Beneficial Fungal Inoculum for Prairie and Wetland Reclamation

LCMR Final Workprogram Abstract ML 95, Chp. 220, Sec. 19, Subd. 13(c)

The restoration of native wetland and prairie communities has received widespread attention in recent years, and local state agencies have expressed considerable interest in the role of plant-mycorrhizae interactions in reclamation efforts. Specifically, the success of a reclamation requires extensive knowledge not only of the appropriate plant community, but of the arbuscular mycorrhizal fungi (AMF) community at a site as well. These symbiotic fungi colonize the roots of most plants and supplement plant nutrition by garnering certain nutrients from the soil and transferring them to their host. Many species of native plants are unable to survive without fungal colonization. Thus, inoculating reclamation sites with mycorrhizae may significantly enhance the re-establishment of desirable native species. Since the fungi may also increase the plant's uptake of heavy metals, inoculation of a metal contaminated site may affect the transfer of metal from the soil to the plant; AMF amendment may thus represent a method for the reclamation and/or remediation of metal-contaminated sites.

Commercial production of AMF is currently expensive, and many of the species produced may not be appropriate for use in Minnesota. In addition, little is known about the AMF populations of this state. The purpose of this research was to A) characterize AMF from several native and restored prairies and wetlands to establish baseline data about these populations; B) screen and adapt known fungal spore production processes to generate native Minnesota AMF inoculum; and C) examine long-term storage effects on AMF propagules.

Soil was collected from 10 different native, reclaimed, and disturbed sites in Minnesota and tested for physical and chemical character. AM fungi isolated from these samples have been characterized and many identified to species. After using this soil in extensive tests on the different pot culture techniques and watering methods used in inoculum production, results suggest that the general inoculum method combined with the Beltsville automated watering system produces more spores and colonized roots than the other methods. The selection of an appropriate host species also significantly affects spore production, as we found native fungi to reproduce much more successfully when a native plant was used as the host. In addition, a refined version of a dual *in vitro* culturing system, successfully established in our lab, has potential commercial application.

Most of the inoculum produced as a result of these experiments has either been installed at reclamation sites, with cooperation and funding support from Mn/DOT, or used in further experiments. Early results from inoculated reclamation sites suggest that the fungal treatment is enhancing the establishment of native grasses without affecting weedy species, many of which are non-mycorrhizal.

Tests on the effects of storage found that over a period of 2 years, storage at 4^0 C had no effect on spore viability. We also found that germination is highly species specific, and that very different conditions may be required by different species.

These results have the potential to significantly increase the success of prairie restoration and reclamation projects in Minnesota, which may represent a significant cost savings for publicly and privately funded projects. This may include increasing the establishment rate of native species in roadside plantings, which has several benefits. A planting of native species requires less mowing and maintenance than one of non-natives, and is thus a less expensive roadside cover. Many prairie plants are deep-rooted and excellent slope stabilizers. The esthetic and wildlife value of native plantings is much higher than that of the non-native species which have established themselves in many areas. This research and its implications for commercial production of AMF inoculum also possesses practical application for the homeowner who wants to do a small-scale restoration on their own property, as early and successful establishment of native species can reduce the cost and maintenance required by their project.

Much of the work presented here is currently being prepared for publication in scientific and professional journals, as well as being supplied to Mn/DOT for dissemination in their technical publications. Through these venues, it will be available to professionals in landscape careers, ecologists, and anyone interested in restoring and preserving Minnesota's native landscape. In addition, work on this project continues, through financial support from Mn/DOT.

Date of Report: July 1, 1997
LCMR FINAL WORKPROGRAM UPDATE REPORT

LCMR Work Program 1995

I. Project Title and Project Number:
Subd. 13c(D12)
Beneficial Fungal Inoculum For Prairie And Wetland Reclamation

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A. Legal Citation: ML 95, Chp.220, Sec.19, Subd.13(C)
Total biennial budget: $100,000
Balance: $0.00

Appropriation Language: This appropriation is from the trust fund to the Commissioner of Transportation for an agreement with the University of Minnesota for the characterization and development of inoculum production methods for soil fungi associated with the roots of native and naturalized Minnesota plants in prairies and wetlands to assist in restoration projects

B. Status of Match Requirement: N/A

II. Project Summary

In nature, vesicular arbuscular mycorrhizal fungi (VAMF) form symbiotic relationships with higher plants, acting as a biofertilizer for these plants in disturbed areas. In exchange for carbohydrates, the fungi provide nutrients, particularly phosphorus, to the plant. Because of the advantages that these beneficial fungi confer on many plants, the establishment of native grasses and forbs amended with VAMF along the highways and at restoration sites is of ecological importance. The first goal is to increase the limited baseline information available about the biodiversity of VAMF in Minnesota prairies and wetlands, especially those near restoration sites. Soil cores collected along transects at these sites will be analyzed for VAMF and soil parameters. Secondly, VAM inoculum production will be set up using the spore samples collected from Minnesota prairies, wetlands, and other sites of interest because of likely tolerance or resistance of the VAMF to salt or heavy metals, conditions found along highways. Culturing of VAMF and determination of the viability of these VAMF spores will be done to generate VAM inoculum to use at restoration sites. The achievement of a high reproduction rate of VAM fungi in pot and/or tissue cultures is one of the most essential steps in a successful restoration process. Our goal is to improve VAMF culturing methods so that large amounts of inocula can be produced commercially.

III. Work Program Summary:

Eleven different native, reclaimed, recently disturbed, or heavy metal contaminated sites in Minnesota were identified as study sites for this project. Crosstown, Feder and Schaefer prairies are remnants of original prairies; whereas St. Croix Prairie and the U of MN site are reclaimed prairies. Sites at Lake Shetek and the JES wetland/prairie complex were recently disturbed and have been reclaimed within the past 10 years or less. In addition, the Country Club wetland north of Cambridge, Minnesota, was identified as an established wetland for sampling and was used as a control for evaluating succession at the JES wetland plots. At the Cedar Creek Natural History Area, upland prairie and oak savanna plots were compared to transitional and wetland plots, and where appropriate, these Cedar Creek locations were also used as control sites for comparing data to disturbed areas. Finally, the Pig's Eye Landfill, a metal-contaminated site within the Twin Cities, was used as a source for AMF which potentially may exhibit heavy metal tolerance.

At each site, soil was collected and subsequently processed for isolation of AM fungal spores, nutrient analysis, and physical and chemical parameters. In addition, roots collected during soil sampling were processed and analyzed for their level of AM fungal colonization. The remaining soil from each site was placed in cold storage and used in subsequent tests on storage effects on inoculum, in watering experiments, and for inoculum production for field reclamation sites. In addition to evaluating the soil and AMF population, plants were identified at three of the sites. Each site was visited at least once and some were several times for additional monitoring and collecting. A list of plants identified at these sites is provided.

Efforts were made to repeat the sampling at each site over at least two years, but in some instances this was not feasible or a site was abandoned due to external factors that influenced the quality of data we were able to obtain.

In general, there are two ways to evaluate the mycorrhizal spores at a site. Using field soil is a valid way to preliminarily identify spore species based on color and size, and yields legitimate estimates for spore numbers and colonization levels. Pot culture is an other method, and is the best way to reproduce spores for species identification. In this method, soil from the site is placed in pots and then a plant is planted on top of this soil inoculum. Big bluestem (*Andropogon gerardii* Vitman) is often used, since it is an obligate host. That is, this native prairie grass will flourish and grow to maturity only if it is colonized by mycorrhizal fungi. Thus, the health of the plant in culture is an excellent preliminary indicator of the mycorrhizal status of the soil inoculum. Plants are maintained in culture for a number of weeks or months, and then either harvested immediately or allowed to senesce, depending the purpose of the culture. The soil in the pot is separated from the roots, and spores are extracted from the soil for identification. Spores produced in this manner are less degraded than those collected directly from the field, and thus identification based on pot culture is far more accurate than that based on field samples. This latter method is, however, significantly more expensive and labor intensive than processing field soil directly.

Soil from some sites was used to propagate AMF using pot cultures, a time- and labor- intensive technique for propagating AMF either for further study or or use as a soil amendment at reclamation sites. The first generation Crosstown Prairie inoculum produced in pot culture with big bluestem as host and grown under a hand watering regime in both the greenhouse and in the growth chambers contained many*Glomus* species as well as *Gigaspora*, *Scutellospora*, and *Entrophospora*. Percent colonization data from the Crosstown pot cultures show a total colonization level near 70%. Total colonization from field soil is around 80%, with a spore count of approximately 70 spores per gram of dried soil.

A comparison of the remnant prairies reveals that Feder soil has much higher levels of calcium and magnesium than Crosstown. The same is true for total nitrogen, percent organic matter, and cation exchange capacity (CEC). These high levels are characteristic of a well-preserved remnant prairie. First generation inoculum produced from Feder soil contained high numbers of spores, especially when native grass was used as a host plant. At the JES wetland site, soil and spore parameters were significantly

different than those at the Country Club undisturbed wetland. Surveys of the JES wetland complex in fall 1994 and the summer of 1995 revealed a low volume of spores at this site, and little to no seasonal variation in numbers. This is consistent with work by other researchers that suggests disturbed sites have low levels of AMF. In contrast, the nearby Country Club control site, a relatively undisturbed wetland in the same drainage, contains high numbers of spores. Here, spore numbers increased later in the season. In addition, the JES wetland site soil was very low in organic matter and carbon in comparison to the Country Club site, and the soil was much more dense. These results are as expected, as the disturbance at the JES site was severe (JES is a Mn/DOT wetland mitigation project, at a site where a new road was created in 1994). Plant diversity at the JES wetlands has been monitored for several years, and new species were located in both 1995 and 1996, indicating that this highly disturbed site is being colonized, and that species diversity continues to increase.

In addition to collecting baseline data about soil and AMF at different types of sites in Minnesota, the effects of several different soil amendments were studied. The JES prairie restoration site, established as part of an earlier Mn/DOT project, was amended with native AM fungi and monitored to assess the effects of AM inoculation on the reestablishment of native plant species. At the Shakopee wet prairie site, part of the area had been treated with biosolids under K. Draeger's 1995-1997 LCMR project (ML 95, Chp.220, Sec.19, Subd.5(t)). Our efforts were focused on monitoring the effects, if any, of biosolid amendment on the numbers and diversity of AM fungi at this Mn/DOT relamation site.

At Shakopee, ten different spore types were distinguished and a comparison of the spore types associated with the biosolid vs. control areas in September,1996 reveals a fairly high degree of similarity. There is slightly more variation when the two treatments from the October sample are compared. The biosolid treatment portions of the Shakopee site display a higher number of spores per gram of dry soil during the growing season and fall, although that difference is no longer statistically significant in the late October 1996 collection, probably due in part to the destruction of the spores by natural processes.

At the JES prairie plots, two years after the area was seeded, plants in the plots amended with AMF had a significantly greater percentage of roots colonized by AM fungi. Percent cover of native prairie species was also significantly greater in the inoculated plots. The increase in percent cover of native grasses suggests that inoculating disturbed soils with AM

fungi can significantly enhance the success of prairie restorations in Minnesota, and that the presence of a diverse, viable population of arbuscular mycorrhizae can be essential to the development of early successional tallgrass prairie communities. Mn/DOT provided funding to monitor this site last year as well as for continued research at this site for the next three years.

A series of experiments with different watering regimes and host species were performed to adjust the culturing conditions to the specific environment of the U of MN CBS greenhouse. The production of handwatered inocula is discussed first followed by the production of inocula using an automated watering system.

Soil collected from the Crosstown Prairie was used in a variety of experiments. To compare the effects of automated versus handwatering on spore production, treatments and controls were established using Crosstown soil as inoculum and big bluestem as a host plant. Pots were maintained in the greenhouse on either a hand-watered or automatic irrigation regime, as well as hand-watered in growth chambers.

After 18 weeks, pots were dried and processed. There was no significant difference in shoot biomass, percentage colonization, or spore production between plants grown in the growth chamber or the greenhouse. There was also no difference in shoot biomass between the inoculated plants and the controls, possibly because plants were grown during the winter, and because the seedlings were quite young when they were transplanted. Results from spore identification show that the spores produced in the greatest number in these experiments are possibly *G. mosseae*, *B. etunicatum*, *G. leptotichum*, *G. occultum*, or immature spores of several other species.

In another experiment, side-oats gramma grass (*Bouteloua curtipendula* (Michx.) Torr.) was used as a host plant and inoculated with soil from Crosstown Prairie. The resulting inoculum contained an average of 20 spores per gram of dry soil, and exhibited moderate levels of colonization. After 1 year in cold storage, this inoculum was used in a second round of culturing with big bluestem as host. The resulting soil, spores, and colonized big bluestem roots (second generation inoculum) were applied to the Shakopee prairie restoration site in June, 1997; the spore numbers cited suggest that this second generation inoculum should be a valuable source of AM at the reclamation site. This inoculum is a new product not

mentioned in our original LCMR agreement, and the installation of it into the field was funded under our current Mn/DOT contract (1997 - 2000).

In a test of hand-watering and wetland inoculum, Canada blue joint grass (*Calamogrostis canadensis* (Michx.) Beauv.) was treated with inoculum from the Country Club wetland site at Cambridge. These plants became infected with a fungus that may have originated from nonsterilized seeds. Hereafter, seeds of wetland plants will be sterilized prior to use in inoculum experiments.

Hand-watering techniques were also used to reproduce AM fungi from heavy metal enriched sites e.g., Pig's Eye landfill, St. Paul, as well as from an uncontaminated prairie site (Feder Prairie, MN). Feder soil was used to obtain strains which could serve as a control. In all experiments, a method of a pot "trap culture" was used, with big bluestem or crimson clover (*Trifolium incarnatum* L.) as host. All cultures were incubated in a growth chamber and watered manually. They provided a high yield of AMF spores, allowing an assessment of the AMF spore diversity at the site of origin. Spores produced thus were also used in further experiments.

In order to decrease the labor required for AMF inoculum production, an automated drip irrigation system was used to supply water or nutrient solution to pot cultures grown in the greenhouse. Because previous experiments had shown that cultures could be contaminated by greenhouse spores, all pots were carefully covered, which made hand-watering more difficult. The automated drip irrigation simplified water delivery to covered pots.

Many experiments were required to determine host effects and appropriate watering regimes to used in the greenhouse. Since the weather conditions during the summer significantly affect the greenhouse environment, the frequency and volume of watering had to be very closely monitored. It should be pointed out that the automated drip irrigation system took a lot of time to establish due to changes in water flow and other problems created when additional pots were added to this system.

In the automated irrigation portion of the experiment with Crosstown soil used as an inoculum with big bluestem, trends and spore types were the same as in the hand-watered trial, above. When comparing the hand-watered and growth chamber treatments to those on automatic irrigation, the hand-watered plants averaged 1 gram of dry shoot mass, versus about 1.7 grams of dry shoot mass for plants maintained on the Beltsville system.

Production of some spore types increased under the automatic watering system versus the hand-watered, and the overall trend seems to be for higher spore production under the automated watering system. Finally, plants grown under automated watering had more root biomass than both the plants from the growth chamber and the hand-watered plants from the greenhouse; this is significant, as colonized roots can be very effective sources of AMF. Some of the inoculum produced in this trial was used later in the full Beltsville experiment, described later in this section.

To test automatic watering with wetland inoculum, Canada blue joint grass pots were treated with inoculum from the Country Club wetland. After about 14 weeks a fungal parasite was found growing on some of the plants, so roots were harvested and preserved. Preliminary analysis indicated that colonization levels in the treated pots averaged about 28%, but varied widely. The controls were not colonized.

