LEGISLATIVE COMMISSION ON MINNESOTA RESOURCES
100 CONSTITUTION AVENUE/ROOM 65/SAINT PAUL, MINNESOTA 55155-1201
PHONE: 651/296-2406 TDD: 651/296-8896 OR 1-800-657-3550 RELAY: 651/297-5350 OR 1-800-627-3529
FAX: 651/296-1321 EMAIL: lcmr@commissions.leg.state.mn.us

John Velin, Director

February 10, 2000

Mr. Eli O. Hunt, Chairman
Leech Lake Band of Ojibwe
6530 Highway 2 NW
Cass Lake, MN 56633

WITHDRAWN

Dear Mr. Hunt:

RE: ML 99, Ch. 231, Sec. 16, Subd 7(I) - Preservation of Native Wild Rice

After my recent letter to you, we were told by the Finance department that they made accounting errors in both the expendable account for the Trust Fund and the Future Resources Fund. The errors produced negative balances for both.

Earlier I suggested that you come in with a proposal for spending slightly different from the past approved project. That suggestion is withdrawn because it is no longer operable. There is no money to recommend this session. Thus the project remains cancelled with no prospect for reviving this session.

I am sorry to have raised your expectations only to then deflate them. This is the reality. Best wishes with whatever part of the project you may be able to accomplish on your own.

Sincerely

John Velin

Director

CC: Senator Len Price, LCMR Chair
Senator Tony Kinkel

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John Velin, Director

February 8, 2000

Mr. Eli O. Hunt, Chairman
Leech Lake Band of Ojibwe
6530 Highway 2 NW
Cass Lake, MN 56633

Dear Mr. Hunt:

RE: ML 99, Ch. 231, Sec. 16, Subd 7(I) - Preservation of Native Wild Rice

Senator Tony Kinkel asked me on several occasions to help him work out alternative solutions to the dilemma you face, namely the cancellation of the 1999 appropriation and the issues of the amount of match and downsizing of the project. As a former LCMR member he understands the legislative process well and works tirelessly for what he feels is right. He also has the good judgement to recognize when he should seek different alternatives.

When I explained to him that it is the state law that required the match amount and the state law that dictates the project cancels unless the match conditions are met, he realized clearly that the 1999 project is over. In my letter to him of Jan 28, 2000, I explained several additional items. I enclose that letter for your reference. We cannot change the law.

How could the project be revitalized? There were two options. First, seek a new project through response to the 2001 RFP we issued last November or seek a recommendation from the LCMR for the 2000 legislative session. The deadline for the RFP response has passed, so that option is gone. The second option is still viable. We did schedule review of this project cancellation for the January 19, 2000 LCMR meeting, but time ran out before we reached this and several other items. We could try to reschedule consideration for LCMR consideration, but that requires a meeting of the LCMR, which is difficult to arrange.

If you wish to pursue a recommendation from the LCMR for action in the 2000 session, I request you submit a letter of request immediately. I will work with you to help develop such a request, but I emphasize the urgency of timing. The session will be short and the schedule quite full.

I must also point out an additional possible barrier. The Department of Finance advised us that the revenues supporting LCMR 1999 recommendations apparently have fallen short. Depending upon the size of the shortage, the option described may not be feasible and we might have to cancel other 1999 projects in order to keep appropriations within revenue projections. I won't know that situation clearly until later this week. The most prudent action would be to develop a letter requesting the changes you seek and then consult with me on the revenue situation.

Cordially,

John Velin

Director

CC: Senator Len Price, LCMR Chair

Senator Tony Kinkel

enclosure

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Date of Report: September 30, 1998 (Revised March 1999)

Date of next status report: January 31, 2000; January 31, 2001; Final report June 30, 2001

Date of Work program Approval:

Project Completion Date: June 30, 2001

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MLK
INTERN

LCMR Work Program 1999

PROJECT TITLE: Preservation of Native Wild Rice Resources

Project Manager: Shirley Nordrum
Affiliation: Leech Lake Reservation
Mailing Address: 6530 Hwy. 2 NW, Cass Lake, MN 56633
Telephone Number: (218) 335-7400 E-Mail: lldrm@mail.paulbunyan.net
fax:(218)335-7430
Web page Address: n/a

