different species or subspecies. The file size limit can conceivably be increased over 10 fold, but has not been tested at this level. Additional subroutines can be incorporated into the program by adding control panels and programming new routines. Programmed with the Visual Basic™ programming package, the Plant Count 1.0 code and forms can be used as a Visual Basic™ Project or compiled as a stand-alone application.

With a point and click interface, Plant Count 1.0 is relatively straightforward. The control panel has buttons for all functions of the program. Two display windows give the user output and program status. Data is saved in output files if the user requests that output be recorded. At present, the program does not have a well-designed user interface, so the novice user may find the program difficult to use. Help menus have not yet been implemented and documentation is not completed, but the basic program is useable with a little trial and error.

The format required for input data files is very specific and must be closely followed (see Figure A). Each Row in a data file represents one plot or transect unit. Each column represents the number of a particular species found in the different plots. At the end of each row (plot) of data is the number 999, which tells the program to begin reading the next row. Following the last row of data is the sequence 998, which indicates that all the data is read. The row following the last data row contains names of each species and ends in the number 9999, which indicates that the program does not need to read any further information. The file must be saved as a comma separated variables file (*.csv) to be read into the computer's memory.

Once running, function buttons on the toolbar control the program. The Ordination function is not implemented at this time and is therefore gray. The bootstrap value can be entered in the text box on the toolbar; it is used only in the saturation function. A small section on the toolbar indicates whether the save prompt is on (green) or off (red). The functions are described below:

Display Input- lists the input data file in the main output window

Save prompt- allows user to turn save prompt on and off . When on, you will be asked to save each analysis that is completed.

Species Count- counts the total number of individuals of a distinct species

Plot Count- counts the number of individuals in a plot

Richness- counts the number of species in each plot

Occurrence- counts the number of plot a species in present in

Diversity- calculates the diversity using the Shannon index

Saturation-calculates the total average number of species encountered if x number of plots was analyzed. (e.g. if you look at 1 plot you find 8 distinct species total, 2 plots 12 species, 3 plots 13)

Similarity- how similar the plots are based on species composition

Ordination- Ordination features are not yet implemented

Print-sends output window to printer

End- ends program immediately

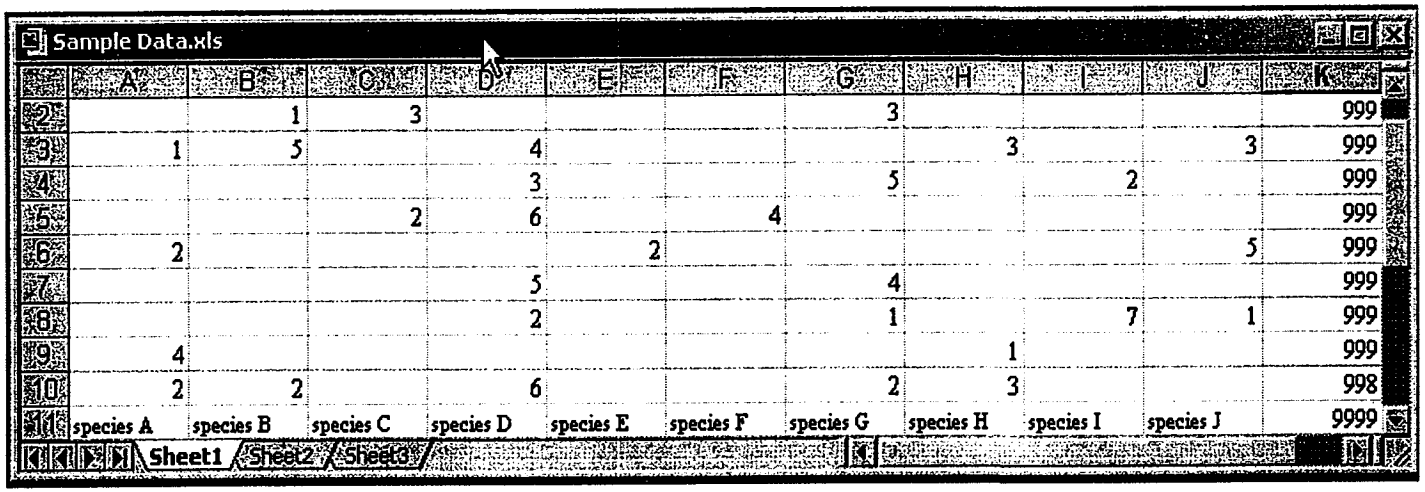


Figure 1. Sample data setup in a Microsoft Excel™ spreadsheet. Important note: File must be saved as a comma separated variable file to be properly read by Plant Count 1.0.