Another experiment used inocula that had been produced from Crosstown Prairie soil in previous experiments and then stored for one year. Pots were treated either with inoculum or with a suspension of Crosstown spores in water, a new experimental method of inoculation. A measured amount of big bluestem *(Andropogon gerardii* Vitman) seeds were sown directly on the inoculum layer and then covered with sand. Plants were grown in greenhouse for 16 weeks and watered with Hoagland's nutrient solution via the Beltsville system.

Pots treated with the spore suspension had the highest shoot mass and pots with soil inoculum produced the least shoot mass. There was no statistically significant difference in root mass between the treatments. Additionally, plants treated with the spore suspension and the controls both exhibited signs of phosphorus deficiency, while the plants grown on soil inoculum showed no such symptoms. At present, the simplest method of inoculum production seems to be to use soil as inoculum. The inoculum produced as a result of this experiment is being tested at a Mn/DOT prairie reclamation project in Shakopee, Minnesota. Additional analysis of this work is currently being funded by Mn/DOT.

Spores of AM fungi isolated from the heavy metal contaminated Pig's Eye landfill site were tested for viability, then used in single-spore inoculation on big bluestem seedlings. Analysis of the big bluestem roots found no AM structures in the investigated roots. Since the spores used in this experiment were known to be viable, the lack of AM colonization in the roots may be attributed either to the separation of AMF spores from

plant roots, or to a failure to establish a functional mycorrhizal association due to specific pot conditions such as the water logging experienced in early in the experiment, when testing the irrigation system. Nonetheless, the lack of mycorrhizal colonization demonstrated that the sterilization techniques used combined with covering the pots prevents contamination by alien spores. This had been a serious concern in manually watered corn plants grown in the greenhouse

In addition to the watering regime, the host species used for reproducing AM fungi has proven to be an important factor affecting the number of spores produced. In an experiment using original soil from the Feder prairie as inoculum, cultures with a native prairie grass (big bluestem) yielded significantly higher spore numbers than did cultures with corn. No such host effect was observed in the case of inoculum from the moderately metal contaminated Pig's Eye landfill site.

In experiments using spores of the same morphotype from different sites (Feder Prairie and the Pig's Eye landfill), a sweet corn variety with a life cycle period shorter than usual has proven very useful as a host for type-cultures, as it has shortened the AMF spore reproduction process from the traditional four months to three months. Spores reproduced in these type-cultures were used to establish *in vitro* dual cultures.

An *in vitro* dual (AM fungus and excised plant roots) culture system offers an exciting perspective for studying various aspects of AMF biology under microbiologically controlled conditions. It can also potentially be used for the production of axenic AM inoculum. To develop a suitable culturing system, a number of experiments were performed to (1) select suitable host roots, (2) develop an AMF spore decontamination procedure, and (3) select AMF species with spores that would germinate under *in vitro* conditions. None of the host root cultures developed and tested in our lab proved applicable for dual cultures. However, a DC1 clone of Ri T-DNA transformed carrot roots developed by G. Bécard and kindly provided by D. Douds resulted in the successful establishment of *in vitro* cultures of *G. etunicatum* and *G. pansihalos*. *G. etunicatum* from St. Paul sporulates easily under the culturing conditions and yields large numbers of viable spores. The *G. pansihalos* used is from a heavy metal enriched mining site in Poland. Due to its heavy metal tolerance, it may have applications in the reclamation of heavy metal contaminated sites. *In vitro* cultures of both AMF species are being maintained for further studies.

Sodium rhodizonate was used to detect lead deposition in AM roots of leek (*Allium porrum* var. Primor) inoculated with *G. etunicatum* and watered with a lead nitrate solution. The red staining of the lead-rhodizonate complex localized lead to plant cell walls in root tissues, as well as to fungal structures. However, the rhodizonate indicator did not detect lead in AM roots of big bluestem plants grown in lead contaminated soil from the Pig's Eye landfill or from a mining site. This may indicate lead deposition below the detection limit of sodium rhodizonate. This suggests that using sodium rhodizonate for lead detection in plants collected from nature should be performed with caution, due to its low sensitivity.

A method to assess whether or not spores are viable is an essential part of any inoculum production process. A variety of experiments were conducted to test the effectiveness of various viability assays.

The traditional method for evaluating viability requires placing a single spore on a host root and subsequently assessing the AM colonization of the root. This method is time consuming and often inaccurate under greenhouse growing conditions, as AM root colonization may result not only from the single spore inoculation but also from the accidental contamination of soil with spores from external sources. In cases of very low spore viability or where soil conditions are unfavorable to the establishment of AM symbiosis, this method is also very inefficient and costly. Therefore, Hepper's germination test, in which spores are germinated in the soil near actively growing host roots (Hepper and Smith, 1976) was selected as a simpler method to test spore viability.

Corn and purple prairie clover (*Dalea purpurea* Vent.) plants were inoculated with single spores, grown for 4 months, and then stored in the cold room for one year. Spore reproduction in this soil was successful, indicating that the original inoculum was viable. Many cultures had few spores, as expected, because spore production in such pots is often low. When high spore numbers were found, the spores were used for identification of the fungus and to generate additional cultures of the individual species. A second experiment, using single spores isolated from samples collected from Crosstown Prairie in 1994 and 1995 and placed on big bluestem, was successful. From this trial, 6 species of *Glomus* were identified, including *G. mosseae* and *G. etunicatum*.

The viability of stored spores from Pig's Eye landfill was tested simultaneously using a single-spore inoculation on big bluestem and

Hepper's viability assay. Roots isolated from the pots of big bluestem showed no evidence of mycorrhizal activity. However, Hepper viability tests indicated that the spores used in this experiment were viable and exhibited germination rates ranging from 2.2 to 41.1%, depending on the species. The lack of AM colonization in roots may be attributed either to the separation of AM spores from the plant roots, or to a failure to establish a functional mycorrhizal association due to specific pot conditions, such as water-logging that occurred early in the experiment while testing the irrigation system.

An experiment to assess the most suitable soil conditions for testing spore viability via "Hepper's germination test" indicated that these conditions do significantly affect spore germination rates, with responses varying among AMF species. When tested under various conditions, spores of *G. etunicatum* germinated best in soil/sand mix without grass seedlings whereas *G. macrocarpum* had the highest germination rate when incubated in sand with seedlings. These results indicate that several experiments may be needed when assessing inoculum viability since the testing environment may affect the results.

The effects of storage time on spore viability was also tested in *G. etunicatum*. Spores of this species were produced in greenhouse pot cultures in the summer of 1994 and they retained high levels of viability (measured by their germination rate near 80%) over a period of 2.5 years. Further experiments on the same stock of inoculum will be performed over several years

Heavy Metal Effects on Spore Germination

In a series of experiments, six species of AMF fungi from various sites with different levels of heavy metal contamination were tested for metal tolerance using the spore germination assay. Spores were buried in disposable Petri plates containing sand, and soaked with increasing concentrations of either cadmium nitrate, cadmium, copper, lead, zinc nitrate, or with water. Two species from two different environments germinated consistently. A St. Paul isolate of *G. etunicatum* was much less tolerant to metal than an isolate of *G. macrocarpum* from the mining site (Pawlowska *et al.*, 1996). The highest metal concentrations under which *G. etunicatum* germinated were 1.0 mg mL^{-1} Cd, 50 mg mL^{-1} Pb and 10 mg mL^{-1} Zn, whereas *G. macrocarpum* was able to tolerate 100 mg mL^{-1} Cd, 20 mg mL^{-1} Cu, 100 mg mL^{-1} Pb and 2000 mg mL^{-1} Zn. The implications

of these different levels of metal tolerance for using AM fungi in the reclamation of heavy metal contaminated sites require further studies.

Several experiments were performed to test the applicability of various stains (phloxine b, FDA, CFDA, and MTT) for the assessment of spore viability in AM fungi. In these assays, spores of *Glomus etunicatum* produced during single spore inoculum trials with spores from the St. Paul site and stored for about 1 year at room temperature were used. Spores were either left intact (nonkilled), or killed by autoclaving or soaking in ethanol for 24 hours, for use as control. The ability of the spores to germinate was assessed via spore germination assays. The stains phloxine B, fluoresceine diacetate (FDA) and carboxyfluorescein diacetate (CFDA) were tested for their suitability to determine spore viability. Tetrazolium bromide (MTT) was used as a control stain.

With Phloxin B, there was no statistically significant difference in stain accumulation between nonkilled and killed spores. Killed and nonkilled spores stained with CFDA exhibited similar levels of flourescence, and flourescence induced by FDA faded so quickly that it was not useful for distinguishing between killed and nonkilled spores. Thus, none of these three stains is appropriate as a viability indicator for the AMF species tested. With MTT, almost all of the living spores stained blue or purple, and most of the control killed spores remained unstained.

Only MTT has proven to be a reliable indicator when used for the assessment of viability in *G. etunicatum* spores reproduced in pot cultures. The numbers of spores staining as viable when using MTT corresponded well with the results of spore germination assays.

Budgeted spending to complete the objectives is listed at the end of each summary. Requests for changes in the budget were made on December 4, 1995, in a letter to the LCMR Director, J. Velin. Funding changes were requested and approved for decreases in In-State Travel, Fees, Other Fxd, and Equipment-miscellaneous, and increases in Salaries and Repair Services. The changes in salaried personnel included hiring a post-doctoral associate with expertise in the identification and systematics of AM fungi. Detailed explanation of the changes were presented in the letter. In addition, a graduate student originally supported by LCMR was awarded a Fellowship by the Graduate School. Part of the LCMR funding originally allocated to this student was used to hire technical assistance, per a1996 memo to J. Velin.

It is important to recognize that the research funded by the LCMR is part of a larger project, aspects of which have been and continue to be funded by Mn/DOT. Specifically, Mn/DOT has funded a 3 year continuation of this research on the two largest parts of our LCMR appropriation: A. Collection and identification of mycorrhizal fungi in established prairies and wetlands in Minnesota, including recently established native sites, and B. Culturing of mycorrhizal fungi from species isolated in recent or past collections. The data processing system and microscope purchased with LCMR funds are essential for the continuation of this work. When combined with results obtained with LCMR funding and with prior Mn/DOT funding, this new data will provide valuable insights into the role of mycorrhizae in Minnesota.

IV. Statement of Objectives:

A. Collection and identification of beneficial soil/root fungi in established prairies and wetlands in Minnesota. Soil root core sampling in Minnesota prairies and wetlands for mycorrhizae. Project included interns and high school student participation.

B. Culturing of mycorrhizal fungi from species isolated in recent or past collections. Production techniques will be established in greenhouse, growth chamber and/or with lab apparatus to determine the best available methods to culture different species of these prairie and wetland fungi.

C. Determination of spore viability from soil samples collected from Minnesota wetlands and prairies in 1991-92 and stored in dried or frozen soil. Little information is available on how long mycorrhizal spores from Minnesota prairies or wetlands can survive when stored for various lengths of time and under different conditions. Such data is essential for many reclamation projects that would benefit from fungal inoculum added during planting. Ideally, fungal inoculum would be produced in the greenhouse during non-growing season or that year. But some may be produced as much as a year before use; consequently, the fungal inoculum would be of different ages.

Timeline for Completion of Objectives(use dates as appropriate):

	7/95	1/96	6/96	1/97	6/97
Objective A and Collection	xxxxxxxxxx	xxxxxxxxx	xxxxxxxx	xxxxxxxxx	

Objective B and Culturing xxxxxxxxxx xxxxxxxxx xxxxxxxx xxxxxxxxx
Objective C and Determination xxxxxxxx xxxxxxxx xxxxxxxx xxxxxxxxx

Each objective (A-C) is continuous throughout the two years, unlike many projects. However, these general objectives have deliverable products that can be completed by specific dates. I have made breaks in the timeline to indicate that we can provide these products at six month intervals.

V. Objective/Outcome

A. **Title of Objective/Outcome:** Collection and identification of beneficial soil/root fungi in established prairies and wetlands in Minnesota.

A.1 Activity: Collection of vesicular arbuscular mycorrhizal fungi (VAMF) from soils of Minnesota prairies and wetlands, especially those near restoration sites, was conducted in the fall of 1995 and of 1996. Some prairies that were initially sampled in 1991 as part of a vesicular arbuscular mycorrhiza (VAM) mini-survey supported by Mn/DOT (Stenlund *et al.*, 1994) were sampled again. Soil cores were taken from the following prairies: Crosstown (Hennepin County), Mn/DOT Materials and Research Laboratory (next to abandoned railroad right-of-way, Ramsey County) and the University of Minnesota (Ramsey County). Other areas likely to have spores of value to the restoration projects initiated by Mn/DOT beginning in the fall of 1994 were identified and sampled.

A.1.a. Context within the project: Because of the advantages that VAMF confer on many plants, the establishment of native grasses and forbs amended with VAMF along the highways is ecologically important. VAMF serves as a biofertilizer for the plants. Objective A will provide valuable baseline data on the kinds of arbuscular mycorrhizae in prairies and wetlands of Minnesota. Without this information we do not know what types of VAMF to culture for restoration. My lab has experience obtaining this type of VAMF field information (Stenlund and Charvat, 1994; Stenlund *et al.* 1994).

The beneficial effects of vesicular arbuscular mycorrhizal fungi (VAMF) in prairie restorations has been well documented (Miller and Jastrow, 1992). VAMF are symbiotic fungi which form mutually beneficial relationships with the roots of many prairie and some wetland plants. Vesicular arbuscular mycorrhiza (VAM) serve as biofertilizer by facilitating the uptake of nutrients, especially phosphorus, by the plant; and the plant provides carbohydrates, the products of photosynthesis, to the fungus. In addition, VAM plants under certain conditions have greater tolerance to drought (Killham, 1994), heavy metals (Dueck *et al.*,1986, Sambandan *et al.*, 1992), and salt (Cooke and Lefor, 1990; Sengupta and Chaudhuri, 1990) than the nonVAM plants.

Some of the spores used to generate inoculum for restoration projects will be selected on the basis of having resistance to heavy metals and the appropriate characteristics to survive and reproduce at restoration and

mitigation sites near highways, to be selected by Mr. Robert Jacobson, Mn/DOT, in 1994-95, and at other restoration sites.

A.1.b. Methods: Prairies, wetlands and other areas likely to have spores of value for restoration projects were identified. A W, or other appropriately shaped transect was set-up at each site. Thirteen, or an appropriate number, soil cores were taken at random points along the transect, and the cores were pooled to obtain a composite sample for the site. The soil cores were obtained using bulk density sampling tubes that produce uniform cores of 1.8 cm x 20 to 30 cm (Staricka *et al.*, 1990). At a limited number of sites plant composition will be determined, as time permits. Collections will be recorded on computer. The data obtained throughout this project was analyzed by appropriate statistical methods, such as those described in our refereed publications (see Stenlund and Charvat, 1994: Meier and Charvat, 1993).

A.1.c. Materials: Equipment that is used in the field to record site information and to obtain plant and soil samples: field corer, quart size Ziplock bags, buckets (for carrying equipment and samples), P-bottles, thermometer, knives, paper bags, tape measure, flags, colored string, plastic stakes, compass, meter stick, shovel, time tape, boots, gloves, plant press, orange vests, first aid kit, coolers and ice, camera, and film (Field Collecting Check List, I. Charvat's lab). The Bulk Density Sampling Tubes (Staricka *et al.*, 1990) will be used to obtain soil samples unless a less expensive soil sampling devise and/or a more efficient one can be found. The computer will be used to map out sites and keep track of materials and dates collected.