Total Biennial Project Budget:

$LCMR	$200,000.00	$Match	$45,000.00
-$LCMR Amount Spent	0	-$Match Amount Spent	0
=$LCMR Balance:	$200,000.00	=$Match Balance:	$45,000.00

A. Legal Citation: ML 1999, Chap. 231, Sec. 16, Subd. 7(i.)
$200,000 is from

APPROPRIATION LANGUAGE: ~~This appropriation is from~~ the future resources fund to the Commissioner of Natural Resource for an agreement with the Leech Lake Reservation to analyze critical factors in different northern wild rice habitats and determine methods to preserve the natural diversity of wild rice. This appropriation must be matched by at least $45,000 of non-state money.

B. **Status of Match Requirement**: Leech Lake Band has committed a $45,000.00 cash match.

II. **Project Summary and Results:**

The project entitled "Preservation of Native Wild Rice Resources" is an orderly, all-encompassing approach to develop and establishing a comprehensive conservation program to protect and preserve Minnesota's natural wild rice resources. The proposal is divided into three projects what we feel are essential for an integrated approach:

(1) **Establish baseline information on biotic and abiotic factors influencing the three study populations of Z. palustris;**

(2) **Determine genetic variability within and among three Z. palustris populations;**

(3) **Refine protocols for long-term storage of grains of Z. palustris in ex situ genebanks.**

The objectives associated with these goals will be administrated by the Leech Lake Band, the Bois Forte Band, The Minnesota Chippewa Tribe, Bemidji State University and The U.S. Department of Agriculture National Seed Storage Facility as well as consultation with Wild Rice Managers from the six member Minnesota Chippewa Tribe, Red Lake Nation and The 1854 Authority.

7(i)

III. **Progress Summary**: No work performed to date.

IV. **Outline of Project Results**

Result 1: Comprehensive Analysis of Three Distinct Wild Rice Ecosystems

An effective conservation plan for Z. *palustris* cannot be designed without some knowledge of the biotic diversity (biodiversity) of the ecosystem. Therefore Leech Lake in cooperation with Bois Forte proposes to undertake a comprehensive analysis of three distinct Z. *palustris* ecosystems. Proposed study sites are U.S. Geological Survey Hydrologic Unit Code: 09030005 (76) the Little Fork River Watershed located on the Bois Forte Reservation and 9030006 (77) Big Fork River Watershed and 7010102 (8) the Leech Lake River Watershed located on Leech Lake Reservation. It is generally accepted that the most influential factors affecting a lake's ability to produce Z. *palustris* are the depth of water, the amount of plant competition, and the level of nutrients available in the sediment. Through this study we will assess landscape based activities; plant and animal assemblages (associations and communities); species; populations; and genes are all potential players for the success or failure of a species. We propose to accomplish comprehensive ecosystem evaluations for the three study areas by collecting data on environmental characteristics such as air temperature, water clarity (secchi disk) water levels (staff gauges will be placed in the beds), water temperature, water pH, dissolved oxygen, conductivity, in flow and out flow volumes of all tributaries, periodic sediment temperature, sediment pH, sediment densities, zooplankton, phytoplankton and benthic invertebrate population indexes. Plant assemblage information such as, number of plants per meter square, number and species of competitive plants as well as detailed morphological characteristics of Z. *palustris* from each specific basin will be analyzed. Water and sediment chemistry will be undertaken to establish a nutrient budget for each ecosystem. Additionally, a common garden experiment will be done to determine if phenotypic differences that are noted in the three wild populations of Z. *palustris* can be replicated in the greenhouse. Replication differences would be a strong argument for genetic differences. Phenotypic similarities would support a position that environmental factors are at play.