The Following is a listing of the program code, which can be used with visual basic to recreate the program:

```
Attribute VB_Name = "Module1"
'DECLARATIONS FOR THE PROGRAM
'
'
'Variable and type                    What it does
'-------------------------------------------------------------------------
Public numsp(80) As Variant           'number of species in array
Public species(80, 80) As Variant     'main data array  (plot, species)
Public Variable As Variant            '
Public Numb As Variant                '
Public Number$                        '
Public infile$                        'name of the input data file
Public saveprompt As Integer          'if val=(0) promt to save file
Public LineOfData$(80)                'string for loading tab-sep. test

Public xstring As Variant             'column marker
Public ystring As Variant             'row marker

Public Statu$                         'display in status box
Public Strng$                         'display in output win
Public display$                       'display in output win 'form2.text1'
Public display2$                      'display in bug win 'form3.text1'

Public speciesnum As Integer          'number of species (columns)
Public numberplots As Integer         'number of plots (rows)
Public speciesname(80) As String      'name of the species

Public ShortString As String * 6      'truncates variables at 6 characters
Public ShorterString As String * 5    'truncates variables at 5 characters
Public entrya$                        'line of text can be displayed

Public speciestotal(80) As Integer    ' total number plants of species x
Public plottotal(80) As Integer       ' total number plants in plot x
Public diversity(80) As Single        ' stores diversity for plot(x)
Public satvalues(80, 3)               ' saturation values
                                      ' 1 = average 2 = hi 3 = low

Public pointer(80, 3)                 'part of randomization and sorting
                                      'stores old and new values, plus 1 data

Public speciespresent(80)             ' keeps track of of 0 values in species
entries

Public sataver                        ' average saturation value for all
bootlegs
Public hisat                          ' tracks the Highest sat value
Public lowsat                         ' tracks the lowest sat value
Public satval As Integer              ' number of species in a given plot

Public howmanyplots(80) As Integer    'number of plots a species occurs in
Public speciesinplot(80)  As Integer  'number of species in a given plot
Public testvariable(80) As Integer    '???

Public sameness(80, 80) As Integer    'stores simillarity scores
Public Header As String * 5           ' simillarity display puts up plot number
```

```
Public Entry As String * 5              ' simmillarity display put value in
matrix
Public sorenson(80, 80) As Single       'stores sorenson data array


VERSION 4.00
Begin VB.Form Form1
   BackColor       =   &H00C0FFFF&
   BorderStyle     =   4  'Fixed ToolWindow
   Caption         =   "Toolbar"
   ClientHeight    =   705
   ClientLeft      =   45
   ClientTop       =   1740
   ClientWidth     =   9795
   ForeColor       =   &H00C0C000&
   Height          =   1395
   Left            =   -15
   LinkTopic       =   "Form1"
   MaxButton       =   0   'False
   MinButton       =   0   'False
   ScaleHeight     =   705
   ScaleWidth      =   9795
   ShowInTaskbar   =   0   'False
   Top             =   1110
   Width           =   9915
   Begin VB.CommandButton Quit
      BackColor       =   &H000080FF&
      Caption         =   "End Program"
      Height          =   375
      Left            =   6000
      TabIndex        =   4
      Top             =   360
      Width           =   1215
   End
   Begin VB.CommandButton Similarity
      Caption         =   "Similarity"
      Height          =   375
      Left            =   6000
      TabIndex        =   7
      Top             =   0
      Width           =   1215
   End
   Begin VB.CommandButton Saturation
      Caption         =   "Saturation"
      Height          =   375
      Left            =   4800
      TabIndex        =   8
      Top             =   360
      Width           =   1215
   End
   Begin VB.CommandButton DiversityH
      Caption         =   "Diversity H'"
      Height          =   375
      Left            =   4800
      TabIndex        =   9
      Top             =   0
      Width           =   1215
   End
   Begin VB.CommandButton Occurance
      Caption         =   "Occurance"
      Height          =   375
      Left            =   3600
```

```
   TabIndex        =   12
   Top             =   360
   Width           =   1215
End
Begin VB.CommandButton Richness
   Caption         =   "Richness"
   Height          =   375
   Left            =   3600
   TabIndex        =   11
   Top             =   0
   Width           =   1215
End
Begin VB.CommandButton PlotCount
   Caption         =   "Plot Count"
   Height          =   375
   Left            =   2400
   TabIndex        =   6
   Top             =   360
   Width           =   1215
End
Begin VB.CommandButton PrintForm2
   Caption         =   "Print"
   Height          =   375
   Left            =   1200
   TabIndex        =   5
   Top             =   360
   Width           =   1215
End
Begin VB.CommandButton speciescount
   Appearance      =   0  'Flat
   BackColor       =   &H80000005&
   Caption         =   "Species Count"
   Height          =   375
   Left            =   2400
   TabIndex        =   3
   Top             =   0
   Width           =   1215
End
Begin VB.CommandButton savealways