<u>New Equipment & reimbursement:</u> A computer-data processing system (approx. $2,700) is needed to efficiently enter and process the data that my graduate students and other trained personnel generate. We now have a Mac SE/30 with limited capable to crunch data numbers. A new computer would be permanently placed in BioSciences Center, R. 762 to be used by my graduate students and other trained personnel to process data from this and similar mycorrhizal research projects in the future. If the use of the new equipment changes, I am committed to reimburse the Fund for an amount equal to the cash value received or if not sold, to the residual value approved by the director of the LCMR.

A.1.d. Budget
Total Biennial LCMR Budget: $4665
LCMR Balance: $0.00

MATCH: $
MATCH BALANCE: $

A.1.e. Timeline:

	7/95	1/96	6/96	1/97	6/97
PRODUCT #1	xxxxxxxxxx		xxxxxxxxx		
(List of sites selected for VAM sampling.)					
PRODUCT #2	xxxxxxxxx		xxxxxxxxx		
(Cores collected from each site)					
PRODUCT #3	xxxxxxx		xxxxxxx		
(Plant composition list from limited number of sites)					

A.1.f. Workprogram Summary

Crosstown, Feder, St. Croix, Schaefer, and University of Minnesota Prairies were identified as established prairies to use for sampling during the course of this experiment. In addition, the Country Club wetland north of Cambridge, Minnesota, was identified as an established wetland for sampling. Plots were also established at the Cedar Creek Natural History Area in Bethel, Minnesota, and monitored for mycorrhizal, soil, and plant parameters. Upland prairie and oak savanna plots were compared to transitional and wetland plots, and where appropriate, these Cedar Creek locations were also used as control sites for comparing data to disturbed areas cited in A.2. Studies at the reclaimed Mn/DOT Materials and Research Laboratories were eliminated, when they were destroyed during construction of a bike path.

Transects or plots were established at each site and soil cores were taken. The soil was transported on ice to the U of MN. Subsamples for spore isolation were dried in the hood and the soil for nutrient testing was placed in the cold room until analyzed. Soil for nitrogen testing was frozen to stabilize the ammonia until analysis, while samples for bulk density and root isolation were refrigerated and processed shortly after collection. The balance of the soil was dried in a forced air oven and then placed in cold storage until needed. The soil from Crosstown Prairie, Feder Prairie, and from the Country Club wetland was used in subsequent tests on storage effects on inoculum, in watering experiments, and for inoculum production.

In addition, plants were identified at three of these locations. Each site was visited at least once and, as time and money permitted and as warranted by preliminary data, some were revisited two to three times or

more for additional monitoring and collecting during the funding period. A list of plants identified at some of the sites follows. (Species identified in 1994 were done so as part of a previous Mn/DOT contract (Charvat *et al.*, 1995); data from both years is listed for purposes of comparison. It should be noted that 1995 data is incomplete, as inventories were sometimes taken late in the season and not all of the plants still had the reproductive structures necessary for accurate identification. This data has been provided to Mn/DOT, as our LCMR liaison.)

Crosstown Prairie

Plant Family	Scientific Name	Common Name(s)	Year
Aceraceae	*Acer negundo*	Boxelder	94, 95
Asclepiadaceae	*Asclepias syrica*	Common milkweed	94, 95
Asteraceae	*Achillea millefolium*	Common yarrow	94
	Ambrosia artemisifolia	Common ragweed	94
	Artemesia ludoviciana	White sage	94, 95
	Aster sp.	Aster	94, 95
	Cirsium vulgare	Bull-thistle	95
	Liatrus sp.	Blazing star	94
	Ratibida pinnata	Globular coneflower	95
	Solidago canadensis	Canada goldenrod	94, 95
	Solidago rigida	Stiff goldenrod	94
Caprifoliaceae	*Symphoricarpos occidentalis*	Wolfberry	95
Equisetaceae	*Equisetum* sp.	Scouring Rush	94, 95
Euphorbiaceae	*Euphorbia* sp.	Spurge	94
Fabaceae	*Lotus corniculatus*	Birdsfoot-trefoil	94
Lamiaceae	*Monarda fistulosa*	Wild bergamot	94, 95
Oleaceae	*Fraxinus* sp.	Ash	94
Onagraceae	*Oenothera biennis*	Evening-primrose	94, 95
Poaceae	*Agrostis gigantea*	Redtop	95
	Andropogon gerardii	Big bluestem	94, 95
	Bromus inermis	Smooth brome	95
	Muhlenbergia racemosa	Muhly	95
	Panicum virgatum	Switchgrass	94, 95
	Phalaris arundinacea	Reed canary grass	95
	Schizachyrium scoparium	Little bluestem	94, 95
	Setaria sp.	Foxtail grass	94, 95
	Sorghastrum nutans	Indian grass	94, 95
Polygonaceae	*Polygonum* sp.	Smartweed, knotweed	94
Ranunculaceae	*Anemone cylindrica*	Thimbleweed	95
Rhamnaceae	*Rhamnus cathartica*	Common buckthorn	95
Rosaceae	*Rosa* sp.	Rose	94
	Rubus sp.	Bramble	94, 95
	Spiraea alba	Meadowsweet	95
Rubiaceae	*Galium* sp.	Bedstraw, cleavers	94
Salicaceae	*Salix* sp.	Willow	95
Scrophulariaceae			

	Veronicastrum virginicum	Culver's root	94
Solanaceae	*Solanum dulcamara*	Bittersweet nightshade	94, 95
Ulmaceae	*Ulmus americana*	American elm	95

Schaefer Prairie - Remnant

Plant Family	Scientific Name	Common Name(s)	Year
Anacardiaceae	*Toxicodendron rydbergii*	Poison-ivy	95
Apiaceae	*Zizia* sp.	Golden alexander	95
Asteraceae	*Achillea millefolium*	Common yarrow	95
	Aster spp.	Asters	95
	Ratibida pinnata	Globular coneflower	95
	Solidago spp.	Goldenrods	95
Lamiaceae	*Monarda fistulosa*	Wild bergamot	95
Poaceae	*Andropogon gerardii*	Big bluestem	95
	Elymus canadensis	Canadian wild rye	95
	Poa sp.	Bluegrass	95
	Schizachyrium scoparium	Little bluestem	95
	Sorghastrum nutans	Indian grass	95
	Spartina pectinata	Prairie cord-grass	95
Rosaceae	*Fragaria virginiana*	Wild strawberry	95
	Rosa sp.	Rose	95

Remnant Wetland

Cambridge - Country Club

Plant Family	Scientific Name	Common Name(s)	Year
Alismataceae	*Sagittaria* sp.	Arrow-head	94
Asclepiadaceae	*Asclepias* sp.	Milkweed	95
Aspleniaceae	*Thelypteris palustris*	Marsh-fern	94
Asteraceae	*Bidens cernua*	Bur-marigold	94, 95
	Cirsium sp.	Thistle	95
	Eupatorium perfoliatum	Boneset	94
	Solidago sp.	Goldenrod	95
Balsaminaceae	*Impatiens* sp.	Touch-me-not	94
Cornaceae	*Cornus racemosa*	Dogwood	94, 95
Cyperaceae	*Carex comosa*	Sedge	94, 95
	Carex utriculata	Sedge	95
	Carex spp.	Sedges	94, 95
Equisetaceae	*Equisetum* sp.	Horsetail	95
Grossulariaceae	*Ribes* sp.	Currants, gooseberries	95
Lamiaceae	*Lycopus uniflorus*	Northern water-horehound	94, 95
Lemnaceae	*Lemna minor*	Lesser duckweed	94, 95
Onagraceae	*Epilobium ciliatum*	American willow-herb	95
Poaceae	*Agrostis gigantea*	Redtop	95
	Bromus ciliatus	Fringed brome	95
	Calamagrostis canadensis	Bluejoint	94, 95
	Phragmites australis	Common reed	95
Polygonaceae	*Polygonum sagittatum*	Arrow-lvd tearthumb	95
	Rumex sp.	Dock	94

Rosaceae	*Rubus* sp.	Bramble	95
Rubiaceae	*Galium asprellum*	Rough bedstraw	95
Salicaceae	*Salix fragilis*	Crack-willow	94
Typhaceae	*Typha angustifolia*	Narrow-leaved cat-tail	94, 95
	Typha glauca	Common cat-tail	94, 95
Urticaceae	*Urtica dioica*	Stinging nettle	94

Budgeted spendings to complete the objective A.1. involved part of a graduate student stipend, salaries for undergraduate and technical help, travel expenses to field sites, field equipment, and supplies. New equipment purchased included a soil corer and a computer. The computer was essential for processing the results from all 3 objectives (A,B, and C), and is presently used to process datea generated under our new Mn/DOT contract. This contract includes a continuation of parts of objectives A and B.

A.2 Activity: VAM was collected from previously and newly established field experimental plots. Additional field experimental plots were set-up in the fall of 1994 as part of the Mn/DOT restoration efforts. At some established sites, such as those near Cambridge, plant composition was determined. Another area was established at a St. Paul landfill site with heavy metal contaminated soils and mycorrhizal plants that may be used to provide inoculum for characterization and restoration in similar sites.

Soil cores were taken from the uncontrolled and controlled field experimental plots established in 1991 when VAM was re-introduced at the Lake Shetek mitigation site (see Stenlund *et al.*, 1994).

Soil cores were taken from the permanent uncontrolled and controlled field experimental plots near Cambridge, MN established by my lab in 1994 at the recommendation of Mr. Robert Jacobson, Mn/DOT. A controlled experimental plot was established at the newly created wetland at the intersection of Highway 65 Cambridge and County Road 30 near Cambridge (called the JES site). An uncontrolled plot was also permanently established. This site, an undisturbed wetland, is found along 357 Avenue NE, a dirt road west of Highway 65. The undisturbed wetland serves as an uncontrolled site for the JES newly created wetland.

A.2.a. Context within the project:

In restoration projects certain characteristics of a site can be analyzed to assess the condition of an ecosystem. Some of these characteristics

include the stage of succession, soil nutrient composition, plant composition and degree of presence of symbiotic fungi (VAMF) in the soil.

VAM spores and roots were isolated from the soil cores so that VAM parameters could be measured. The VAM parameters that were determined include the number of VAM spores and the % of VAM colonization inside the roots.

A.2.b. Methods: The type of transect or method of collection varied with the history and and size of the site. At the newer sites, such as the plots near Cambridge, a W shaped transect was established. The size of the W transect varied with the area of the site. Thirteen soil cores were taken at random points along the transect, and the cores pooled to obtain a composite sample for the site. The soil cores obtained using bulk density sampling tubes that produce uniform cores of 1.8 cm x 20 to 30 cm (Staricka *et al.*, 1990).

Straight-line transects were established at the Lake Shetek mitigation site as described by Stenlund *et al.* (1994). Five subplots were sampled, each separated by 50m along a transect for each of the plots: the buffer, low density VAM, high density VAM, residual VAM, and undisturbed. The samples from the subplots were combined to form a composite sample for each plot. The original plots established in 1991 are too narrow to sample effectively with a W transect.

The site information (location and plant composition) and the cores collected were recorded on computer.

A.2.c. Materials: The same equipment that is listed in A.1.c to be taken to the field was also used here, along with the bulk density sampler and a computer.

A.2.d. Budget
Total Biennial LCMR Budget: $4662
LCMR Balance: $0.00
MATCH: $
MATCH BALANCE: $

A.2.e. Timeline:

7/95	1/96	6/96	1/97	6/97

PRODUCT #1	xxxxxxxxx	
(List of new research plots established)		
PRODUCT #2	xxxxxxxxxx	xxxxxxxxxx
(List of sites selected for VAM sampling)		
PRODUCT #3	xxxxxxxxxx	xxxxxxxxxx
(Soil core list collected from research plots)		
PRODUCT #4	xxxxxxxxx	xxxxxxxxxx
(Plant composition lists from limited number of sites)		

A.2.f. Workprogram Summary

A variety of experimental sites were selected and monitored. Three of them, the two Cambridge sites (the JES wetland/prairie restoration complex and the Country Club, a control site for JES wetland) and the Lake Shetek mitigation site had been previously established as part of an earlier Mn/DOT project (Charvat *et al.*, 1995). The JES wetland/ prairie restoration complex and the Lake Shetek mitigation site (Stenlund *et al.*, 1994) were amended at different times with native AM fungi, and were being monitored to assess the effects of AM inoculation on the reestablishment of native plant species. At the Shakopee wet prairie site, part of the area had been treated with biosolids, as part of K. Draeger's 1995-1997 LCMR project (ML 95, Chp. 220, Sec.19, Subd.5(t) administered by Steve Stark and Tom Vesley at the Met Council). Our efforts were focused on monitoring the effects, if any, of biosolid amendment on the numbers and diversity of AM fungi. In addition, plots or transects at the Cedar Creek Natural History Area, Schaefer Prairie, and other natural areas near experimental sites were established for use as controls (see A.1).

At each site, soil cores were removed, bagged, and transported on ice to the University of Minnesota. Subsamples were taken from each bag for spore isolation, root isolation, bulk density, soil nutrient analysis, and nitrogen testing. The remaining soil was dried and placed in cold storage until needed.

Plants were also identified at some experimental sites. At the JES site, several new species were found in 1995, which indicated that this highly disturbed site is being colonized by more plant species, including some native species. The JES wetland site and Country Club were reexamined under a Mn/DOT contract during 1996, and the plant diversity at JES continue to increase (Gould *et al.*, 1997a; Gould *et al.*, 1997b). JES plant data is presented at the end of this section. Data from the undisturbed sites, Crosstown Prairie, Schaefer Prairie and Country Club, is presented in A.1.

U of MN Legume Fields

New transects also were set up in the fall of 1996 at a field planted with native prairie legumes at the University of Minnesota, to determine whether these plants are mycorrhizal. Five different straight line transects were established, each in a row of different plants. These plants were: Illinois Bundleflower, Wild Blue Indigo from root stock, Wild Blue Indigo from seed, False Indigo, and Wild Senna. Subsamples were collected using the same procedure as used at other sites. The samples were transported on ice and stored as described for other sites (see A.1)

St. Paul Pig's Eye Landfill Site

In order to study the role of AM fungi in the mineral nutrition of plants exposed to moderate metal contamination, soil and root samples were collected from the experimental plots established by M. Chin and R. L. Chaney at the St. Paul, Minnesota Pig's Eye landfill, a site contaminated with cadmium, copper, and lead. Details about the presence of AM fungi in the rhizosphere of selected plant species, and the effects of nitrogen fertilizers and sulfur amendments on the intensity of mycorrhizal colonization in the roots of metal-accumulating corn plants, were presented by Charvat *et al.*, 1995 and Pawlowska *et al.* 1995. Soil samples from this landfill site served also as a source of AMF inoculum for reproducing AMF which may potentially exhibit increased tolerance to heavy metals. Strains of AM fungi isolated from the site are being maintained at the University of Minnesota and will be used in further studies.