Completion date: June 30, 2001

LCMR Budget:	$ 77,545.00	Match	$ 34,066.00
Balance:	$ 77,545.00	Match Balance	$ 34,066.00

Result 2. Assess Genetic Diversity

Leech Lake will be consulting with Bemidji State University Staff and well and The U. S. Department of Agriculture National Seed Storage Facility staff to determine if phenotypically distinct populations of Z. *palustris* can be identified on the basis of genotypic difference. We expect this work would allow us to identify DNA polymorphism in the three populations of Z. *palustris* being studied. This information, in turn may be of value in managing wild Z. *palustris* stands by allowing restoration of appropriate strains of wild rice, based on genotyping and correlation of phenotype with the environment in which the particular strain is most suited.

Completion date: June 2001

LCMR Budget:	$103,919.00	Match	$13,200.00
Balance:	$103,919.00	Match Balance	$13,200.00

Result 3. Seed Storage

The objective of this result is to provide protocols for storage of seeds from wild populations of Z. *palustris* that are cost-effective and maintain highly vigorous seeds with minimum preserve for several decades. To achieve these objectives, we must determine the interaction between water content, temperature and survival of seeds harvested from these three study populations, optimize drying protocols, and evaluate longevity at different storage temperatures. Cryopreservation protocols were previously developed using Z. *palustris* accessions harvested from cultivated varieties. The proposed research will use similar biophysical and physiological approaches to test efficiency of these protocols in terms of survival (confirmation data) and longevity (innovation) on wild populations. In addition, we will determine whether drying time and moisture levels can be manipulated to increase the levels of desiccation tolerance in immature embryos (innovation). This result will be used to enhance the cryostorability of accessions of Z. *palustris*.

From the results of the project we expect to provide generalized protocols for conservation of all wild populations of *Z. palustris* as well as provided a framework for the cryopreservation of recalcitrant seeds in general. It is our goal that preservation techniques are cost-effective, reliable, simple and applicable to a wide range of *Z. palustris* populations. This work will be useful to conservationists as well as breeders.

Completion date: June 2001

LCMR Budget:	$18,536.00	Match	$.00
Balance:	$18,536.00	Match Balance	$.00

V. **Dissemination:**

Results from the seed storage will be published in peer review journals, this will be the first program to preserve recalcitrant seeds and information will be useful for other endangered aquatic species. Ecosystem analysis information will be disseminated to other tribal, federal, state and local natural resource management agencies. All primer and microsatellite sequences will be freely available to be used in assessments of other Z. *palustris* populations and to monitor changes or infusions of wild populations with "foreign" pollen sources.

VI. **Context:**

A. Significance: The goal of Tribal people has been to restore, enhance and protect wild rice and its habitat in Minnesota. Wars have been fought and initiatives undertaken to protect wild rice throughout the history of Minnesota. Within its core range in Minnesota, wild rice is the single most important native grain for the Ojibwe people; it has spiritual, cultural and economic importance. The plant and its seeds are also heavily consumed by waterfowl; it provides habitat for breeding and brood-rearing waterfowl and other species. The significance of this portion of the combined project is that this research proposes to establish enough physical, biological and chemical information to assure wild rice managers have the information necessary to assure the promulgation of natural wild rice production in all ecologically fit habitats within the state of Minnesota.

Objectives of the project are direct: Habitats for *Zizania spp* are being destroyed at an alarming rate in the United States and there is concern that the genetic diversity of this important genus will be irretrievably lost. Continued loss of habitat and genetic manipulation research could lead to genetic shifts and eventual loss of genetic diversity unless wild populations are protected in a comprehensive conservation program. We are proposing to establish that conservation program. By integrating in situ and ex situ strategies, we will attempt to protect the biological diversity of three stands of *Z. palustris* located on the Leech Lake Reservation and the Bois Forte Reservation in northern Minnesota. These strains remain relatively undisturbed by human intervention except for traditional harvesting by Tribal members and those non-Indians living within the boundaries of these Reservations. There are a number of challenges to establishing a conservation program and the knowledge we gain from this work will allow us to propose conservation efforts to protect these and other populations of Zizania and aquatic species in general. This project will also provide those natural resource managers charged with the conservation Minnesota's native species, and wild life habitat by supplying habitat information that gives insight into the influencing

components of a healthy natural Z. *palustris* ecosystem and the preservation of genetic diversity important to provide genes for insect and disease resistance, tolerance to abiotic factors.