   Appearance      =   0  'Flat
   BackColor       =   &H80000005&
   Caption         =   "Save Prompt"
   Height          =   375
   Left            =   1200
   TabIndex        =   2
   Top             =   0
   Width           =   1215
End
Begin VB.TextBox Text2
   Height          =   285
   Left            =   7560
   TabIndex        =   10
   Text            =   "5"
   Top             =   360
   Width           =   375
End
Begin VB.CommandButton displayinput
   Caption         =   "Display Input"
   Height          =   375
   Left            =   0
   TabIndex        =   1
   Top             =   360
```

```
   Width           =   1215
End
Begin VB.CommandButton Load
   Appearance      =   0  'Flat
   BackColor       =   &H80000005&
   Caption         =   "Load File"
   Height          =   375
   Left            =   0
   TabIndex        =   0
   Top             =   0
   Width           =   1215
End
Begin VB.Label Label3
   Alignment       =   2  'Center
   BackStyle       =   0  'Transparent
   Caption         =   "Bootleg Value"
   Height          =   255
   Left            =   7200
   TabIndex        =   15
   Top             =   120
   Width           =   1095
End
Begin VB.Label Label1
   BackColor       =   &H00FF80FF&
   ForeColor       =   &H00000000&
   Height          =   735
   Left            =   7200
   TabIndex        =   14
   Top             =   0
   Width           =   1215
End
Begin MSComDlg.CommonDialog CommonDialog2
   Left            =   7680
   Top             =   -120
   _ExtentX        =   847
   _ExtentY        =   847
   _Version        =   327681
End
Begin VB.Label Label2
   Alignment       =   2  'Center
   BackColor       =   &H000000FF&
   Caption         =   "Save OFF"
   BeginProperty Font {0BE35203-8F91-11CE-9DE3-00AA004BB851}
      Name            =   "MS Sans Serif"
      Size            =   9.75
      Charset         =   0
      Weight          =   700
      Underline       =   0   'False
      Italic          =   0   'False
      Strikethrough   =   0   'False
   EndProperty
   Height          =   375
   Left            =   8400
   TabIndex        =   13
   Top             =   0
   Width           =   1455
End
Begin VB.Menu File_Options
   Caption         =   "File"
   Begin VB.Menu load_file
      Caption         =   "Load"
      Shortcut        =   ^L
```

```
      End
      Begin VB.Menu save_window
         Caption         =   "Save Window"
         Shortcut        =   ^S
      End
      Begin VB.Menu print_file
         Caption         =   "Print"
         Shortcut        =   ^P
      End
      Begin VB.Menu exit_prog
         Caption         =   "Exit"
         Shortcut        =   ^X
      End
   End
   Begin VB.Menu actions
      Caption         =   "Actions"
      Begin VB.Menu rich
         Caption         =   "Richness"
      End
      Begin VB.Menu species_count
         Caption         =   "Species"
      End
   End
   Begin VB.Menu show_wins
      Caption         =   "Windows"
      Begin VB.Menu main_win
         Caption         =   "Main output"
         Shortcut        =   ^M
      End
      Begin VB.Menu user_win
         Caption         =   "User Information"
         Shortcut        =   ^I
      End
   End
   Begin VB.Menu help_menu
      Caption         =   "Help"
      Begin VB.Menu help_file
         Caption         =   "Plant Count"
         Shortcut        =   ^H
      End
      Begin VB.Menu prog_info
         Caption         =   "About Plant Count"
      End
   End
End
Attribute VB_Name = "Form1"
Attribute VB_Creatable = False
Attribute VB_Exposed = False

Private Sub Command1_Click()

End Sub

Private Sub CommonDialog2_Click()
End Sub

Private Sub bootlabel_Click()

End Sub

Public Sub loadprob_Click()
```

```
Show debugA

debugA.variablea = "errormessage"
debugA.valuea = errormessage
debugA.variablee = "infile$"
debugA.valueE = infile$
debugA.message = Text$
debugA.valuec = hasskiploaded
debugA.variablec = "hasskiploaded"

CommonDialog2.filename = ""

disor:
    Text$ = "An error has occured during the loading" & wrap$
    Text$ = Text$ & " of a file.  The cancel button may have been pressed." &
wrap$
    Text$ = Text$ & " please try loading the file again." & wrap$
    Text$ = Text$ & wrap$
    debugA.message = Text$
    errormessage = 0

End Sub

Private Sub displayinput_Click()
Rem ***********************************************
Rem This proceedure properly displays the array   *
Rem in a textbox using linewraps and spaces       *
Rem ***********************************************
Cls
wrap$ = Chr$(13) + Chr$(10)

Rem ************************************************************
Rem set display output of colomn labels to textbox 1 on form 2 *
Rem************************************************************
entrya$ = entrya$ + "plot "
Form2.Text1 = entrya$

Rem ***********************************************
Rem adds each truncated name to line of text      *
Rem ***********************************************
For x = 1 To speciesnum
    ShorterString = speciesname(x)
    ShortString = ShorterString
    entrya$ = entrya$ & ShortString
Next x
entrya$ = entrya$ & wrap$

Rem ***********************************************
Rem  Display main data                            *
Rem ***********************************************
For x = 1 To numberplots
    ShortString = Str(x)
    entrya$ = entrya$ & ShortString
    For y = 1 To speciesnum
        ShortString = species(x, y)
        entrya$ = entrya$ & ShortString
        If y = speciesnum Then entrya$ = entrya$ & wrap$
    Next y
Next x
Form2.Text1 = entrya$
```

```
End Sub

Private Sub DiversityH_Click()