Plant Species from Select Experimental Sites

Reclaimed Wetland
Cambridge - JES

Plant Family	Scientific Name	Common Name(s)	Year
Asclepiadaceae	*Asclepias* sp.	Milkweed	94
Asteraceae	*Ambrosia* sp.	Ragweed	94
	Arctium sp.	Burdock	94
	Bidens cernua	Bur-marigold	94, 95
	Cirsium sp.	Thistle	94
	Hieracium sp.	Hawkweed	94
	Rudbeckia hirta	Black-eyed Susan	94, 95
	Taraxacum officinale	Common dandelion	94
Brassicaceae	*Berteroa incana*	Hoary alyssum	94
	Capsella bursa-pastoris	Shepherd's purse	94
Caryophyllaceae	*Silene* latifolia	White campion	94, 95
Cyperaceae	*Carex* sp.	Sedge	94
	Carex comosa	Sedge	94

	Scirpus americanus	Bulrush	94, 95
Equisetaceae	*Equisetum* sp.	Horsetail	94
Fabaceae	*Medicago lupulina*	Black medick	94
	Trifolium hybridum	Alsike clover	94
	Trifolium pratense	Red clover	94, 95
	Trifolium repens	White clover	94
Hydrocharitaceae	*Elodea* sp.	Water-weed	95
Juncaceae	*Juncus effusus*	Soft rush	94, 95
Lemnaceae	*Lemna minor*	Lesser duckweed	94, 95
Onagraceae	*Epilobium ciliatum*	American willow-herb	94, 95
	Oenothera sp.	Evening-primrose	94
Plantaginaceae	*Plantago major*	Common plantain	94, 95
Poaceae	*Agrostis gigantea*	Redtop	95
	Avena sativa	Oats	94
	Echinochloa muricata	Barnyard-grass	94, 95
	Elymus canadensis	Canada wild rye	95
	Eragrostis spectabilis	Purple lovegrass	95
	Glyceria grandis	American mannagrass	95
	Lolium perenne	Italian ryegrass	94, 95
	Muhlenbergia sp.	Muhly	94
	Panicum virgatum	Switchgrass	94
	Phalaris arundinacea	Reed canary grass	94, 95
	Phleum pratense	Timothy	94
	Setaria viridis	Green foxtail	95
	Triticum aestivum	Wheat	95
Polygonaceae	*Polygonum* sp.	Smartweed, knotweed	94
	Rumex acetosella	Red sorrel	94
	Rumex crispus	Curly dock	94, 95
Ranunculaceae	*Ranunculus recurvatus*	Hooked crowfoot	94
Rosaceae	*Fragaria virginiana*	Wild strawberry	95
	Potentilla norvegica	Strawberry-weed	94
Salicaceae	*Populus deltoides*	Cottonwood	94
	Salix nigra	Black willow	94, 95
Saxifragaceae	*Penthorum sedoides*	Ditch-stonecrop	95
Scrophulariaceae	*Verbascum thapsus*	Common mullein	94, 95
Typhaceae	*Typha* sp.	Cat-tail	94, 95
Urticaceae	*Urtica dioica*	Stinging nettle	94
Verbenaceae	*Verbena* sp.	Vervain	94

Restored Prairie
Lake Shetek

Plant Family	Scientific Name	Common Name(s)	Year
Apiaceae	*Zizia* sp.	Golden alexanders	94
Asclepiadaceae	*Asclepias* sp.	Milkweed	94
Asteraceae	*Achillea millefolium*	Common yarrow	94
	Aster spp.	Asters	94
	Cirsium sp.	Thistle	94
	Helianthius sp.	Sunflower	94
	Ratibida sp.	Coneflower	94
	Rudbeckia hirta	Black-eyed Susan	94

Fabaceae	*Lotus corniculatus*	Birdsfoot-trefoil	94
	Melilotus alba	White sweet clover	94
Lamiaceae	*Monarda fistulosa*	Wild bergamot	94
Onagraceae	*Oenothera* sp.	Evening-primrose	94
Poaceae	*Andropogon gerardii*	Big bluestem	94
	Bouteloua curtipendula	Side-oats gramma	94
	Elymus canadensis	Canada wild rye	94
	Panicum virgatum	Switchgrass	94
	Phleum pratense	Timothy	94
	Spartina pectinata	Prairie cord-grass	94
Polygonaceae	*Polygonum* sp.	Smartweed, knotweed	94

Budgeted spendings to complete the objective A.2. involved part of a graduate student stipend, salaries for undergraduate and technical help, travel expenses to field sites, field equipment, and supplies. New equipment purchased included a soil corer and a computer. The computer was essential for processing the results from all 3 objectives (A,B, and C), and is presently used to process datea generated under our new Mn/DOT contract. This contract includes a continuation of parts of objectives A and B.

A.3 Activity: Characterization of VAM collected from soils of prairies, wetlands and sites near restoration projects will be by VAM spore isolation (numbers and kinds listed by size and color) and by determination of percent VAM colonization of the roots. At some sites soil parameters, such as pH and nutrient content, and plant composition may be determined and correlated with the VAM data.

A.3.a. Context within the project: The characteristics of VAM isolated from locations most likely to serve as the sources for generating inocula for restoration projects will be done. The most appropriate VAM samples will serve as inoculum to generate more VAM for restoration projects.

In nature, VAMF form symbiotic relationships with higher plants. In exchange for carbohydrates, the fungi provide nutrients, particularly phosphorus, to the plant (Cox *et al.*, 1975). The site of this exchange is believed to be the arbuscule (Cox & Tinker, 1976). Due to the vital importance of arbuscules, some authors consider them to be the definitive sign of a symbiosis (McGonigle *et al.*, 1990). For this reason, (1.) % colonization of VAM structures inside the roots will be done as an indicator of the VAM activity in the ecosystem. (2.) VAMF spores will be isolated from the soil as another way of assessing the level of activity of these fungi in the soil.

A.3.b. Methods: The VAM spores will be isolated from the soil samples using a method modified from Tommerup and Kidby (1979). The number of VAM spores/gram of dried soil and the kind of spores will be determined according to the procedures outlined in Stenlund *et al.* (1994). The percent colonization of mycorrhizal fungi inside the roots of the plant cover will be determined by either of these methods: Giovanetti and Mosse (1980) for pre-screening or McGonigle *et al.* (1990) for post-screening as time permits.

Statistical analyses will be performed on the spore isolation and % colonization results using Statview SE+Graphics, v. 1.03 or a newer version to provide an analysis of varience. The mean separation will be determined using the least significant difference (LSD) criteria (see Stenlund *et al*, 1994). Additional statistical analyses may be done as outlined in our refereed publications (see Stenlund and Charvat, 1994; Meier and Charvat, 1993).

Soil samples will be sent to the U of M, Research Analytical Lab for analysis of nutrient content. Other soil parameters such as pH, bulk density, % water will be measured and analyzed in our lab according to McKeague (1978). Statistical analyses of soil parameters will be done according or similar to Stenlund, 1990.

A.3.c. Materials Sieves (38, 90, and 250 mm), screw top erlenmyer flasks, balance, Eberback Mechanical Shaker, Calgon, sucrose, Fisher filter apparatus, nitro-cellulose filter, centrifuge, centrifuge tubes, scale, petri plate, Gelman Sciences Analyside storage containers will be used for the spore isolation procedures modified from Tommerup and Kirby, 1979. For percent colonization, the following equipment will be used: P-bottles, tweezers, petri plates, slides, coverslips, chemicals (such as potassium hydroxide, hydrochloric acid, stains, sucrose) dissecting and compound microscopes, and a computer (for analysis of data).

A.3.d. Budget
Total Biennial LCMR Budget: $17,838
LCMR Balance: $0.00
MATCH: $
MATCH BALANCE: $

A.3.e. Timeline:

	7/95	1/96	6/96	1/97	6/97
PRODUCT #1 (Spore isolation data)	xxxxxxx	xxxxx	xxxxxxx	xxxx	
PRODUCT #2 (% VAM colonization data)	xxxxxxx	xxxxx	xxxxxxx	xxxx	
PRODUCT #3 (Other soil parameters-such as pH, bulk density)	xxxxxxxxxx		xxxxxxxxxxx		
PRODUCT #4 (Soil nutrient analysis)	xxxxxxxxxx		xxxxxxxx		

A.3.f. Workprogram Summary:

In general, there are two ways to evaluate the mycorrhizal spores at a site. Using field soil is a legitimate method to preliminarily identify spore species based on color and size, and yields valid estimates for spore numbers and colonization levels. Pot culture is another method, and is the best way to reproduce single spores for species identification (Morton *et al.*, 1993). In this method, soil from the site is placed in pots and then a plant host that requires the presence of mycorrhizae is planted on top of this soil inoculum. Big bluestem (*Andropogon gerardii* Vitman) is often used, since it is an obligate host. That is, this native prairie grass will flourish and grow to maturity only if it is colonized by mycorrhizal fungi. Thus, the health of the plant in culture is an excellent preliminary indicator of the mycorrhizal status of the soil inoculum. Plants are maintained in culture for a number of weeks or months, and then either harvested immediately or allowed to senesce, depending the purpose of the culture. The soil in the pot is separated from the roots, and spores are extracted from the soil for identification. Spores produced in this manner are less degraded than those collected directly from the field, and thus identification based on pot culture is far more accurate than that based on field samples (Morton *et al.*, 1993).

Crosstown Prairie

The first generation inoculum produced in pot culture with big bluestem as host and grown under a hand watering regime in both the greenhouse and in the growth chambers contained large and small spores in 4 different colors; Dr. Hamdy Agwa, a postdoctoral associate and visiting professor who specializes in the systematics of AM fungi, has identified the following species: *Glomus occultum*, *Glomus intraradices*, *Glomus mosseae*, *Glomus etunicatum*, *Glomus constrictum*, *Gigaspora gigantea*, *Scutellospora pellucida*, *Entrophospora infrequens*, as well as several other *Glomus*,

Gigaspora, and *Scutellospora* species that have yet to be identified. Dr. Agwa presented these results at the First International Mycorrhizal Conference at Berkeley (Agwa and Charvat, 1996; Agwa and Charvat, 1997). A manuscript on his work is now in preparation. Dr. Agwa has currently returned to Egypt, and in July 1997 will resume his work identifying the spore species from native Minnesota sites, with support from Mn/DOT.

Percent root colonization data from the Crosstown pot cultures show a total colonization level near 70%, including fungal hyphae (the majority of the colonization), arbuscules, and vesicles (trace amounts). Total colonization from field soil is around 80%, with a spore count of approximately 70 spores per gram of dried soil.

St. Croix Prairie

The St. Croix Prairie can be divided into two parts: one which has been burned regularly, and one which has not been subject to burning. As burning is a common management practice for native prairies and at prairie reclamation sites, an understanding of the effects that surface burning has on AMF populations may in turn contribute to our understanding of how burns affect prairies. In turn, this may guide future management decisions. Thus, samples were taken from both the burned and unburned portions of the site for comparison.

Spores isolated from site soil fell into 1 of 4 color categories, and were scored as either large or small for a total of 8 spore types. Small brown spores were by far the most abundant, regardless of site history. Summer spore population at the unburned site was lower than both the summer spore count for the burned site and the fall counts for burned and unburned. Differences in percent colonization were statistically significant only when comparing the unburned prairie summer and fall samples, where the summer percent colonization levels were significantly higher. These patterns of colonization and spore number in the unburned section are consistent with reports by Bentivenga and Hetrick (1992). Colonization is highest in the warm months, when plants are actively growing and photosynthates are abundantly available for the fungus. When plant growth slows during the fall, the decrease in food supply stimulates the fungus to produce spores, which are better adapted to stress.

Feder Prairie

Feder soil has much higher levels of calcium and magnesium than Crosstown. The same is true for total nitrogen, percent organic matter, and cation exchange capacity (CEC). These high levels are characteristic of a

well-preserved remnant prairie. Soil from this remnant prairie was used as pot culture inoculum with either big bluestem or corn as host species. The first-generation inoculum contained high levels of spores, with a noticeable difference in the numbers produced with different host plants. Using corn as host, an average of nearly 60 spores were produced per gram of dry soil. With big bluestem, the number increased dramatically, to nearly 300 per unit of soil. The methods and results for this experiment are discussed fully in B.2.

Schaefer Prairie

Part-way into this project, we found that some areas of the Schaefer Prairie old field-prairie complex which were being reclaimed had been subjected to a variety of manipulations, including herbicide treatments. These were designed to potentially increase the rate of succession of old field back to native prairie. We were unable to identify which specific treatments had been applied to the areas we were sampling, and without this information, any data we obtained would be meaningless. Thus, we abandoned our efforts at this site.

U of MN Prairie

This 10 year old prairie reclamation is located along a northern portion of the St. Paul campus of the University of Minnesota. Samples were collected from two points within the system, once in mid-summer and once in the fall. This provided an opportunity to study seasonal fluctuation in spore numbers and colonization levels at a restoration site. Overall spore numbers at this site were higher than at the St. Croix Prairie restoration, especially during the fall. One of the sample points also showed significantly higher spore levels in the fall versus the summer collections, whereas the other point showed almost no seasonal difference.

Cedar Creek

The experimental plots established at Cedar Creek Natural History Area in Bethel, Minnesota, were monitored for mycorrhizal, soil, and plant parameters. Upland prairie and oak savanna plots were compared to transitional and wetland plots. Percent water at the site increased from upland to transition. NO_3 levels and pH were similar in all the plots and ranged from 0.5ppm - 0.7ppm and 3.9 - 4.2 respectively. The levels of soil phosphorus, ammonium, calcium and magnesium showed a similar trend of being greatest in the upland, lowest in the transition and intermediate in the wetland. The ranges for these soil nutrients are as follows: phosphorus, 23 ppm - 79 ppm; ammonium, 7.8 ppm - 20.7 ppm; calcium, 852 ppm - 3504 ppm; magnesium, 112 ppm - 270 ppm. The levels of soil

potasium and sodium were greatest in the transition zones, lowest in the upland and intermediate in the wetland (155 ppm - 353 ppm and 10 ppm - 25 ppm respectively) (Smith *et al.*, 1997c).

Percent colonization of roots by mycorrhizae was also compared between the plots. No arbuscules were found in any of the plots. Colonization by hyphae was greatest in the upland (mean = 49.8) intermediate in the transition (mean= 23.5) and lowest in the wetland (mean= 11.9). Percent colonization by vesicles was lowest in the upland (mean= .83) intermediate in the wetland (mean= 1.33) and highest in the transition (mean= 1.55). Mycorrhizal spore numbers were highest in the upland soil, intermediate in the transition soil and lowest in the wetland soil (Smith *et al.*, 1997c).

Country Club Wetland

This site is discussed with JES, its related experimental site, in the following section.

Budgeted spending to complete objective A.3 involved part of a graduate student stipend, salaries for undergraduate and technical help including a postdoc with expertise in identification of AMF (see letter to J. Velin, 12/4/95), and laboratory expenses.

A.4 Activity: Evaluation of the presence of VAM from existing research sites such as at (1) Lake Shetek, (2) plots established adjacent to Highway 65 near Cambridge, MN and (3) a land fill near St. Paul, MN. At some sites soil parameters including pH, bulk density, % water and nutrient analysis will be done. Plant composition will be determined at certain sites and the plant and soil results will be correlated with the VAM data.

Stenlund *et al.* (1994) recommended the continued monitoring of the existing Lake Shetek plots to assess the effectiveness over a longer period of the VAM re-introduced in 1991 at this Mn/DOT mitigation site. Soil cores and site evaluation will be done in 1995 and /or 1996.

Characterization and monitoring of certain soil and VAM/plant parameters of the newly created (JES site) and undisturbed wetlands near Cambridge, MN will be done. A comparison of future results to the results of the preliminary study done in the summer of 1994 and in the future will be valuable to assess the condition of the JES restoration site at progressive stages of succession.

Samples of soil collected from a St. Paul landfill in the fall/winter of 1993 will continue to be analyzed for VAMF, particularly to identify any VAMF tolerant to heavy metals. New samples will not be obtained from this site; so it is not listed under A.2.