B. Time: This work plan will be accomplished in two (2) years.

C. Budget Context: No related budget history for this project.

1.**Budget**

Personnel:	$56,797 (1 Wild Rice Technician) $56,797 (1 Research Technician), $15,360.00 (Plant Researcher) $900 (a student researcher) $26,122.00 (1 Assistant Researcher)
Equipment:	0
Supplies:	$14,000.00 pippetors, electrophoresis boxes, general laboratory supplies
Contractual:	$7,748.00 (1 Research Consultant), $20,000.00 analytical work on water and sediments
Acquisition:	$0
Development	$0
Other	$0
Total	$200,000.00

There are considerable in-kind contributions from the National Seed Storage Facility, The Minnesota Chippewa Tribe, and The Leech Lake Band.

2. Budget details submitted as attachment "A"

VII. **Cooperation:** This project will require the cooperation of John Perssel of The Minnesota Chippewa Tribe Water Quality Laboratory, Chris Holm of the Boise Forte Band of Chippewa, Dr. Patrick Guilfoil of Bemidji State University, and Dr. Christina Walters of The U.S. Department of Agriculture Seed Storage Facility. Consultation will take place with the wild rice managers for the six member Minnesota Chippewa Tribe, The Red Lake Nation and The 1854 Authority.

VIII. **Location:** Leech Lake Reservation, Itasca and Cass Counties and Boise Forte Reservation St. Louis and Koochiching Counties but this research has state wide applications.

IX . **Reporting Requirements:** Work program progress reports will be submitted no later that **January 31, 2000** and **January 31, 2001.** A final work program report and associated products will be submitted by **June 30, 2001**, or by the completion data set in the appropriation.

Attachment "A" Deliverable Products and Related Budget				
LCMR Project Biennial Budget				
	Result 1	Result 2	Result 3	
Budget Item	Wild Rice Ecosystem Analysis	Assess Genic Diversity	Seed Storage	Row Total
Wages, salaries & Benefits	$56,797.00	$82,919.00	$16,260.00	$155,976
Space rental, maintenance& utilities				
Printing & Advertising				
Communications, telephone, mail etc.				
Contracts				
Professional/technical	$20,748.00	$7,000.00		$27,748.00
other contracts			$2,276.00	$2,276.00
Local automobile mileage paid				
Other travel expenses in Minnesota				
Travel outside Minnesota				
Office Supplies				
Other Supplies		$14,000.00		$14,000.00
Tools and Equipment				
Other capital equipment				
Other direct costs				
Land Acquisition				
Land rights acquisition				
Buildings and other land improvements				
Legal fees				
Column Total	$77,545.00	$103,919.00	$18,536	$200,000.00

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X. Research Projects

Result 1: Comprehensive Analysis of Three Distinct Wild Rice Ecosystems

Principal: Zizania is an aquatic grass that provided habitat for breeding and brood rearing and has a historical, cultural and spiritual ties to the Ojibwe people as well as being enjoyed as a delicacy by many. There are three species endemic to North America: *Z. palustris*, *Z. aquatica* and *Z. texana*. Habitats for *Zizania spp* are being destroyed at an alarming rate in the United States and there is concern that the genetic diversity of this important genus will be irretrievably lost. *Z. texana* is already listed as a species in danger of extinction on the Federal Register. Some stands of Z. *palustris* have been heavily managed by humans and this species is also intensely bred for uniformly maturing, no-shattering grains. These manipulations will lead to genetic shifts and eventual loss of genetic diversity unless wild populations are protected in a comprehensive conservation program. To date, there have been no major scientific investigations to address potential effects that differing environmental pressures have on the production of Z. *palustris*. Leech Lake Reservation and several other Tribes in Minnesota and Wisconsin have collected baseline data for standard chemical and physical parameters however, We are proposing to further investigate the Z. *palustris* ecosystem to not only improve our understanding of causative effects of environmental factors influencing Z. *palustris* but to develop and establish a conservation program that would benefit wild rice managers across the board.

i. Monitoring program time frame: The environmental monitoring program discussed in Result 1 will consist of the following criteria: The proposed study areas are as follows: Nett Lake, in the Rainy River watershed; Squaw Lake in the Big Fork River watershed; and Leech Lake in the Leech Lake River watershed. These sites were chosen on the availability of baseline water quality data and baseline Z. *palustris* monitoring data indicating that these were environmentally distinct Z. *palustris* ecosystems. There will be 10 sample stations per Z. *palustris* stand with the following sample variables being assessed.