Rem*************************************************
Rem This function and its sub-functions calculate *
Rem the diverstiy of plots. Steps are as follows  *
Rem                                               *
Rem   Count the total species in a plot           *
Rem   Start with first species                    *
Rem     If the species is present perform calc    *
Rem     If not go to next species                 *
Rem   Loop through to other plots                 *
Rem   Save to disk                                *
Rem                                               *
Rem*************************************************
wrap$ = Chr$(13) + Chr$(10)
x = 0: y = 0: z = 0
display$ = "  Calculating plot diversity " & wrap$ & wrap$
display$ = display$ & "Data Source File: " & infile$ & wrap$ & wrap$
Form2.Text1 = display$


Rem ***********************************************
Rem 1) count number of individual plants          *
Rem 2) then go back and for each species occuring *
Rem    calculate pi (proportion of that plant     *
Rem    in plot)                                   *
Rem 3) keep running total of pi and pi*log (pi)   *
Rem 4) if species number= 0 then skip species     *
Rem    if species number= 999, -sum pi*log pi     *
Rem***********************************************
For x = 1 To numberplots
    For y = 1 To speciesnum
      plottotal(x) = plottotal(x) + species(x, y)
    Next y
    Form2.Text1 = Form2.Text1 + "."
    For z = 1 To speciesnum
      If species(x, z) > 0 Then pi = (species(x, z) / plottotal(x))
      If species(x, z) < 0 Then skip = 1
      If species(x, z) > 999 Then skip = 1
      If species(x, z) = 0 Then skip = 1
      Do While skip = 0 And species(x, z) > 0
        diversity(x) = diversity(x) - (pi * Log(pi))
        skip = 1
      Loop
      skip = 0
Next z
Next x

Rem ***********************************************
Rem Display the diversity                         *
Rem ***********************************************
For Counter = 1 To numberplots
    Form2.Text1 = Form2.Text1 & wrap$ & "Plot " & Counter & " Diversity = " &
diversity(Counter)
Next Counter

Rem ***********************************************
Rem Save file if user wants it saved              *
Rem ***********************************************
shouldsave = 1
If saveprompt = 0 Then shouldsave = 0
```

```
If saveprompt = 1 Then shouldsave = 1
Do While shouldsave = 0
    CommonDialog2.filename = "diversity.csv"
    CommonDialog2.Filter = "output (*.csv)|*.csv"
    CommonDialog2.ShowSave
    Open CommonDialog2.filename For Output As #2
    Print #2, "Input File: ", infile$
    Print #2, "Plot Diversity Measure: Shannon Index"
    For Counter = 1 To numberplots
        Print #2, "Plot ", Counter, " Diversity = ", diversity(Counter)
    Next Counter
    Close #2
    shouldsave = 1
Loop

End Sub

Private Sub exit_prog_Click()
 Call Quit_Click
End Sub

Private Sub Form_Load()
Form2.Show
Form3.Show
saveprompt = 1

'**************************************
'*  disable these buttons until a file *
'*  is loaded                          *
'**************************************
Occurance.Enabled = False
Similarity.Enabled = False
Saturation.Enabled = False
PlotCount.Enabled = False
speciescount.Enabled = False
DiversityH.Enabled = False
Richness.Enabled = False
displayinput.Enabled = False

'**************************************
'*  Disable functions not yet         *
'*  Not yet implimented               *
'**************************************

End Sub

Private Sub Load_Click()
Rem ***********************************************
Rem  This sub-routine loads data from a text file *
Rem  The maximum data set is 80 species by 80     *
Rem  plots.  This can be changed by increasing    *
Rem  the array size in the declaration and        *
Rem  increasing the top numbers in the x and y    *
Rem  loops                                        *
Rem                                               *
Rem ***********************************************
wrap$ = Chr$(13) + Chr$(10)
errormessage = 0
loadfile = 0
infile$ = ""
Form2.Text1 = ""
Form3.Text1 = ""
```

```
Text$ = ""
Statu$ = ""
CommonDialog2.filename = ""
Close #1
On Error GoTo skipload

Rem ***********************************************
Rem Reset data array                                *
Rem ***********************************************

    For x = 1 To 80
         For y = 1 To 80
            species(x, y) = 0
        Next y
    Next x

    For x = 1 To 80
        speciesname(x) = 0
    Next x

    x = 0
    y = 0

Rem ***********************************************
Rem Get filename and location from user             *
Rem ***********************************************
CommonDialog2.Filter = "comma delimited data- bas (*.csv)|*.csv"
CommonDialog2.ShowOpen
infile$ = CommonDialog2.filename
Form3.Text1 = infile$

Rem ***********************************************
Rem This is the loading section.                    *
Rem ***********************************************
Open CommonDialog2.filename For Input As #1
Do While loadfile = 0
    Form2.Text1 = "Loading"
    For x = 1 To 80
        Form2.Text1 = Form2.Text1 & wrap$
         For y = 1 To 80
            Let numsp(x) = y
            Form2.Text1 = Form2.Text1 & "."
            Input #1, indata
            Variable = Val(indata)
            If indata = "" Then Variable = 0
            species(x, y) = Variable

            If species(x, y) = 999 Then numsp(x) = y - 1: y = 80
            If species(x, y) = 998 Then numsp(i) = y - 1: numbplot = x: x = 80:
y = 80


        Next y

    Next x
    speciesnum = numsp(1)
    numberplots = numbplot