A.4.a. Context within the project:

Because of the advantages that VAMF confer on many plants, the establishment of native grasses and forbs amended with VAMF along the highways is ecologically important. A previous Mn/DOT research project done in my lab dealt with characterization of VAMF from selected prairies in the twin cities metropolitan area and in southwestern Minnesota (Stenlund *et al.*, 1994). Some of the collected prairie soil samples were used to generate VAM inoculum in host plants (little blue stem, *Schizachyrium scoparium*, and sideoats grama, *Bouteloua curtipendula*) grown in the greenhouse. When the Mn/DOT wetland/prairie mitigation site at Lake Shetek in Murray County was seeded, the greenhouse produced VAM inoculum was added to the seeder and incorporated into two plots in an effort to provide biofertilizer for the new plant cover (Stenlund *et al.*, 1994). The spore inoculum cost approximately $0.10/1000 spores to produce. The material for actual spore production per pot varied between $12.50 to $50.00. The inoculum, which included spores and also root fragments, was incorporated into an area of 33 meters by 250 meters. In fall, 1994, we collected soil cores from the low and high density areas which we will be analyzing in 1995 for spore parameters as part of our 1994-95 Mn/DOT project (Charvat, 1994).

As suggested by Stenlund *et al.* (1994), we will continue to monitor the existing Lake Shetek plots over a longer period to assess the effectiveness of the VAM re-introduced in 1991. Robert Jacobson (Mn/DOT) plans to have this restored prairie burned in the spring/summer of 1995; so the evaluation of VAM parameters in the fall of 1994 (see Charvat, 1994) and again in 1995 and/or 1996 will permit us to determine the effectiveness of this initial effort at VAM re-introduction. The spore number for restoration would be the existing number of spores per gram of soil in an established prairie. This number has been shown to vary widely in Kansas and elsewhere, and we need more data from Minnesota prairies to make a reasonable estimate of what VAM spore values represent successful re-introduction. (This data will be obtained under Objective A.)

Vesicular arbuscular mycorrhizae provide numerous benefits to plants, essentially serving as biofertilizers. The contributions that VAM make to improving the soil conditions is less well know but important in promoting

plant growth and development (Miller and Jastrow, 1991; Quintero-Ramos, *et al.*, 1993). The advantages conferred by VAM to native grasses and other plants during germination and growth in the inhospitable environment found along the highway rights-of way make re-introduction of VAM a positive addition as part of restoration projects. This research project involves the isolation and characterization of VAM from prairies and wetland and the use of the VAM as inoculum to generate additional inoculum for native grass and forb establishment amended with VAM.

The areas adjacent to the new Highway 65, Cambridge were selected by Mn/DOT for such establishment. Hence, my lab started a preliminary study during the summer of 1994 at the newly created JES and the undisturbed wetland sites near Cambridge, MN described in A.2.a.. We have continued this research for the three years, first as part of the 1994-95 Mn/DOT project (see Charvat, 1994); then as part of the LCMR funded research (1995-97). All the data collected on soil, plant and VAM parameters will be processed to address the question of the level of active VAM activity at these sites.

A.4.b. Methods: The same methods described in part A.3.b. are applicable here. Soil cores collected along a transect at these sites has been analyzed for several parameters. The number and kind of spores/gram of dried soil as well as the percent colonization of mycorrhizal fungi inside the roots of the plant cover has been determined by means of either of these methods: Giovanetti and Mosse (1980) for pre-screening or McGonigle *et al.* (1990) for post-screening as time permits. The spore number and % colonization results will be compared to those obtained in 1991 from Lake Shetek and reported in Stenlund *et al.* (1994) as well as the data collected in 1994 from the Cambridge sites (Charvat, 1994).

The same tests were administered on the undisturbed and the newly created wetland at the JES site that were done in the summer, 1994 so that comparisons can be made. The soil parameters will be measured according to McKeague (1978).

The same types of statistical analyses that are described in part A.4.b. were used here.

New equipment: My lab is in dire need of an excellent compound microscope with a camera attachment (approximately $8,800). The scope and camera attachment are as important for recording results as the computer is for numerical data. The only microscope with a camera

attachment in my lab is over 25 years old. My lab does continuous microscope work, and the sophistication of our experimental design depends on access to a microscope with fluorescent, phase, dark-field and Nomarski optics and camera attachment. The microscope would be put in my lab and remain there at the end of two years for continued use in sophisticated light microscopic research on mycorrhizae.

A.4.c. Materials: The same materials that were used in part A.3.c were used here for spore isolation, percent colonization, and soil parameters.

A.4.d. Budget
Total Biennial LCMR Budget: $17,835
LCMR Balance: $0.00
MATCH: $
MATCH BALANCE: $

A.4.e. Timeline:

	7/95	1/96	6/96	1/97	6/97
PRODUCT #1	xxxxxxx	xxxxx	xxxxxxx	xxxx	
(Spore isolation data)					
PRODUCT #2	xxxxxxx	xxxxx	xxxxxxx	xxxx	
(% VAM colonization data)					
PRODUCT #3	xxxxxxxxxx		xxxxxxxxxxx		
(Other soil parameters-such as pH, bulk density)					
PRODUCT #4	xxxxxxxxxx		xxxxxxxxx		
(Soil nutrient analysis)					

A.4.f. Workprogram Summary

JES and Country Club

Surveys of the JES wetland complex in September and October of 1994, and June, July, and August of 1995 revealed a low volume of spores at this site, and little to no seasonal variation in numbers. This is consistent with work by other researchers that suggests disturbed sites have low levels of AMF (Abbott and Robson, 1991; Louis, 1990; Jasper, Abbott and Robson, 1989). In contrast, the nearby Country Club site, a relatively undisturbed wetland in the same drainage, contains very high numbers of spores. Here, spore numbers increased later in the season. Species diversity was high at both locations. The categories of spores were the same at both sites (4

colors, with large and small spores within each color category), with comparable relative abundance of the different types although absolute abundance was different.

Soil parameters differed between the two sites. JES had slightly more available phosporus but significantly less total P than Country Club. Nitrate levels at Country Club were lower than those at JES. Ammonia levels however, were higher at Country Club, and the percent carbon at Country Club was 20 times the level at JES. In addition, the percent organic matter at Country Club was 40 times that at JES. The pH values were approximately neutral at both sites, though Country Club was slightly more acidic. The bulk density of the two areas differed significantly, with JES soil nearly 8 times as dense as the soil from Country Club (Charvat *et al*, 1995).

JES Upland Prairie Reclamation

The effect that arbuscular mycorrhizal inoculum has on the development of an early successional tallgrass prairie reclamation was investigated in field plots at the JES site in Cambridge, Minnesota. Mycorrhizal inoculum reproduced from Crosstown prairie (see task B, side oats gramma inoculum) was placed below a mix of prairie seed and monitored over a two year period. Two sets of control plots were established, those with seed only and those with seed and a sterilized soil. By the end of the second year, plants in the inoculated plots had significantly greater percentage of roots colonized by AM fungi. Inoculation had no effect on total percent cover of plants. Percent cover of native prairie species, however, was significantly greater in the inoculated plots than in the two sets of controls. The percent cover of weedy species was unaffected by inoculation because inoculum was placed in the furrows and not broadcast over the entire plots, and also because many weedy species are non-mycorrhizal. The increase in percent cover of native grasses indicates that inoculating disturbed soils with AM fungi can significantly enhance the restoration success of prairies in Minnesota. Our findings suggest that the presence of a diverse, viable population of arbuscular mycorrhizae can be essential to the development of early successional tallgrass prairie communities (Smith *et al*., 1997a; Smith *et al*., 1997b).

In addition to studying the effects of inoculation on the percent cover of native and weedy species, some of the spores from the site have been isolated and identified. At least two genera are represented, *Scutellospora* and *Glomus*, including *G. mosseae* and *G. occultum*, two common species. In 1996, *G. occultum* was found only in the uninoculated plots, while *G.*

mosseae was present in the treated as well as the untreated locations. The *Scutellospora* species were also present throughout the site. Other *Glomus* species were present in the inoculated plots, but have yet to be identified to species.

Lake Shetek

We found little variation in percent colonization for the different treatment areas at the Lake Shetek prairie/wetland mitigation site in both 1994 and 1995 (Stenlund *et al*, 1994; Charvat *et al.*, 1995). Similarly, spore values showed little difference between different treatment areas. Interestingly, spore numbers declined between1991 and 1994. This may result from the area's history as a hay field, which would not have been treated with chemicals. Now that the Lake Shetek area has been reestablished as prairie, lower AM values would be expected, as indicated by other prairie studies (Hetrick and Bloom, 1983.) The lack of variation in the AM inoculum amended low and high density plots as compared to plots not treated, the buffer plots, and undisturbed transects may be due to high levels of soil phosphorus, which are known to inhibit the growth and reproduction of many mycorrhizal fungi. The increases in plant-fungi interactions at inoculated plots at JES are likely enhanced by the naturally low levels of available phosphorus at that site.

Shakopee

Spores isolated from site soil were scored based on size and color. Ten different spore types were distinguished (Charvat *et al*,1997a), and a comparison of the spore types associated with the biosolid vs. control areas in September, 1996 reveals a fairly high degree of similarity. There is slightly more variation when the two treatments from the October sample are compared. The biosolid treatment portions of the Shakopee site display a higher number of spores per gram of dry soil during the growing season and fall, although that difference is no longer statistically significant in the late October 1996 collection (Charvat *et al.*, 1997a), probably due in part to the destruction of the spores by nematodes and other organisms. The most frequently found spore types were the small hyaline (younger) in the biosolid area and the crusty and large brown (more mature) in the control area.

Soil levels of percent carbon, percent organic matter, available phosphorus, total phosphorus, metal levels, and pH values show little difference between the two treatment areas. Biosolid levels of ammonium, nitrate, calcium, and sodium were nearly twice as high as those from the untreated areas.

Based on these results, it appears that the biosolid application supplements nitrogen and calcium, nutrients that, when levels are low, limit plant growth (Charvat *et al.*, 1997b). In addition, the biosolid area may supply micornutrients, such as copper, to the plant via the mycorrhizal fungi.

U of MN Legume Fields

Spores were isolated from U of MN fields planted with native legumes, and initial data indicated that there were very few mycorrhizal spores present. In addition, there were so few roots at this site that accurate determination of percent mycorrhizal colonization was not possible. This site has a crop production history, and it is possible that the repeated soil disruptions and chemical treatments used on an agricultural field have removed nearly all of the AMF population and prevents their reestablishment.

St. Paul Pig's Eye Landfill Site

Spores were isolated from this site were used in AMF culturing experiments discussed in B.1 and B.2.

Budgeted spending to complete objective A.4 involved part of a graduate student stipend, salaries for undergraduate and technical help including a postdoc with expertise in identification of AMF, and laboratory expenses. Total microscope expenditure was less than the amount originally budgeted, as shortly after our proposal was funded we had the opportunity to hire Dr. Hamdy Agwa as a post-doctoral associate. He has provided invaluable assistance in identifying the mycorrhizal fungi species present at the sites we investigated in objectives A.3 and A.4. Dr. Agwa's hiring was approved by LCMR personnel (see letter to J. Velin, 12/4/95). As a result, we needed additional lab technician hours to complete the tasks agreed to in the LCMR contract. This ultimately was more expensive, thus less funding was available for a microscope purchase. The microscope body was purchased with LCMR funds, and the required optical parts were purchased on other funding sources.

It is important to recognize that the research funded by the LCMR is part of a larger project in our lab, aspects of which have been and continue to be funded by Mn/DOT. Thus, work on many of the tasks funded by LCMR continues to be an important part of our lab. Specifically, we have Mn/DOT funding through the year 2000 to continue work on objectives A and B. A high-quality microscope is an essential part of this continuing research.

B. **Title of Objective/Outcome:** Culturing of mycorrhizal fungi from species isolated in recent or past collections.

B.1 Activity: VAM inoculum production was set up using the spore samples collected from Minnesota prairies, wetlands, and other sites of interest because of likely tolerance or resistance of the VAMF to salt or heavy metals.

B.1.a. Context within the project: Three methods have been utilized to produce VAM fungi for commercial purposes. These methods are the growth of VAM inoculated: (1) plants on solid substrates, (2) plants in hydroponic culture (Millner and Kitt, 1992) and (3) root organ (tissue) cultures (Sullia, 1991). All inoculum generated in our earlier study (Stenlund *et al.*, 1994) was done by growing inoculated plants on solid substrates (soil/sand mixtures) and providing them with nutrient solution once/week (Hoagland and Snyder, 1933). This traditional method (1) has the advantage of being a proven one for generating spores of many different species. However, the number of spores produced per gram of dried soil is highly variable. Then too, several desirable species do not produce spores using this set up.

Mycorrhizal inoculum production is frequently expensive and time consuming because VAM must be cultured in living roots. Although Stenlund *et al.* (1994) successfully produced inoculum for VAM introduction at the Lake Shetek wetland mitigation site in Murray Country Minnesota, a period of three months was required to generate the VAM in the roots of the host plants in the greenhouse. Even then, the larger sized spores, such as *Glomus mosseae* and *Glomus gigantea*, showed a decrease in number which may have resulted from several factors including: the greater production of smaller sized spores over the larger ones under the culturing conditions used or the lack of the ability of the larger sized spores to mature under the culturing conditions employed. We need to improve the production process by decreasing the time involved and increasing the spore number and species produced.

B.1.b. Methods: The most appropriate samples collected as part of Objective A.1 will serve as inocula to generate more VAM for restoration projects. The native prairie host plants inoculated with VAM will be grown in a soil/sand mixture in the greenhouse and growth chambers in the fall and winter to generate inoculum for the spring and summer restoration plantings (see Stenlund *et al.*, 1994).

B.1.c. Materials: Growth chambers, pots, sand, soil, computer, and chemicals (such as those needed for making nutrient solution)

B.1.d. Budget
Total Biennial LCMR Budget: $7813
LCMR Balance: $0.00
MATCH: $
MATCH BALANCE: $

B.1.e. Timeline:

	7/95	1/96	6/96	1/97	6/97
PRODUCT #1 (List of plants inoculated with VAM and growing in the greenhouse or growth chambers.)	xxxxxxxxxxxx	xxxxxxx	xxxxxxxx	xxxxxxx	
PRODUCT #2 (% colonization of inoculated plants)	xxx	xxxxxxx	xx	xxxxxxxxx	
PRODUCT #3 (VAM spore number from inoculated plants)	xxx	xxxxxxx	xx	xxxxxxxxxx	

B.1.f. Workprogram Summary:

Crosstown Inoculum, with Big Bluestem as Host: Handwatered

Fresh soil collected in the fall of 1995 from the Crosstown Prairie was dried, sieved and used as a general inoculum without a prior cold treatment. A layer of inoculum soil was placed between moist layers of sterile 1:1 sand:soil mix in 30 labeled pots. Clumps of big bluestem were planted in each pot. Two types of controls were set-up: one had inoculum without plants (10 pots) and another had plants without inoculum (10 pots). Equal numbers of pots were placed in the growth chamber and the greenhouse. The pots were hand-watered with distilled water and weeded as needed. Light intensity was monitored and the pots were rotated weekly.

After 18 weeks, watering ceased and core samples were taken. Roots, extracted from each core sample, were stained with Chlorazol Black, according to Koske and Gemma (1989) and Brundrett *et al* (1983). Percentage colonization was determined according to McGongile *et al.* (1990). After drying in the pots, the shoots were removed, dried, and weighed, and the soil was placed in plastic bags and stored under refrigeration. Spores were isolated from each sample using a modified

sucrose density centrifugation method and separated by size and color (Tommerup and Kidby, 1979).