Environmental Characteristics: Air temperature, water clarity (secchi disk) water levels (staff gauges will be placed in the beds), water temperature, water pH, dissolved oxygen, conductivity, in flow and out flow velocities, sediment temperature, sediment pH, sediment firmness, zooplankton, phytoplankton and benthic invertebrate population indexes.

Plant assemblages: wild rice, number of plants per meter square, number and species of competitive plants.

Plant Morphological Characteristics: Random sample of wild rice from meter plots will be worked up and morphological characteristics will be root length (including 1st roots, 3rd roots, etc), runner length, leaf length (including 1st leaf, 2nd leaf, 3rd leaf, etc.) Node intervals, number of seeds per head, total kernel length, beard length, kernel weight.

Common Garden Experiment Approach: *Z. palustris* seeds will be collected from the three study areas. Complete morphological characteristics of plants selected will be recorded to be aligned with similar or dissimilar developmental characterizations. If the phenotypic differences that are noted in wild populations of *Z. palustris* are replicated in the greenhouse, there could be a strong argument for genetic differences. If not, it supports a position that environmental factors are at play.

Water and Sediment chemistry: Nutrient budgets will be development for Squaw Lake and Nett Lake. A 1997 water quality project developed a nutrient budget for Leech Lake (LLDRM and MCT 1997). Water chemistry will consist of total phosphorus, total Kjeldahl Nitrogen, Total chlorophyll, true color, and turbidity. Tributaries will be monitored monthly, lake and precipitation stations quarterly. This will be done for one year. Sediment samples from the 10 random sampling sites per bed will be analyzed for total Phosphorus and Total Kjeldahl Nitrogen four times a year for an 18-month period. This information will be correlated with the plant characteristics to determine if nutrient availability is a limiting factor in wild rice productivity.

ii. Quality assurance plan and procedures: A quality assurance plan incorporating all aspects of sample collection and evaluation is important to insure that all routinely generated data are scientifically valid and defensible, and are of known precession and accuracy. A plan describing quality assurance activities mentioned in result one is on file with the Leech Lake Division of Resources Management and The Minnesota Chippewa Tribe Water Research Lab.

The Plan will follow all criteria set forth by the United States Environmental protection Agency (EPA-814B-92-002, September 1992). The Plan will address major topics and sub-related topics including but not limited to the following: sample procedures; sample handling; sample tracking and integrity; chain-of-command procedures; analytical procedures; instrument calibration procedures; frequency of instrument use; data validation; data reduction and reporting procedures; preventative maintenance procedures; corrective action etc. The plan will be specific to the project in that it will identify total number of samples to be collected for each parameter; time lines for collection of samples; special conditions for handling and transportation of samples; preservation and holding times for each sample parameter of interest; and chain of custody procedures for transmittal of samples for field to the laboratory personnel as relevant to this particular project.

iii. Sample collection procedures: Care in handling and transport is essential to maintain the integrity of the data it contains. All samples collected will be maintained in containers specified as appropriate by Standard Methods (19th Ed.). Samples collected will be maintained on ice and transported to the Laboratory as soon as possible after collection. Testing for specific parameters will occur within times specified appropriate by Standard methods. All procedures related to this topic will be defined in the Quality Assurance Project Plan.

iv. Physical water chemistry data: Technical staff will be monitoring a number of physical, biological and chemical parameters throughout the life of this project all aspects of chemical, physical and biological collection, analysis and reporting will be addressed in the Quality Assurance Project Plan as discussed in section *ii quality assurance plan and procedures*.

v. Sediment and plant tissue nutrient content evaluation: Sediment and Z. *palustris* plant tissue from each ecosystem will be dried, burned, and the ash tested for total organic content, Total Kjeldahl Nitrogen (TKN) and Total Phosphorus (TP). Nitrogen and Phosphorus will be considered as nutrients central to production of plant growth and lake productivity.