If species(numberplots, (speciesnum + 1)) <> 998 Then errormessage = 1

Rem ***********************************************
Rem This section loads the species names            *
Rem ***********************************************
```

```
    For x = 1 To speciesnum
        Input #1, spnam$
        speciesname(x) = spnam$
        specieslist$ = specieslist$ & speciesname(x) & wrap$
    Next x


Rem ***********************************************
Rem Displays load status and activates buttons    *
Rem ***********************************************

    Statu$ = Statu$ & "Data File loaded" & wrap$
    Statu$ = Statu$ & "Plots loaded: " & numberplots & wrap$
    Statu$ = Statu$ & "Species loaded: " & numsp(1) & wrap$
    Text$ = Statu$ & wrap$ & Text$
    Text$ = infile$ & wrap$ & Text$
    Form3.Text1 = Text$
    Form3.Text1 = Form3.Text1 & specieslist$
    Occurance.Enabled = True
    Similarity.Enabled = True
    Saturation.Enabled = True
    Ordination.Enabled = False
    PlotCount.Enabled = True
    speciescount.Enabled = True
    DiversityH.Enabled = True
    Richness.Enabled = True
    displayinput.Enabled = True
    loadfile = 1
Loop

skipload:
    loadfile = 1
    hasskiploaded = 1
Resume Next

Rem ***********************************************
Rem If there is an error this func is activated   *
Rem the error message is displayed, loading halts *
Rem ***********************************************

If errormessage = 1 Then Call loaderror

End Sub

Private Sub Load_file_Click()
    Call Load_Click
End Sub

Private Sub Occurance_Click()
wrap$ = Chr$(13) + Chr$(10)

Rem***********************************************
Rem* This function counts number of plots        *
Rem* containing a given species                  *
Rem***********************************************




Rem ***********************************************
Rem zeros arrays, displays and variables          *
Rem ***********************************************
```

```
 Form2.Text1 = ""
 display$ = ""


 If infile$ < "" Then loadfile = 1
Do While errormessage = 1
    Text$ = "An error has occured during the loading" & wrap$
    Text$ = Text$ + " of a file.  The cancel button may have been pressed." &
wrap$
    Text$ = Text$ + " please try loading the file again." & wrap$
    Text$ = Text$ + "error message" & errormessage & wrap$
    Text$ = Text$ + wrap$
    Form2.Text1 = Text$
Loop



display$ = " Occurance of Each Species " & wrap$ & wrap$
display$ = display$ & "Data Source File: " & infile$ & wrap$ & wrap$


Rem ********Zero Counting array ********
  For x = 1 To speciesnum
    howmanyplots(x) = 0
  Next x

Rem *********Count plots (y) containing species (x)
For x = 1 To speciesnum
    For y = 1 To numberplots
        If species(y, x) > 0 And species(y, x) < 998 Then present = 1
        howmanyplots(x) = howmanyplots(x) + present
        present = 0
    Next y
    lineout$ = speciesname$(x) & " is present in " & howmanyplots(x) & " plots"
& wrap$
    display$ = display$ + lineout$
    savefile$ = savefile$ & speciesname$(x) & ", is present in, " &
howmanyplots(x) & ", plots" & wrap$
Next x

Form2.Text1 = display$

Rem ***********************************************
Rem Save file if user wants it saved               *
Rem ***********************************************
If saveprompt = 0 Then shouldsave = 0
If saveprompt = 1 Then shouldsave = 1
Do While shouldsave = 0
    CommonDialog2.filename = "simil.csv"
    CommonDialog2.Filter = "output (*.csv)|*.csv"
    CommonDialog2.ShowSave
    Open CommonDialog2.filename For Output As #2
    Print #2, "Input File: ", infile$
    Print #2, savefile$
    Close #2
    shouldsave = 1
Loop
End Sub

Private Sub Ordination_Click()
Form4.Visible = True
```

```
End Sub

Private Sub plotcount_Click()
Rem*************************************************
Rem This function lists the number of plants         *
Rem in each plot                                     *
Rem                                                  *
Rem*************************************************
wrap$ = Chr$(13) + Chr$(10)

Rem *****zeroes counting array*****
For y = 1 To numberplots
    plottotal(y) = 0
Next y

For y = 1 To numberplots
    For x = 1 To speciesnum
      plottotal(y) = plottotal(y) + species(y, x)
      totalplants = totalplants + species(y, x)
    Next x
    lineout$ = "plot" & y & " has " & plottotal(y) & " individuals" & wrap$
    display$ = display$ + lineout$
  Next y

lineout$ = "total number of plants= " & totalplants & wrap$
display$ = display$ & lineout$
Form2.Text1 = display$

Rem ***********************************************
Rem Save file if user wants it saved              *
Rem ***********************************************
shouldsave = 1
If saveprompt = 0 Then shouldsave = 0
Do While shouldsave = 0
    CommonDialog2.filename = "diversity.csv"
    CommonDialog2.Filter = "output (*.csv)|*.csv"
    CommonDialog2.ShowSave
    Open CommonDialog2.filename For Output As #2
    Print #2, "Input File: ", infile$
    Print #2, display$
    Close #2
    shouldsave = 1
Loop

End Sub

Private Sub Print_Click()

End Sub

Private Sub print_file_Click()
    Call PrintForm2_Click

End Sub

Private Sub PrintForm2_Click()
Printer.Print Form2.Text1
Printer.EndDoc
End Sub

Private Sub Quit_Click()
```

```
    End
End Sub

Private Sub Richness_Click()
Rem*************************************************
Rem This function lists the number of plants         *
Rem in each plot                                     *
Rem                                                  *
Rem*************************************************
wrap$ = Chr$(13) + Chr$(10)