There was no significant difference in shoot biomass, percentage colonization, or spore production between plants grown in the growth chamber or the greenhouse. There was also no difference in shoot biomass between the inoculated plants and the controls, possibly because plants were grown during the winter, and because the seedlings were quite young when they were transplanted. Results from spore identification show that the spores produced in the greatest number in these experiments, the hyaline and yellow spores, are possibly *G. mosseae, B. etunicatum, G. leptotichum, G. occultum,* or immature spores of several other species. The data also show that the control pots that did not contain inoculum did not become contaminated with greenhouse spores. However, weeds, including foxtail (*Setaria glauca)*, germinated in the pots that contained inoculum soil and willow herb *(Epilobium ciliatum)* contaminated the pots in the greenhouse towards the end of the experiment. In B.2, these results are compared to those from the automatic watering treatments. Some of the inoculum produced in this trial was used later in the full Beltsville experiment, described in B.2.

Crosstown Inoculum With Side Oats Gramma As Host

In this experimental set-up, side-oats gramma grass (*Bouteloua curtipendula* (Michx.) Torr.) was planted into 20 pots for use as a host plant for soil inoculum from Crosstown Prairie. The inoculation procedure was the same as that given in the big bluestem treatments, above.

After growing for 16 weeks, the soil from the pots was bagged and placed in cold storage for nearly 1 year. Then, soil samples were removed from each bag and combined to make a composite first generation inoculum. This was then used to inoculate 40 pots of big bluestem and 10 controls. The resulting soil, spores, and colonized big bluestem roots (second generation inoculum) were applied to the Shakopee prairie restoration site; this inoculum is a new product not mentioned in our original LCMR agreement. The installation of inoculum into the field was funded under our current Mn/DOT contract (1997 - 2000).

The original soil from Crosstown Prairie has been processed for spore isolation and identification. At least 8 types of spores are represented at Crosstown, 4 colors and 2 sizes. Of these, small hyaline spores comprise nearly 50% of the population. Large hyaline, large yellow, and small brown spores are also fairly common. Spores were also present in a first generation inoculum produced with side-oats gramma, with an average of 20 spores per gram of dry soil and correspondingly moderate colonization

values. These numbers suggest that the second generation inoculum now applied at Shakopee should be a valuable source of VAM at the reclamation site.

Country Club Wetland Inoculum with Canada Blue Joint Grass as Host

Canada blue joint grass (*Calamogrostis canadensis* (Michx.) Beauv.) pots were treated in late 1995 with inoculum from the Country Club wetland site at Cambridge. These plants became infected with a fungus that may have originated from nonsterilized seeds. Hereafter, seeds of wetland plants will be sterilized prior to use in inoculum experiments.

Inocula Generated from Heavy Metal Sites

A number of experiments were performed in order to reproduce AM fungi from heavy metal enriched sites (Pig's Eye landfill, St. Paul, MN (Charvat *et al.*, 1995; Pawlowska *et al.*, 1995; Pawlowska and Charvat, 1997a); zinc mining site, Poland (Pawlowska *et al.*, 1996)) as well as from an uncontaminated prairie site (Feder Prairie, MN), which was used to obtain strains which could serve as a control in further experiments. In all experiments, a method of a pot "trap culture" was used. Soil from the site of interest was combined in a 1:1 proportion with sterile sand and used as inoculum for the host species (big bluestem, *Andropogon gerardii* Vitman, and crimson clover, *Trifolium incarnatum* L.). All cultures were incubated in a growth chamber and watered manually. Trap cultures provided a high yield of AMF spores, allowing an assessment of the AMF spore diversity at the site of interest. The spores produced thus were also used in further experiments.

Budgeted spendings to to complete the objective B.1 involved: a graduate student stipend, salaries for undergraduate and technical help, growth chamber fees, and laboratory supplies. Equipment required included a microscope for studying mycorrhizal structures. It is important to recognize that the research funded by LCMR is part of a larger project in our lab, aspects of which have been and continue to be funded by Mn/DOT. Thus, work on many of the tasks funded by LCMR continues to be an important part of our lab. Specifically, we have Mn/DOT funding through the year 2000 to continue work on objectives A and B. A high-quality microscope is an essential part of this continuing research.

B.2 Activity: Propagation of VAM fungi isolated from prairie and wetlands as well as special sites, such as those containing

heavy metals and appropriate control sites, via spore cultures associated with roots of host plants using principles of a Beltsville method (Millner and Kitt, 1992). The Beltsville method is a newer hydroponic procedure that uses a computerized drip nutrient feeding set-up and that also regulates the pH of the nutrient solution ensuring the production of acidophilic and basophilic VAM isolates.

B.2.a. Context within the project: Establishment of the efficient method of the production of VAM inoculum of known origin and defined characteristics is important for the production of fungal strains of desired features to be used in restoration practices, such as along highways and at former mining sites. The Beltsville method, using sand as a potting medium, greatly facilitates recovery of newly produced spores reducing significantly time of spore isolation (Millner and Kitt, 1992). As a soilless technique, it protects spores from excessive contamination typical for soil grown spores. This feature is very important for spores to be used further in *in vitro* cultures. Host plants are nourished by watering with a modified Haogland's solution allowing to maintain a stable pH level, which is essential for the development of some VAM fungi (Tommerup, 1994).

B.2.b. Methods: Spores isolated by wet sieving and decanting followed by sucrose centrifugation (Tommerup and Kidby, 1979) from the prairies, wetlands, heavy metal containing and appropriate control sites will be used as spore inoculum for the seedlings planted individually in pots containing sand watered with Hoagland's solution of a low phosphorus content promoting development of VAM. Various plants species, native prairie grasses and corn, *Zea mays*, will be tested to find the most efficient host plant to reproduce both, native prairie and heavy metal tolerant VAM fungi.

B.2.c. Materials: Spores of VAM fungi isolated from undisturbed prairie/wetland sites and heavy metal contaminated sites, seeds of host plants, pots, chemicals, growth chambers, sand, soil, and computer.

B.2.d. Budget
Total Biennial LCMR Budget: $7812
LCMR Balance: $0.00
MATCH: $
MATCH BALANCE: $

B.2.e. Timeline:

	7/95	1/96	6/96	1/97	6/97
PRODUCT #1	xxxxxxxxxxxx	xxxxxx	xxxxxxx	xxx	
(List of plants inoculated with VAM grown using the Beltsville method.)					
PRODUCT #2	xx	xxxxxxxxx	xxxxxxxx	xxxxx	
(% colonization of inoculated plants)					
PRODUCT #3		xxxxxxxxxx	xxxxxxx	xxxxxxx	
(VAM spore number from inoculated plants)					
PRODUCT #4		xxxxxxxxxx	xxxxxxx	xxxxxxx	
(Spores of VAM to be used in further experiments)					

B.2.f. Workprogram Summary:

In order to decrease the labor intensity of AMF inoculum production, we decided to apply an automated drip irrigation to provide water or nutrient solution to host plants grown in greenhouse pot cultures. The automated drip irrigation greatly facilitated water delivery to pots where the surface of the potting medium was covered with a protective cover. Possible inoculation of pot cultures by greenhouse indigenous AM fungi and cross-contamination between pots inoculated with dissimilar inoculum were our great concerns based on the past experience of culturing AM fungi (Charvat *et al.*, 1995). A series of experiments with different watering regimes and host species were performed allowing the adjustment of the culturing conditions to the specific environment of the U of MN CBS greenhouse. Since the greenhouse environment is greatly affected by the external weather conditions during the hot summer months, the frequency and volume of watering has to be very closely monitored.

The automated watering system significantly increased AM spore production compared to hand-watered pots grown in either the greenhouse or the growth chamber (Nichols *et al.*,1997). Further characterization of spore species is underway. The set-up is described in B.1.f. It should be pointed out that the automated drip irrigation system took a lot of time to establish due to changes in water flow and other problems created when additional pots were added to this system.

Crosstown Inoculum Grown in Soil:Sand Mix with Big Bluestem as Host: Automated Drip Irrigation System (Modified Beltsville)

The soil from Crosstown Prairie used to inoculate big bluestem plants in B.1 was also used here to inoculate 15 experimental pots and 5 controls. These pots were connected to a modified Beltsville automated drip

irrigation system, in the greenhouse. The plants were inoculated and cared for the same way as those discussed in B.1.

There was no significant difference in shoot biomass between the inoculated plants and the controls, possibly due to growing the plants during the winter, and to the young age of the seedlings when they were transplanted. Results from spore identification show that the spores produced in the greatest number in these experiments, the hyaline and yellow spores, are possibly *G. mosseae, G. etunicatum, G. leptotichum, G. occultum,* or immature spores of several other species. The data also show that the control pots that did not contain inoculum did not become contaminated with greenhouse spores. However, weeds, including foxtail *(Setaria glauca)*, germinated in the pots that contained inoculum soil and willow herb *(Epilobium ciliatum)* contaminated the pots in the greenhouse towards the end of the experiment. Some of the inoculum produced in this trial was used later in the full Beltsville experiment, described later in this section.

When these results are compared to the similar B1 experiment, the greenhouse plants had significantly greater shoot biomass than the plants grown in the growth chambers. A significant difference in shoot biomass was also apparent when comparing hand-watered plants to those grown under the automatic drip system, as the hand-watered plants averaged 1 gram of dry shoot mass, versus about 1.7 grams of dry shoot mass for plants maintained on the Beltsville system. Production of some spore types increased under the automatic watering system versus the hand-watered, and the overall trend seems to be for higher spore production under the Beltsville system. Finally, plants grown under automated watering had more root biomass than both the plants from the growth chamber and the hand-watered plants from the greenhouse.

Country Club Wetland Inoculum on the Automated Drip Irrigation System (Modified Beltsville)

Soil collected at the Cambridge Country Club wetland site in the fall of 1995 was processed in the same way as the Crosstown Prairie soil and used as a general inoculum. The inoculation procedure was the same as that discussed in B.1. Twenty experimental pots were used, with Canada blue joint grass (*Calamagrostis canadensis* (Michx.) Beauv. as host. The pots were connected to the automatic irrigation system in the greenhouse and cared for in the same way as discussed in B.1. After about 14 weeks a fungal parasite was found growing on some of the plants, so roots were harvested and preserved. Preliminary analysis indicates that colonization levels in the treated pots average about 28%, but vary widely. The controls were not colonized.

Crosstown Inoculum on the Automated Drip Irrigation System (Full Beltsville)

Inocula produced from Crosstown Prairie soil (see B.1 and B.2) was stored for one year, then used as a soil inoculum and source of spores for this experiment. Sixty pots were partially filled with silicon minispheres, then either had first generation Crosstown Prairie inoculum added to them or received a treatment of Crosstown Prairie spores suspended in water. The spore suspension is a new experimental method of inoculation. A measured amount of big bluestem *(Andropogon gerardii* Vitman) seeds were sown directly on the inoculum layer and then covered with sand. Plants were grown in the College of Biological Sciences Greenhouse for 16 weeks. Temperature and light data were recorded. Plants were watered with a nutrient solution (Hoagland and Snyder, 1933) using the Beltsville automatic watering system.

After 16 weeks, plants were harvested. The tops were used for biomass measurements, and roots were either preserved for percent mycorrhizal colonization studies or dried for biomass analysis.

In the data analysis, we found that the pots treated with the spore suspension had the highest shoot mass and pots with soil inoculum produced the least shoot mass. There was no statistically significant difference in root mass between the treatments. Additionally, both the plants treated with the spore suspension and the controls exhibited signs of phosphorus deficiency, while the plants grown on soil inoculum showed no such symptoms (Tallaksen *et al.*, 1997). At present, the simplest method of inoculum production seems to be to use soil as a growth medium. The inoculum produced as a result of this experiment is being tested at a Mn/DOT prairie reclamation project in Shakopee, Minnesota. Additional analysis of this work is currently being funded by Mn/DOT, and results will be publicly available in 3 years, when our Mn/DOT contract expires.

Feder Prairie and Pig's Eye Landfill Inoculum Grown in Soil:Sand Mix (Note: The single-spore inoculation portion of this experiment fulfills requirements for C.1, but is presented here for clarity as it also tests the Beltsville irrigation method.)

Clusters of big bluestem seedlings were inoculated with single spores of AM fungi isolated from the heavy metal contaminated Pig's Eye landfill site. The ability of AMF spores to germinate was tested according to Hepper (1979). The results indicated that the spores were viable and potentially able to inoculate host plants (Charvat *et al.*, 1995). To set up

the inoculation experiment, first garden soil from the CBS greehouse was steamed and sand was autoclaved, as prior experiments had indicated that special precautions should be taken to ensure that soil is sterilized and that spores are not transferred between pots or introduced from the environment (Charvat *et al.*, 1995). Inoculated big bluestem seedlings were planted in the sterile mix and watered automatically. To prevent both incidental inoculation with indigenous greenhouse AM spores and cross contamination between, pots were covered with aluminum foil. To monitor the establishment of AM colonization in big bluestem roots, soil cores were collected from each pot twice: one month and four months after planting. The recovered roots were stained to visualize fungal structures, and AM root colonization was assessed. Analysis of the big bluestem roots indicated a lack of AM structures in the investigated roots. Since the spores used in this experiment were known to be viable and able to germinate under conditions similar to the pot environment, the lack of AM colonization in analyzed roots may be attributed either to the separation of AMF spores from plant roots, or to a failure to establish a functional mycorrhizal association due to specific pot conditions such as the water logging experienced in early in the experiment, when testing the irrigation system. Nonetheless, the lack of mycorrhizal colonization demonstrated that (1) steam sterilization of soil and (2) covering pots with aluminum foil protects plants from cross-contamination between pots, and from contamination by indigenous greenhouse spores. This had been a serious concern in manually watered corn plants grown in soil-sand mix in the greenhouse (Charvat *et al.*, 1995).

The selection of a host species for the reproduction of AM fungi has proven to be an important factor that affects the number of AMF spores produced. In an experiment using original soil from the Feder prairie as inoculum, cultures with big bluestem, a native prairie grass, yielded significantly higher spore numbers than did cultures with corn. No such host effect was observed in the case of inoculum that had originated from the moderately metal contaminated Pig's Eye landfill site (Charvat *et al.*, 1995 and Pawlowska *et al.*, 1995; Charvat and Pawlowska, 1997a).

A sweet corn variety with a life cycle period shorter than usual has proven very useful as a host for AMF type-cultures, where plants were inoculated with a small number (10-20) spores of the same AMF spore morphotype, from either Feder prairie or the Pig's Eye landfill. It has shortened the AMF spore reproduction process from the traditional four to three months. Spores reproduced in spore type-cultures were used to establish *in vitro* dual cultures, as described in B.3.

Budgeted spendings to complete the objective B.2 involved: a graduate student stipend, salaries for undergraduate and technical help, cost of soil chemical analyses, and laboratory supplies including the partial cost of an automated drip irrigation system. Equipment required included a microscope for studying mycorrhizal structures (see B.1).

B.3 Activity: Establishment of *in vitro* dual (VAM fungus and plant roots) culture system for production of VAM inoculum and studies on the effects of selected heavy metal ions, such as those found at sites likely to undergo reclamation, on VAM.