vi. Data and records management: Samples processed by tribal laboratory facilities will be documented in data information log books specific for this purpose. Field and Laboratory results collected by Leech Lake will be maintained in a data base at the Leech Lake Division of Resource Management and shared with the Wild Rice Managers from the six member Minnesota Chippewa Tribe, Red Lake Nation and 1854 Authority.

vii. Data reporting: Data will be presented in report form to the LCMR and participating agencies on dates specified in the work plan. A summary report that will summarize all activities, findings and recommendations of this project will be provided to the LCMR at the end of the project period.

Result 2. Assess Genetic Diversity

Principal: In conjunction with the Ecological sturdy described above, we will determine if phenotypically distinct populations of wild rice can be identified on the basis of genotypic difference. The genotyping, in turn, may be valuable for management decisions regarding replanting beds of wild rice, if the genotypes and phenotypes correlate with differences in the ability of the rice to survive and propagate in different environments.

I. Background and Hypothesis: We postulate that phenotypic difference in wild rice has at least a partial underlying genetic basis. We further hypothesize that identifying genetic differences between populations of plants may be useful in the management of wild rice. A great deal of genetic marker information is currently available for cultivated rice (Oryza sativa). We plan to exploit this information as a base for identifying potential genotyping tools for wild rice (Zizania sp.) Initially, we will plan to do PCR, using primer pairs from cultivated rice that amplify polymorphic loci in wild rice (Dr. Mike Antolin, Colorado State University, personal communication). If additional work is needed, we will use random amplification of polymorphic DNA (RAPD) and c DNA probes from cultivated rice (which hybridize to wild rice, Dr. Kennard, personal communication) for our genotyping efforts.

II. Description of methodology: We will plan to combine PCR and amplified fragment length polymorphism analysis to genotype the wild rice populations. Southern hybridization and DNA sequencing will also be used for genotyping if necessary.

III. Timeframe: The population sampling could be completed during the summer of 1999. The genetic analysis

would require an additional year.

i. Quality Assurance: Two techniques will be used to assure that the results are reliable. First, at least 10% of the PCR reactions will be run in duplicate to assure that of these reactions are reproducible. Second, each series of reactions will be made from a PCR master mix, and there will be at least one "No DNA" control in each series to verify that we are not getting any false positive reaction.

ii. Sample collection: Leaves from at least 50 plants will be collected and analyzed from each of the three lakes in the study. The samples will be collected randomly as described in the ecological analysis, above. Comparison will be made of the genetic variability of populations within as well as between the lakes.

iii. PCR Analysis:
Dr. Mark Antolin, (Colorado State University) has been doing genetic analysis on Zizania texana using PCR. He is willing to provide us with the primer sequences to allow us to do a similar analysis with Zizania palustris populations. These primers amplify tandem repeat sequences (Microsatellite) that vary between individuals at high frequency. We will use these primer sets to amplify polymorphic DNAs. By using 3-6 primer pairs per plant, we should be able to distinguish population, if those differences exist. We assume this analysis will be sufficient to provide sufficient genetic information to identify the different populations, if genetically distinct populations do exist.

DNA will be isolated from Zizania palustris leaves that have been immediately frozen after collection. We will use the Qiagen Dneasy Plant Mini Kit for the DNA isolation.

We will initially conduct pilot reaction to verify that the primers do reproducibly amplify the DNA. We will use Qiagen HotStar Tag, Clontech Advantage Tag, or Amplitag Gold Tag polymerase to minimize artificial banding patterns from premature DNA amplification. We will use duTP in our PCR cocktails to provide a method for preventing contamination of PCR reactions (using Uracil DNA glycosylase) if contamination becomes a problem. PCR reactions conditions will be based on those that Dr. Antiloin has developed for the various primer pairs.

In case additional genetic information is required, we will use the method described in the next two paragraphs, below.