display$ = "Species richness of plots" & wrap$ & wrap$
display$ = display$ & infile$ & wrap$ & wrap$

savefile$ = "Species richness of plots" & wrap$ & wrap$
savefile$ = savefile$ & infile$ & wrap$ & wrap$



Rem *****zeroes counting array*****
For y = 1 To numberplots
    speciesinplot(y) = 0
Next y
present = 0

Rem ******* Counting sub-routing ***********
For y = 1 To numberplots
    For x = 1 To speciesnum
      If species(y, x) > 0 And species(y, x) < 998 Then present = 1
      speciesinplot(y) = speciesinplot(y) + present
      present = 0
      totalplants = totalplants + species(y, x)
    Next x
    lineout$ = "Plot " & y & " has " & speciesinplot(y) & " species" & wrap$
    display$ = display$ + lineout$
    savefile$ = savefile$ & "Plot," & y & ",has, " & speciesinplot(y) &
",species" & wrap$
  Next y
Form2.Text1 = display$

Rem ***********************************************
Rem Save file if user wants it saved              *
Rem ***********************************************
If saveprompt = 0 Then shouldsave = 0
If saveprompt = 1 Then shouldsave = 1
Do While shouldsave = 0

    CommonDialog2.filename = "richness.csv"
    CommonDialog2.Filter = "output (*.csv)|*.csv"
    CommonDialog2.ShowSave
    Open CommonDialog2.filename For Output As #2
    Print #2, savefile$
    Close #2
    shouldsave = 1

Loop

End Sub

Private Sub saturation_Click()
wrap$ = Chr$(13) + Chr$(10)
```

```
Rem***********************************************
Rem This function and its sub-functions calculate *
Rem the diverstiy of plots. Steps are as follows  *
Rem                                               *
Rem   Count the total species in a plot           *
Rem   Start with first species                    *
Rem     If the species is present perform calc    *
Rem     If not go to next species                 *
Rem   Loop through to other plots                 *
Rem   Save to disk                                *
Rem                                               *
Rem***********************************************

Randomize (Timer)
display$ = ""
Form2.Text1 = ""
Cls

Rem ***********************************************
Rem   Describes the function in the text box      *
Rem ***********************************************

outline$ = "this routine provides graph data for the"
display$ = display$ + outline$ + wrap$
outline$ = "species saturation at a given site.  It uses"
display$ = display$ + outline$ + wrap$
outline$ = "the number of species observed in a set of "
display$ = display$ + outline$ + wrap$
outline$ = "same sized sampling plots.  Please enter the "
display$ = display$ + outline$ + wrap$
outline$ = "number of times you would like your data "
display$ = display$ + outline$ + wrap$
outline$ = "sampled for each # of plots in the bootleg"
display$ = display$ + outline$ + wrap$
outline$ = "box on the toolbar window"
display$ = display$ + outline$ + wrap$
Form2.Text1 = display$

    bs$ = Form1.Text2
    bootstrap = Val(bs$)
    display$ = display$ & "Satuation curve data for plots" & wrap$ & wrap$
    display$ = display$ & "Data Source File: " & wrap$ & wrap$
    display$ = display$ & "Bootstraping: " & Str(bootstrap) & wrap$ & wrap$

    For plotstoscan = 1 To numberplots
        hisat = 0: lowsat = 50: totalsat = 0
        For sample = 1 To bootstrap      ' number of times to sample data

            For lup = 1 To 60           'randomizes plots
                pointer(lup, 2) = Rnd   ' assigns random values in column 2
                pointer(lup, 3) = lup   ' assigns unchangeable plot # in column
3
            Next lup

            a = 1
            sort = 1
                              'reorders plots
            Do While sort = 1       'sorts according to rand on pointer()
                ShouldShuffle = 0          ' if sort = 1 then need to continue
sorting
                For a = 1 To 60
                If pointer(a + 1, 2) > pointer(a, 2) Then shuffle = 1
```

```
                    Do While shuffle = 1
                        tempA2 = pointer(a, 2)       ' if pointer (a,2) is
greater
                        tempA3 = pointer(a, 3)       ' than pointer (a+1<2) then
                        tempB2 = pointer(a + 1, 2)   ' put the values in temp
values
                        tempB3 = pointer(a + 1, 3)   ' and swap a for a+1
                        pointer(a, 2) = tempB2
                        pointer(a, 3) = tempB3
                        pointer(a + 1, 2) = tempA2
                        pointer(a + 1, 3) = tempA3
                        sort = 1                     ' tells plot rand that
sorting
                        ShouldShuffle = 1                 ' has not yet
finished
                        shuffle = 0
                    Loop
                If a = 59 And ShouldShuffle = 0 Then sort = 0
                Next a
            Loop


            satval = 0
            For M = 1 To speciesnum      'calculates the saturation val for
                x = 0                    'a given plot
                speciestotal(M) = 0
                For x = 1 To plotstoscan
                    y = species(pointer(x, 3), M)
                    speciestotal(M) = y + speciestotal(M)
                    If speciestotal(M) > 0.9 Then speciespresent(M) = 1
                    If speciestotal(M) < 1 Then speciespresent(M) = 0
                Next x
                If speciespresent(M) = 1 Then satval = satval + 1
            Next M


            totalsat = totalsat + satval
            If hisat < satval Then hisat = satval
            If lowsat > satval Then lowsat = satval
            'display$ = display$ & wrap$ & "plot:" & plotstoscan & " sample:" &