B.3.a. Context within the project:
Successful attempts to establish *in vitro* dual cultures of plant roots and VAM fungus (e.g. Mosse and Hepper, 1975; Bécard and Fortin, 1988) created a tool for an easy visual assessment of the developmental stages of mycorrhiza including spore production. *In vitro* dual cultures provide also an excellent system for studying of the effects of heavy metals on development of VAM. The fungal species tolerant to heavy metal contamination can be further used as the inoculum for reclamation of sites containing higher concentrations of heavy metals, such as found along some highways and at former mining sites.

We will use agricultural species beginning with corn because its large roots are easy to inoculate with VAMF, and because corn has been used successfully as a host for many VAMF species. Once the production problems have been solved, other plants will be used as hosts.

B.3.b. Methods: Root cultures of monocotyledonous (e.g. corn, *Zea mays*) and dicotyledonous species (e.g. carrot, *Daucus* and tomato, *Lycopersicon*) will be used for the establishments of *in vitro* dual cultures with spores produced by the single spore pot culture method described in part B2.

B.3.c. Materials: Materials required include sterile Petri plates, chemicals, growth chambers, microscopes, and computer.

B.3.d. Budget
Total Biennial LCMR Budget: $7813
LCMR Balance: $0.00
MATCH: $

MATCH BALANCE: $

B.3.e. Timeline:

	7/95	1/96	6/96	1/97	6/97
PRODUCT #1 (Data on propagation of VAM fungi on monocot roots via in vitro dual cultures)	xxxxxxxxx	xxxxxxxx	xxxxxxx	xxxxx	
PRODUCT #2 (Data on propagation of VAM fungi on dicot roots via in vitro dual cultures)	xxxxxxxxx	xxxxxxxx	xxxxxxxx	xxxx	

B.3.f. **Workprogram Summary**

An *in vitro* dual (AM fungus and excised plant roots) culture system offers an exciting perspective for studying various aspects of AMF biology under microbiologically controlled conditions. It can also potentially be used for the production of axenic AM inoculum. In pursuit of the development of a suitable culturing system, a number of experiments were performed to (1) select suitable host roots, (2) develop an AMF spore decontamination procedure, and (3) select AMF species with spores that would germinate under *in vitro* conditions. None of the host root cultures developed and tested in our lab (corn, carrot, *Lotus corniculatus*, tomato) proved applicable for dual cultures. However, a DC1 clone of Ri T-DNA transformed carrot roots developed by G. Bécard and kindly provided by D. Douds resulted in successful establishment of *in vitro* cultures of *G. etunicatum* and *G. pansihalos* (Pawlowska *et al.*, 1997a; Pawlowska *et al.*, 1997b). *In vitro* cultures of both of these species are of great scientific and practical value. *G. etunicatum* from St. Paul sporulates easily under the devised culturing conditions and yields large numbers of viable axenic spores. The *G. pansihalos* used is from a heavy metal enriched mining site in Poland (Pawlowska *et al.*, 1996; Pawlowska *et al.*, 1997b). Due to its enhanced heavy metal tolerance, it may have great applications in the reclamation of heavy metal contaminated sites. *In vitro* cultures of both AMF species are maintained in our laboratory and will be used in further studies.

Budgeted spending to complete the objective B.3 involved: a graduate student stipend, salaries for undergraduate and technical help as well as laboratory supplies. Equipment required included a microscope for studying mycorrhizal structures (see B.1).

B.4 Activity: Assessment of the effects of selected heavy metal ions (Cd, Pb, Zn, Cu) on VAM development depending on the origin of VAM fungus (prairie/wetland vs. sites containing heavy metals) in *in vitro* dual cultures. Detection of lead localization in mycorrhizal and nonmycorrhizal roots will be attempted using a histochemical indicator (Garty and Theiss, 1990).

B.4.a. Context within the project: VAM has been shown to increase the uptake of micronutrients such as copper and zinc when present in soil at low concentrations (Lambert *et al.*, 1979). Furthermore, VAM may protect the host plant from excessive uptake of some heavy metals (Gildon & Tinker, 1983). On the other hand, VAM enhance absorption of metals even if they occur in excess, which may have a detrimental effect on the host plant (Killham & Firestone, 1983). Successful reclamation of heavy metal contaminated sites, such as along highways may depend on the introduction of VAM fungal strains able to play a protective role against metal stress. Exposure of *in vitro* dual cultures to increased concentrations of metal ions may provide indication of the role of the tested VAM fungal strains in metal uptake and distribution.

Employment of *in vitro* dual cultures for studying the effects of heavy metal ions on the development of mycorrhiza is environmentally safer in terms of their disposal after course of experiments than usage of the intact plants grown in artificially heavy metal contaminated potting media.

B.4.b. Methods: In vitro growing dual cultures of VAM fungi from prairies and heavy metal contaminated sites and plant roots will be exposed to increased concentration of Cd, Pb, Zn, Cu. Usage of histochemical stains to detect deposition of lead (Pb) within mycorrhizal and control nonmycorrhizal roots will be tested (Garty and Theiss, 1990).

B.4.c. Materials: Petri plates, chemicals, microscopes computer

B.4.d. Budget
Total Biennial LCMR Budget: $6562
LCMR Balance: $0.00
MATCH: $
MATCH BALANCE: $

B.4.e. Timeline:

	7/95	1/96	6/96	1/97	6/97
PRODUCT #1 (Data on the effect of heavy metals on the development of VAM)	xxxxxxxxxxxxx	xxxxxx	xxxxxxxxx		
PRODUCT #2 (Data on applicability of histochemical stains to detect deposition of heavy metals in VAM roots)	xxxxxxxxxxxxx	xxxxxx	xxxxxxxxx		

B.4.f. Workprogram Summary

Excised Ri T-DNA transformed carrot roots developed by G. Bécard, which have proven to be able to support *G. etunicatum* mycorrhizal colonization (see B.3.f), were only able to tolerate low concentrations of metal in the growth medium (10 µM Cd, 10 µM Cu, 100 µM Pb and 100 µM Zn). Therefore, only the effects of low concentrations of metal on AM development could be tested with this experimental system. Preliminary results indicated that although *G. etunicatum* spore germination rates under these conditions did not differ from a control treatment, AM colonization was delayed in roots grown on plates with 10 µM Cu, 100 µM Pb, and 100 µM Zn. This suggests that plants under metal stress may not be able to obtain the full nutritional benefit from AM colonization, due to the slow rate of establishment of AM colonization.

A histochemical indicator, sodium rhodizonate, was used to detect lead deposition in AM roots of leek (*Allium porrum* var. Primor) inoculated with a *G. etunicatum* and grown for several weeks in a soil-sand mix and watered with lead nitrate solution. The red staining of the lead-rhodizonate complex localized lead to plant cell walls in the root epidermis, cortex, endodermis and vascular cylinder, as well as to fungal structures including arbuscules and extramatrical hyphae. However, the rhodizonate indicator did not detect lead deposition in AM roots of big bluestem plants grown in lead contaminated soil from the Pig's Eye landfill (Charvat *et al.*, 1995 and Pawlowska *et al.*, 1995) and a mining site (Pawlowska *et al.*, 1996). Inability to detect lead may be explained by the low availability of lead to the AM roots, resulting in lead deposition below the detection limit of sodium rhodizonate. Our experience indicates therefore that application of sodium rhodizonate as a histochemical indicator for detection of lead deposition in plants collected from nature should be performed with caution, due to its low sensitivity.

Budgeted spending to complete the objective B.4 involved: a graduate student stipend, salaries for undergraduate and technical help as well as laboratory supplies. Equipment required included a microscope for studying mycorrhizal structures (see B.1).

C. **Title of Objective/Outcome:** Determination of spore viability from soil samples collected from Minnesota wetlands and prairies in 1991-96 and stored in dried or frozen soil.

C.1 Activity: Test for spore viability by inoculating a plant with a single or small group of spores stored dried or frozen; grow the plant for 4 weeks or longer and determine % colonization of the roots and/or isolate the spores from the pot.

C.1.a. Context within the project:
Although VAM spores may be produced by the plants, they may not remain viable when stored for reclamation projects. VAM inoculation of a host plant is a traditional, lengthy method for determining spore viability that we need to use to corroborate the more experimental methods to be tried in parts C.2 and C.3.

C.1.b. Methods: Inoculation of plants with VAM spores; growth of the plant in the greenhouse/growth chamber for 4 weeks or longer; determination of % VAM colonization (Giovanetti and Mosse, 1980) and/or spore isolation (Tommerup and Kidby, 1979). For statistical analyses, see A.3.b.

C.1.c. Materials: Materials required include growth chambers, microscopes, and computer plus all supplies to grow plants, determine % colonization and spore isolation (see part A.3.b.).

C.1.d. Budget
Total Biennial LCMR Budget: $8337
LCMR Balance: $0.00
MATCH: $
MATCH BALANCE: $

C.1.e. Timeline:

	7/95	1/96	6/96	1/97	6/97

PRODUCT #1 xxxxxxxxxx xxx xxxxxx xx
(List of plants inoculated with VAM)
PRODUCT #2 xxxxxxx xxxxxx xxxxxxxx xxxxxxxx
(Data from VAM % colonization)
PRODUCT #3 xxxxxx xxxxxxx xxxxxxx xxxxxxxx
(Data from spore isolation)

C.1.f. Workprogram Summary

Assessment of Spore Viability

An accurate assessment of spore viability is of primary importance, both for scientific experiments and for commercial spore production. The traditional method for evaluating viability requires placing a single spore on a host root and subsequently assessing the AM colonization of the root. This method is time consuming and often inaccurate under greenhouse growing conditions (see B1 and B2), as AM root colonization may result not only from the single spore inoculation but also from the accidental contamination of soil with spores from external sources. In cases of very low spore viability or where soil conditions are unfavorable to the establishment of AM symbiosis, this method is also very inefficient and costly. Therefore, Hepper's germination test, in which spores are germinated in the soil near actively growing host roots (Hepper and Smith, 1976) was selected as a simpler method to test spore viability.

Viability Tests of AMF Spores Using Single Spore Inoculum
Crosstown Prairie

Corn and purple prairie clover (*Dalea purpurea* Vent.) plants were inoculated with single spores, grown for 4 months, and then stored in the cold room for one year. Spore reproduction in this soil was successful, indicating that the original inoculum was viable. Many cultures had few spores, as expected, because spore production in such pots is often low. When high spore numbers were found, the spores were used for identification of the fungus and to generate additional cultures of the individual species.

A second experiment, using single spore inoculation with spores isolated from samples collected from Crosstown Prairie in 1994 and 1995 and placed on big bluestem, was successful. From this trial, 6 species of *Glomus* were identified. Of those 6, four have been identified to species, including *G. mosseae* and *G. etunicatum.*

Pig's Eye Landfill

The viability of stored spores from Pig's Eye landfill was tested using a single-spore inoculation on big bluestem, described fully in the automated watering experiments in B.2. Spore viability was simultaneously tested using the Hepper's viability assay (Charvat *et al.*, 1995). Soil cores were removed from each pot one month after planting, and again 4 months after planting. Roots isolated from these cores and evaluated for colonization showed no evidence of mycorrhizal activity. However, under conditions similar to the pot environment, Hepper viability tests had shown that the spores used in this experiment were viable and exhibited germination rates ranging from 2.2 to 41.1% depending on the species. The lack of AM colonization in roots may be attributed either to the separation of AM spores from the plant roots, or to a failure to establish a functional mycorrhizal association due to specific pot conditions, such as water-logging that occurred early in the experiment while testing the irrigation system (see B.2).

Assessment of Spore Viability Under Different Conditions Using "Hepper's Germination Method"

An experiment to assess the most suitable soil conditions for testing spore viability via "Hepper's germination test" indicated that these conditions do significantly affect spore germination rates, with responses varying among AMF species. Spores sandwiched between filters were placed in photographic slide frames (Hepper and Smith, 1976) and buried horizontally in a sterile soil-sand mix. Grass seedlings were planted above each germination unit. A flat containing the germination experiment was placed in a growth chamber under controlled conditions and watered as needed. After several weeks, the germination units were recovered from the soil, gently rinsed, and stained to visualize fungal hyphae (Koske and Gemma, 1989). After staining, the sandwiches were carefully opened and spore germination was scored using a dissecting microscope. When tested under various conditions (sand or soil/sand mix with or without grass seedlings), spores of *G. etunicatum* germinated best in soil/sand mix without grass seedlings whereas *G. macrocarpum* had the highest germination rate when incubated in sand with seedlings. These results indicate that several experiments may be needed when assessing inoculum viability since the testing environment may affect the results.

Effects of Storage Time

The effects of storage time on spore viability was also tested in *G. etunicatum.* Spores of this species were produced in greenhouse pot cultures in the summer of 1994 and they retained high levels of viability

(measured by their germination rate near 80%) over a period of 2.5 years. Further experiments on the same stock of inoculum will be performed over several years.

Budgeted spending to complete the objective C.1 involved: a graduate student stipend, salaries for undergraduate and technical help as well as laboratory supplies. Equipment required included a microscope for studying mycorrhizal structures (see B.1).

C.2 **Activity:** Selection of VAM fungi collected from native prairie, other natural areas and heavy metal contaminated sites for tolerance to heavy metal contamination using Hepper's spore germination test (Hepper 1979).

C.2.a. **Context within the project:** Germination of VAM fungal spores collected from sites not exposed to heavy metal contamination is strongly impaired by the excessive presence of metal ions as Mn^{2+}, Cu^{2+}, Zn^{2+} (Hepper and Smith, 1976), which may contribute to failure in the re-establishment of plant communities on heavy metal contaminated sites such as found along many highways. A simple test allowing to assess the ability of VAM spores to germinate under stress conditions was developed by Hepper (1979). Unlike the *in vitro* germination procedures, Hepper's method exposes spores to the conditions naturally occurring in soil evaluating hence spore utility as inoculum for reclamation procedures.

C.2.b. **Methods:** Stored spores or spores produced by the single spore pot culture method described in part B1 will be placed between two layers of Millipore membrane held together by a photographic slide binder and buried in the native nonsterile soil of various concentrations of heavy metals.

C.2.c. **Materials:** Millipore membrane, photographic slide binders, soil, chemicals, computer, microscope, growth chambers.

C.2.d. **Budget**
Total Biennial LCMR Budget: $8333
LCMR Balance: $0.00
MATCH: $
MATCH BALANCE: $

C.2.e. **Timeline:**

	7/95	1/96	6/96	1/97	6/97
PRODUCT #1	xxxxxxxxxx	xxxxxxx	xxxxxxxx		
(Data on germination of stored spores)					
PRODUCT #2	xxxxxxxxxx	xxxxxxx	xxxxxxxx		
(Data on spore germination under heavy metal stress)					

C.2.f. **Workprogram Summary**

Heavy Metal Effects on Spore Germination

In a series of experiments, six species of AMF fungi from various sites with different levels of heavy metal contamination were tested for metal tolerance using the spore germination assay. Spores were sandwiched separately between filters held together by photographic slide frames. These frames were buried in disposable Petri plates containing sand, and soaked with increasing concentrations of either cadmium nitrate, cadmium, copper, lead, or zinc nitrate. Spores treated with deionized water served as a control. Two species from two different environments germinated consistently, thus exhibiting different levels of metal tolerance (Pawlowska and Charvat, 1996). A St. Paul isolate of *G. etunicatum* was much less tolerant to metal exposure than the isolate of *G. macrocarpum* from the mining site (Pawlowska *et al.*, 1996). The highest metal concentrations under which *G. etunicatum* germinated were 1.0 $\mu g\ mL^{-1}$ Cd, 50 $\mu g\ mL^{-1}$ Pb and 10 $\mu g\ mL^{-1}$ Zn, whereas *G. macrocarpum* was able to tolerate 100 $\mu g\ mL^{-1}$ Cd, 20 $\mu g\ mL^{-1}$ Cu, 100 $\mu g\ mL^{-1}$ Pb and 2000 $\mu g\ mL^{-1}$ Zn. The implications of these different levels of metal tolerance for using AM fungi in the reclamation of heavy metal contaminated sites require further studies.