If additional genetic markers are needed, we will next perform RAPD analysis on DNA from the three populations of Zizania palustris. Decamer primers will be used for this procedure (Operon technologies, Alameda CA and we will vary the thermocycling parameters to get reproducible patterns (e.g. Schwedder et al., 1995 Effect of transition interval between melting and annealing temperatures on RAPD analysis. Bio Techniques 19:38)

If necessary, we will use RFLP analysis to identify additional polymorphic DNAs. Probes will be cDNA from cultivated rice (obtained from Susan McCounch at Cornell or Ron Phillips at the University of Minnesota). DNA form RFLP analysis will be isolated using Dneasy Plant Mini Kits (Qiagen, Chatsworth, CA). DNA will be digested with various restriction enzymes, run on an agarose gel, blotted to a nylon membrane, hybridized with the appropriate DNA probes, and detected by autoradiography or chemiluminescent detections.

iv. Data and records management: Samples processed by laboratory facilities will be documented in data information log books specific for this purpose. Laboratory results collected will be maintained the Leech Lake Band at the Minnesota Chippewa Tribe Research Lab.

v. Data reporting: Data will be presented in report form to the LCMR and participating agencies on dates specified in the work plan. A summary report that will summarize all activities, findings and recommendations of this project will be provided to the LCMR at the end of the project period.

Result 3. Seed Storage:
Because much of the preliminary research developing cryopreservation techniques used *Zizania palustris* as a model species, we know quite a bit about its development and cryopreservation potential (described below). In addition, colleagues at the National Seed Storage Laboratory have an independent project to cryopreserve seeds from wild

populations. *Z. palustris* and *Z. texana* began in the summer and fall of 1998. Samples of *Z. palustris* were subdivided into different maturity classes. Greater that 100% survival is routinely obtained when the water contents of the most mature embryos (hard brown) are adjusted to between 0.5 and 0.7 g. H20/g and embryos are cooled at rates of 500C/min or greater. Studies with less mature embryos have not been done. Our colleagues report that the optimum water content of cryopreservation of Z. texana (at full maturity resembles *Z. palustris* at the hard green state) is between 0.9 and 1.1 g H20/g, but that the cooling at rates in excess of 5000 c/sec yields only 20% survival. We are concerned by this low survival rate in what appears to be less mature tissues and feel that comparisons of the glass forming properties of *Z. palustris* at different stages of maturity may be critical.

Storage of Seeds in ex situ gene banks.

I. Abstract: The objective of this project is to provide protocols for storage of seeds from wild populations of *Zizania palustris* that are cost effective and maintain highly vigorous seeds with minimum selection pressures for several decades. To achieve these objectives, we must determine interaction between water content, temperature and survival of seeds harvested from the three study populations, optimize drying protocols, and evaluate longevity at different storage temperatures. Cryopreservation protocols were previously developed using A. palustris accessions harvested from cultivated varieties. The proposed research will use similar biophysical and physiological approaches to test the efficacy of these protocols in terms of survival (confirmation data) and longevity (innovation) on wild populations. In addition, we will determine whether drying time and moisture level can be manipulated to increase the levels of desiccation tolerance in immature embryos (innovations). This result will be used to enhance the cryostorability of accessions of *Z. palustris.*

II. Background and Hypotheses: Storing *Z. palustris* in ex situ genebanks is difficult because the seeds produced by Zizania spp. are "recalcitrant" (Probert and Longley 1989; Probert and Brierly, 1989; Still et al., 1994, Vertucci et all, 1994, 1995; Walters 1998b). Recalcitrant seeds do not survive drying and so cannot be stored using the conventional storage protocols of 5=2% moisture content and -18C recommended for orthodox (desiccation tolerant) seeds (IBPGR, 1985). Recent research suggests that highly viable *Z. palustris* seeds can be maintained for 1 year by storing them in flooded peat moss at 3C (Oelke, 1997). While this preservation practice may be suitable for producers of paddy grown *Z. palustris*, it does not provide adequate longevity for breeding programs (longevity of about 10 years is required) or conservation programs (longevity 25 years desired).