sample & " satval:" & satval & " hival:" & hisat & " lowsat:" & lowsat & "
totalsat: " & totalsat
        Next sample
        sataver = totalsat / bootstrap

        Print " "
        display$ = display$ & wrap$ & "Saturation for " & plotstoscan & " plots
is :" & sataver
        'display$ = display$ & wrap$ & "hisat for " & plotstoscan & " plots is
:" & hisat
        'display$ = display$ & wrap$ & "lowsat for " & plotstoscan & " plots is
:" & lowsat & wrap$
        'display$ = display$ + " " + wrap$

          satvalues(lup2, 1) = sataver
        satvalues(lup2, 2) = hisat
        satvalues(lup2, 3) = lowsat

        sataver = 0
        hisat = 0
```

```
        lowsat = 0
    Next plotstoscan

Form2.Text1 = display$

Rem ***********************************************
Rem Save file if user wants it saved              *
Rem ***********************************************
shouldsave = 1
If saveprompt = 0 Then shouldsave = 0
Do While shouldsave = 0
    CommonDialog2.filename = "diversity.csv"
    CommonDialog2.Filter = "output (*.csv)|*.csv"
    CommonDialog2.ShowSave
    Open CommonDialog2.filename For Output As #2
    Print #2, "Input File: ", infile$
    Print #2, display$
    Close #2
    shouldsave = 1
Loop

End Sub

Private Sub save_window_Click()
    temp$ = Form2.Text1
    CommonDialog2.filename = "output.txt"
    CommonDialog2.Filter = "output (*.txt)|*.txt"
    CommonDialog2.ShowSave
    Open CommonDialog2.filename For Output As #2
    Print #2, "Input File: ", infile$
    Print #2, temp$
    Close #2
End Sub

Private Sub savealways_Click()
toggle = saveprompt

If toggle = 1 Then
Label2.Caption = "Save ON"
Label2.BackColor = &HC000&
saveprompt = 0
End If

If toggle = 0 Then
Label2.Caption = "Save OFF"
Label2.BackColor = &HFF&
saveprompt = 1
End If

End Sub

Private Sub SaveForm2_Click()
Close #4
wrap$ = Chr$(10) + Chr$(13)
On Error GoTo skipsave

CommonDialog2.Filter = "JT's Stat Prog (*.jsp)|*.jsp"
CommonDialog2.ShowSave

Open CommonDialog2.filename For Output As #4
If saveerror <> 999 Then
    save$ = Form2.Text1
```

```
    Print #4, save$
    Close #4
End If

skipsave:
Form2.Text1 = Form2.Text1 & "File not saved- try again" & wrap$
saveerror = 999

End Sub

Private Sub Similarity_Click()

'this subroutine will calculate the Similarity of plots
'It will proceed in several steps
'step 1) assign and zero arrays
'step 2) looping structure assigns two plot numbers
'   step 3) similarity calculation
'   step 4) assign value to array
'step 5) return to looping structure
'step 6) display and save functionfunction

'*********************************************
'*    Set arrays and assign values           *
'*********************************************
wrap$ = Chr$(13) + Chr$(10)
plota = 0           ' First plot to compare
plotb = 0           ' Secondplot to compare

'*********************************************
'*    Looping Start                          *
'*********************************************
For x = 1 To numberplots
    For y = (x + 1) To numberplots
        plota = x
        plotb = y

'*********************************************
'*    Calculation function                   *
'*********************************************

For M = 1 To speciesnum

valuea = species(plota, M)
valueb = species(plotb, M)

If valuea > 0 And valueb > 0 Then present = 1
If valuea > 0 And valueb < 1 Then inplotone = 1
If valuea < 1 And valueb > 0 Then inplottwo = 1

a = a + present
b = b + inplotone
C = C + inplottwo

inplotone = 0
inplottwo = 0
present = 0
'display$ = display$ & valuea & "  " & valueb & "  " & a & Wrap$

'display$ = display$ & valuea & "  " & valueb & "  " & sore & Wrap$

Next M
sore = (2 * a) / ((2 * a) + b + C)
```

```
If temp$ <> "" Then Print #6, plota, plotb, a, b, C, sore

'display$ = display$ & plota & "   " & plotb & "   " & sore & wrap$
'Form2.Text1 = display$

'*************************************************
'*    set up array of results                    *
'*************************************************

sameness(plota, plotb) = a
sorenson(plota, plotb) = sore

a = 0: b = 0: C = 0

    Next y

Next x

'form2.text1 = display$
'*************************************************
'*    Display array and save to a file           *
'*************************************************

display$ = "Simillarity of :" & infile$ & wrap$ & wrap$ & "      "

For t = 1 To numberplots
    Header = Str(t)
    display$ = display$ & Header
Next t

display$ = display$ + wrap$ + "       "

For t = 1 To numberplots
    Header = "------"
    display$ = display$ & Header
Next t

display$ = display$ + wrap$

For t = 1 To numberplots
    Header = Str(t)
    display$ = display$ & Header & "|"
    For u = 1 To numberplots
        Value = sorenson(t, u)
        Entry = Str(Value)
        If t = u Then Entry = "-"
        If Value = 0 Then Entry = "      "
        display$ = display$ & Entry


    Next u
display$ = display$ + wrap$
Next t

Form2.Text1 = display$

Rem *************************************************
Rem Save file if user wants it saved                 *
Rem *************************************************
shouldsave = 1
skiploop = 0
temp$ = 0
```

```
If saveprompt = 0 Then shouldsave = 0
If saveprompt = 1 Then shouldsave = 1

Do While shouldsave = 0
    For t = 1 To numberplots
        v = t + 1
        For u = v To numberplots
            Value = sorenson(t, u)
            temp$ = temp$ & "plot " & "," & t & "," & "plot " & "," & u & ","
            temp$ = temp$ & " similarity: " & "," & Value & wrap$
        Next u
    Next t

    CommonDialog2.filename = "simil.csv"