Budgeted spending to complete the objective C.2 involved: a graduate student stipend, salaries for undergraduate and technical help as well as laboratory supplies. Equipment required included a microscope for studying mycorrhizal structures (see B.1).

C.3 **Activity:** Development of a simple, reliable viability stain assay allowing for easy assessment of the vitality of the VAM inoculum to be used in restoration practices.

C.3.a. **Context within the project:** Currently used viability assays of VAM spores involve staining techniques often yielding ambiguous results (Meier and Charvat, 1993). Therefore, identifying a simple test would be of value to ecological and restoration projects. Candidates include tetrazolium, phloxine B, and other sensitive fluorescent stains.

The phloxine b stain incorporated into the growth media has been routinely used to assess viability of both bakery yeast, *Saccharomycees cerevisiae* (Cannon *et al*, 1986) and fission yeast, *Schizosaccharomyces pombe* (Fantes, 1981) staining the dead cells red. This stain, if it works, would permit fast identification of dead VAM spores.

C.3.b. **Methods:** Usage of phloxine b and other stains, such as sensitive fluorescent ones, as a viability marker in VAMF spores will be tested. The data will be statistically analyzed according to methods published in Meier and Charvat, 1993.

C.3.c. **Materials:** VAMF spores, chemicals, computer, microscope

C.3.d. **Budget**
Total Biennial LCMR Budget: $8330
LCMR Balance: $0.00
MATCH: $
MATCH BALANCE: $

C.3.e. **Timeline:**

	7/95	1/96	6/96	1/97	6/97
PRODUCT #1	xxxxxxxxxxx				
(Evaluation of the phloxine b stain as a viability stain for VAMF)					
PRODUCT #2			xxxxxx	xxxxxxx	xxxxxxxxx
(Evaluation of other stains)					

C.3.f. **Workprogram Summary**

Several experiments were performed to test the applicability of various viability tests (phloxine b, FDA, CFDA, and MTT) for the assessment of spore viability in AM fungi.

In these germination and viability assays, spores of *Glomus etunicatum* produced during single spore inoculum trials with spores from the St. Paul site and stored for about 1 year at room temperature were used. Spores were either left intact (nonkilled), or killed by autoclaving or soaking in ethanol for 24 hours, for use as control. The ability of the spores to germinate was assessed as described earlier (C.1) via spore germination assays. The stains phloxine B, fluoresceine diacetate (FDA) and carboxyfluorescein diacetate (CFDA) were tested for their suitability to determine spore viability. Tetrazolium bromide (MTT) was used as a control stain.

Phloxine B

Nonkilled and killed spores of *G. etunicatum* were incubated in phloxine B at several concentrations for 24, 48 and 72 hours. Then, the spores were examined for stain accumulation. Although the percentage of stained spores increased with higher stain concentrations, there was no statistically significant difference between nonkilled and killed spores. Greater stain accumulation in nonkilled versus killed spores does not correspond to patterns observed in yeast cells, where live cells do not stain and stain accumulates instead in dead cells. This, together with lack of consistent patterns in phloxine B accumulation by the AMF spores, indicates that this stain should not be recommended as a viability stain for distinguishing between live and dead AMF spores.

FDA and CFDA

Nonkilled and killed spores of *G. etunicatum* were incubated in FDA or CFDA for 24 hours (Stewart and Deacon, 1995). Spore subsamples were collected at 6 hour intervals and examined for green fluorescence using an inverted fluorescent microscope. At the tested incubation times, no difference in fluorescence was observed between nonkilled and killed spores. FDA does not seem to be suitable for spore staining because its fluorescence fades very rapidly, making spore counts difficult. Although the CFDA persisted long enough to count flourescing spores, the stain did not distinguish between killed and nonkilled spores.

MTT

Nonkilled and killed spores of *G. etunicatum* were incubated in MTT (Meier and Charvat, 1993) for several hours in tightly sealed ELISA plates. Staining was monitored every 24 hours using a dissecting microscope. After 76 hours of incubation, almost all of the living spores stained blue or purple, and most of the control killed spores remained unstained.

Only MTT has proven to be a reliable indicator when used for the assessment of viability in *G. etunicatum* spores reproduced in pot cultures. The numbers of spores staining as viable when using MTT corresponded well with the results of spore germination assays.

Budgeted spending to complete the objective C.3 involved: a graduate student stipend, salaries for undergraduate and technical help as well as laboratory supplies. Equipment required included a microscope for studying mycorrhizal structures (see B.1).

VI. Evaluation: The achievement of a high reproduction rate of VAM fungi in pot/tissue cultures is one of the most essential steps in a successful restoration process. Both newly generated spores and VAM colonized host roots may be used as an inoculum for reclamation practices. Choice of the proper culture parameters such as a host plant species, concentration of nutrients, and watering, to promote extensive root colonization and/or spore formation is therefore of great importance. We want to improve VAMF culturing methods so that large amounts of inocula can be produced commercially.

The development of a technique allowing for the establishment of easily subculturing vigorous root cultures, which can be inoculated with VAM spores (from prairie and heavy metal contaminted sites), develop VAM colonization and produce spores will indicate applicability of this technique for restoration practices.

The assessment of effects of metal ions on VAM in dual cultures will provide a confirmation that organ culture of VAM fungi may be used as a tool for studying of these phenomena without employing intact plants, which is a more environment threatening type of experiment. Detection of lead distribution in VAM roots will confirm the applicability of histochemical indicator in mycorrhizal systems.

Application of Hepper's germination test will allow us to select VAMF strains tolerant to enhanced concentrations of metal ions, which could be used in reclamation practices of heavy metal contaminated sites.

The final part of the project will assess the applicability of phloxine b or other stains as viability tests for VAM spores. If successful, the stain will be of a great importance for improvement of quality of mycorrhizal

inoculum, which could be easily tested for its viability and used at restoration sites.

VII. Context within field: In restoration projects certain characteristics of a site can be analyzed to assess the condition of an ecosystem. Some of these characteristics include the stage of succession, nutrient composition, plant composition and degree of presence of symbiotic fungi (VAMF) in the soil.

One goal of this project is to identify the best sources of indigenous field VAM inoculum for producing inocula for use at restoration sites. The VAM samples must be collected to serve as the original inoculum for generating inoculum for re-introduction at restoration sites lacking VAM propagules. Part of the VAM collected will be use to inoculate host plants to produce more VAM. If additional VAM inoculum is needed after pre-screening the initial collections for VAM, more collections will be made at natural areas near the selected restoration sites or at sites having VAM species of value to the restoration project.

All three methods for production of VAM inoculum have advantages and disadvantages; however, the use of this combination of methods should increase the number of spores/species and the number of VAM species available for Minnesota restoration projects. At future restoration sites, we want to re-introduce VA mycorrhizae, from a nearby location, that have the ability to form functional mycorrhizae with the native species.

The second goal is to increase the limited baseline information available about the biodiversity of VAM in Minnesota prairies and wetlands (Pfleger and Stewart, 1989; Johnson, 1991; Stenlund and Charvat, 1994). The changes that occur in these prairies, wetlands and other nature areas over both short and long periods need to be determined. The variation in the successional patterns in VAM communities is the type of information needed to successfully monitor the VAM populations at restoration sites from beginning to completion of the projects.

Because of the advantages that VAMF confer on many plants, the establishment of native grasses and forbs amended with VAMF along the highways is ecologically important. A previous Mn/DOT research project done in my laboratory dealt with characterization of VAMF from selected prairies in the twin cities metropolitan area and in southwestern Minnesota (Stenlund *et al.*, 1994). Mn/DOT has an on going interest in the

use of VAM at restoration sites, and they has awarded a contract of $25,000 to my lab for the 1994-95 academic year. This contract is for funding for students to continue a part of the VAM biodiversity study and to participate in the establishment along the highways of native grasses and forbs amended with VAM. A new graduate student, Michael Smith, will be funded for the academic year and the other students will be funded for the fall quarter (refer to part b.7.a).

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VIII. **Budget context:**

a) Funding from June, 1994 to June, 1995

Funding source	Period of funding	Amount	Primary use
1. NSF Aquatic Undergrad Program	6/94-8/94	$2,500	Undergrad internship for summer
2. MN/DOT	9/94-6/95	$25,000	Grad Res Assistant for academic year

b) University of Minnesota Cost Sharing during the academic years, 1994-1996.

Iris Charvat, Ph.D, will provide the necessary effort to train the undergraduate and graduate students who are participating in this research project or will do the research herself. I. Charvat's salary for the 1994-95 academic year is about $52,000.

IX. Dissemination: The results obtained will be presented at symposia sponored by one or more of the following groups: Mn/DOT, Minnesota Academy of Sciences, national scientific meetings (most likely American Institute of Biological Sciences, Mycological Society of America and/or Botanical Society of America) as well as at seminars and poster sections at the University of Minnesota. The published abstracts of the results in national journals or newsletters will be available to everyone. Ultimately, scientific papers will be submitted to national and international journal that will give everyone access to the results.

Fungal pot cultures and the spore repository will be stored at the University of Minnesota, St. Paul, MN, at the Biological Sciences Center, R. 759, 762 and 765, rooms assigned to I. Charvat, or in the cold room (R. 788). The data will be shared with Mn/DOT and other interested agencies.

X. Time: For the objectives outlined, we will be able to complete the project in two years. However, a limited number of Minnesota prairies and wetland sites will be sampled for VAMF under objective A, and we will be looking for additional funding to continue this survey work, which is similar to the County Survey for Plants funded by LCMR. The completion

of the VAM culturing research outlined in Objective B will provide data on better methods for producing VAM inoculum in small amounts; however, additional research will be required for the production of large quantities.

XI. Cooperation: I. Charvat's, Program Manager, effort will be directed towards training undergraduate and graduate students who are participating in objectives A, B and C or doing part of that research, particularly objectives A.1., A. 2 and C. The percent of effort of the Program Manager will vary depending on the needs of the students for training and help.

The role of Mn/DOT is described here. Cooperation for objectives A and B. will be obtained from Mr. Robert Jacobson (Mn/DOT Department of Environmental Services), the technical liaison for the Mn/DOT funded 1994-95 project to I. Charvat. Mr. Jacobson will spent 15 % of his effort on objective A. identifying sites of interest to Mn/DOT for VAM fungal inoculum characterization, providing management services at our field sites and equipment such as bright orange vests to be worn at our sites along the highways. For objective B, he will identify and provide seeds of native grasses and forbs to serve as VAM host plants, about a 5% effort.

Although another cooperator was listed on the original LCMR proposal to provide field samples, Mr. Wayne Feder is not listed here because the extra funding provided by Mn/DOT for 1994-95 permitted us to check those samples this year.

XII. Reporting Requirements: Semiannual six-month workprogram update reports will be submitted not later than January 1, 1996, July 1, 1996, January 1, 1997, and a final six-month workprogram update and final report by June 30, 1997.

XIII. REQUIRED ATTACHMENT:

1. Qualifications:

Iris D. Charvat, Ph.D.

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CURRICULUM VITAE

EDUCATION

B.S., 1963, Biology, University of Illinois, Urbana
M.S., 1964, Botany, University of Illinois, Urbana
Ph.D., 1973, Biology, University of California, Santa Barbara

ACADEMIC APPOINTMENTS

1967-1968, Teaching Assistant, University of California, Santa Barbara

1972-1973, Research Position, University of California, Santa Barbara

1973-1979, Assistant Professor, Department of Botany, University of Minnesota

1979-present, Associate Professor, Department of Plant Biology, University of Minnesota

RESEARCH INTERESTS

•Rhizosphere microorganisms, especially arbuscular mycorrhizal fungi, in native and naturalized plants in Minnesota prairies and wetlands.

•Mycorrhizal associations in prairie and wetland plants: physiological/ecological studies

•Seed bank and seed germination studies on wetland species including *Lythrum salicaria*.

•Regulation of phosphatases in *Schizophyllum commune*, a wood rotting fungus.

•Localizations of hydrolases to mark the lysosomal compartments in fungi and mycorrhizal associations.

PROFESSIONAL SOCIETIES

•American Society for Cell Biology
•American Society of Plant Physiologists
•Botanical Society of America
•Minnesota Native Plant Society
•Mycological Society of America
•Sigma Xi

RECENT GRANTS AND FUNDING OBTAINED FOR RESEARCH AND RELATED ACTIVITIES

1994-1995 Native Grass and Forb Establishment Amended with VAM. Minnesota Department of Transportation-Research Project Contract. Ends June, 1995. $25,000. PI Iris Charvat.

1994-1996 Refinement of Dazomet Application in Forest Nurseries. United States Department of Agriculture. Ends December 31, 1996. $142,000. PIs Jennifer Juzwik and Iris Charvat.

1994 University of Minnesota Undergraduate Research Program. Summer Internship for Judi Tamasi. Funded by National Science Foundation and DuPont Corporation. Ten weeks Summer Program. $2,500.

1994. University of Minnesota, Undergraduate Research Opportunities Program. (UROP), Quantification of VAM Colonization: A comparison of two procedures. For Jennifer White to work in I. Charvat's Lab. $1005.00

1991-1994 College of Biological Sciences High School Summer Science Research Program. NSF/ Howard Hughes Program. $15,000 (8 scholars).

1989-1991 The Control and Eradication of *Lythrum salicaria*: Seed Germination, Microflora of the Seed Bank and Seedling Growth. Minnesota Department of Natural Resources. $42,500.

PRESENTATIONS: Selected

Charvat, I. 1994. Phosphorus utilization curve and mycorrhizal condition of *Lythrum salicaria*. Fifth International Mycological Congress.

Charvat, I. 1994. Mycorrhizae in Wetland Plants. Symposium sponsored by the Minnesota Native Plant Society. March, 1994

Charvat, I. 1993. Mycorrhizae: Role in Different Ecosystems. Biology Colloquium, University of Minnesota.

areas on roadsides. Results will be applicable for restoring Parks and Wildlife Management Areas, etc.

Inter-agency Roadside Committee (IRC) Co-chair. The IRC is composed of representatives from Mn/DOT, MnDNR, and MDA. The group seeks to coordinate activities, projects etc., between the state agencies at the field and technical level. Our objective is to reduce herbicide and pesticide usage in land management practices, especially roadsides, by using ecological practices and biological controls. The IRC sponsors an annual symposium on Integrated Vegetation Management.

Restoration: Mn/DOT restores approxiamtely 1,500 acres to native vegetation annually under the supervision of the Turf Establishment & Erosion Prevention Unit.

SEMINARS/WORKSHOPS GIVEN IN 1994

Subject: Seeding Nature Species on Highway Construction Projects. 1) SCS Workshop for Metro Area Planners, April 1994; 2) Center for Transportation Studies Conference, May 1994; 3) Association of American Road Builders Conference, September 1994; 4) Pheasants Forever Annual Meeeting, September 1994.

Subject: Obtaining ISTEA Enhancement Funding & Project Ideas. The Nature Conservancy's International Skills Workshop in Washington, D.C., April 1994.

PROFESSIONAL ASSOCIATIONS:

Minnesota Native Plant Society, Minnesota Erosion Control Association, The Nature Conservancy