    CommonDialog2.Filter = "output (*.csv)|*.csv"
    CommonDialog2.ShowSave
    Open CommonDialog2.filename For Output As #2
    Print #2, "Input File: ", infile$
    Print #2, temp$
    Close #2
    shouldsave = 1
Loop

End Sub

Private Sub speciescount_Click()
  Rem*************************************************
  Rem This function counts the number of plants        *
  Rem of each species                                  *
  Rem                                                  *
  Rem*************************************************
wrap$ = Chr$(13) + Chr$(10)
totalplants = 0
display$ = "Number of Individuals of Species found" & wrap$ & wrap$
display$ = display$ & "Data Source File: " & infile$ & wrap$ & wrap$
savefile$ = display$


Rem ********Zero Counting array *********
For x = 1 To speciesnum
    speciestotal(x) = 0
Next x
Form2.Text1 = Form2.Text1 + Str(speciesnum) + Str(numberplots)

For x = 1 To speciesnum
    For y = 1 To numberplots
      speciestotal(x) = speciestotal(x) + species(y, x)
      totalplants = totalplants + species(y, x)
    Next y
    lineout$ = speciesname$(x) & " has " & speciestotal(x) & " individuals" &
wrap$
    display$ = display$ + lineout$
    savefile$ = savefile$ & speciesname$(x) & ", has ," & speciestotal(x) & ",
individuals" & wrap$

Next x
  Print "total number of plants= "; totalplants

Form2.Text1 = display$

Rem ************************************************
Rem Save file if user wants it saved              *
```

```
Rem ***********************************************
If saveprompt = 0 Then shouldsave = 0
If saveprompt = 1 Then shouldsave = 1
Do While shouldsave = 0

    CommonDialog2.filename = "sppcount.csv"
    CommonDialog2.Filter = "output (*.csv)|*.csv"
    CommonDialog2.ShowSave
    Open CommonDialog2.filename For Output As #2
    Print #2, savefile$
    Close #2
    shouldsave = 1

Loop

End Sub

Public Function loaderror()
wrap$ = Chr$(13) + Chr$(10)
debugA.Hide

debugA.variablea = "errormessage"
debugA.valuea = errormessage
debugA.variablee = "infile$"
debugA.valueE = infile$
debugA.message = Text$
debugA.valuec = hasskiploaded
debugA.variablec = "hasskiploaded"

    Text$ = "An error has occured during the loading" & wrap$
    Text$ = Text$ & " of a file.  The cancel button may have been pressed." &
wrap$
    Text$ = Text$ & " please try loading the file again." & wrap$
    Text$ = Text$ & wrap$
    debugA.message = Text$

    Occurance.Enabled = False
    Similarity.Enabled = False
    Saturation.Enabled = False
    Ordination.Enabled = False
    PlotCount.Enabled = False
    speciescount.Enabled = False
    DiversityH.Enabled = False
    Richness.Enabled = False
    displayinput.Enabled = True

errormessage = 0

Form2.Text1 = "An error has occured during loading"
Form3.Text1 = ""

End Function


VERSION 4.00
Begin VB.Form Form2
   BackColor       =   &H0080C0FF&
   Caption         =   "Main Output Window"
   ClientHeight    =   7095
   ClientLeft      =   75
   ClientTop       =   1020
   ClientWidth     =   7950
```

```
   Height          =   7500
   Left            =   15
   LinkTopic       =   "Form2"
   ScaleHeight     =   7095
   ScaleWidth      =   7950
   Top             =   675
   Width           =   8070
   Begin VB.TextBox Text1
      BeginProperty Font {0BE35203-8F91-11CE-9DE3-00AA004BB851}
         Name            =   "Courier"
         Size            =   9.75
         Charset         =   0
         Weight          =   400
         Underline       =   0   'False
         Italic          =   0   'False
         Strikethrough   =   0   'False
      EndProperty
      Height          =   7095
      Left            =   0
      MultiLine       =   -1  'True
      ScrollBars      =   3  'Both
      TabIndex        =   0
      Top             =   0
      Width           =   7935
   End
End
Attribute VB_Name = "Form2"
Attribute VB_Creatable = False
Attribute VB_Exposed = False


VERSION 4.00
Begin VB.Form Form3
   Caption         =   "User Information Window"
   ClientHeight    =   7050
   ClientLeft      =   8145
   ClientTop       =   1035
   ClientWidth     =   3390
   Height          =   7455
   Left            =   8085
   LinkTopic       =   "Form3"
   ScaleHeight     =   7050
   ScaleWidth      =   3390
   Top             =   690
   Width           =   3510
   Begin VB.TextBox Text1
      Height          =   7095
      Left            =   0
      MultiLine       =   -1  'True
      ScrollBars      =   2  'Vertical
      TabIndex        =   0
      Top             =   0
      Width           =   3375
   End
End
Attribute VB_Name = "Form3"
Attribute VB_Creatable = False
Attribute VB_Exposed = False


VERSION 4.00
```

```
Begin VB.Form Form5
   Appearance      =   0  'Flat
   BackColor       =   &H80000005&
   Caption         =   "Welcome to Plant Count 1.0"
   ClientHeight    =   2580
   ClientLeft      =   4560
   ClientTop       =   2850
   ClientWidth     =   6510
   FillStyle       =   0  'Solid
   Height          =   2985
   Left            =   4500
   LinkTopic       =   "Form5"
   MaxButton       =   0   'False
   MinButton       =   0   'False
   NegotiateMenus  =   0   'False
   Picture         =   "Form5.frx":0000
   ScaleHeight     =   2580
   ScaleWidth      =   6510
   ShowInTaskbar   =   0   'False
   Top             =   2505
   Width           =   6630
End
Attribute VB_Name = "Form5"
Attribute VB_Creatable = False
Attribute VB_Exposed = False

Private Sub Form_Load()

Form5.Show
welcome$ = "welcome to Joel's Stat's program"

For x = 1 To 1000
    For y = 1 To 10000
    Next y
Next x
Form5.Hide
menubar.Show

Toolbar.Show
Form2.Show
Form3.Show

End Sub
```