The genetic composition of accession of seeds stored for germplasm conservation purposes should match the genetic composition of the population from which it was collected. We foresee 2 problems in accomplishing this goal in field collected samples of Zizania palustris. Because *Z. palustris* produces recalcitrant seeds, storage procedures are intrinsically difficult and some mortality is expected. Because grains from wild populations usually vary in maturity status we also expect mortality during preservation since maturity status affects ability to survive desiccation (Vertucci and Ferrant, 19950 freezing (Vertucci et al., 1991, 1994, 1995) and long term storage (Walters, 1998a). It is likely that some genotypes are more susceptible to freezing or desiccation damage and that other genotypes require longer seed maturation times (so only the immature sees are harvested). Unless cryopreservation procedures give high survival rates for all genotypes, selection pressures will be imposed and will cause genetic shifts in the ex situ collection. To avoid these selection pressures, we must understand the nature of sensitivity to desiccation, freezing and aging stresses in *Z. palustris* grains and how maturation influences the level of sensitivity.

Seeds acquire tolerance of desiccation during development (reviewed by Vertucci and Ferrant, 1995; Walker 1998B). The early stages of embryogenesis appear to be similar for both desiccation tolerant and sensitive seed types. During dry matter accumulation the water potential for the embryo in situ remains about -0.8 MPa and sees are damaged if dried to water potentials less that about -4 MPa (reviewed by Walter, 1998b). Within a few days after abscission, orthodox embryos acquire complete tolerance of desiccation and recalcitrant embryos acquire tolerance to water potentials as low as about -12 MPa. Some seeds with intermediate characteristics tolerate water potentials of -75 MPa.

The exact mechanism by which seeds acquire partial or complete tolerance of desiccation is unknown. Many suspect that vascular abscission or the associated decrease in water potential stimulate the production of protective compounds such as stress proteins (Gala et al., 1991; Chandler and Robertson, 1994; Chermidae, 1995) or soluble

carbohydrates (reviewed by Obendorf, 1997). It is intriguing that water potentials of less that -1.4 MPa are needed to stimulate productions of stress proteins in wheat seedlings (Walters et al., 19970 and that these proteins are associated with acquisition of tolerance in these tissues (Ried and Walker- Simmons, 1993) Similar proteins are found in grains of Zizania spp., even through embryos do not achieve the same level of desiccation tolerance acquired by orthodox embryos (Bradford and Chandler, 1992; Still et al., 1994; Gee et al., 1994; Ferrant et al., 1996) It has also been established that production of these proteins are stimulated in *Z. palustris* grains by drying (Bradford and Chandler , 1992, Still et al., 1994, Gee et al., 1994). Composition of sugars in developing Zizania embryos is comparable to those in more desiccation tolerant seeds (Still et al., 1994; Walkers, unpublished; reviewed by Walkers 1998b).

Finch-Savage (1992) suggested that recalcitrant embryos do not complete the embryogenic program and so not to acquire full tolerance of desiccation. Instead, recalcitrant seeds accumulate some protectants, but not is sufficient levels to confer tolerance. We hypothesize that if the embryogenic program is prolonged in *Z. palustris* seeds by either slight drying or in vitro culture at -1.5 MPa, greater quantities of protectants bay be accumulated. Similar strategies are used routinely to enhance quality of orthodox seeds (reviewed by Chermidae, 1995; Hong and Ellis, 1997) as well as in the production of somatic embryos (e.g. McKersie, 1995). In a previous experiment with *Z. palustris*, slow drying did not appear to change tolerance of desiccation (Probert and Brierly, 1989_ but these authors may not have targeted the appropriate window of water potentials at which protectants are produced or the given the proper time at those water potentials. Consistent with our hypotheses, lines *Z. palustris* bred for cultivation in California mature to 5-15 days later that genetic stocks used for shorter growing season and grains of California-grown *Z. palustris* appear to be more tolerant of desiccation (Kovach and Bradford, 1992; Berjak et al., 1994; Vertucci et al., 1995; Ntuli et al., 1997).

The level to which seeds can be dried is also affected by temperature, with seeds exposed to lower temperatures requiring more water (i.e. Walker 1998a). The trend for increasing critical water contents with decreasing temperature has been establish for Zizania embryos (Kovach and Bradford, 1992, Berjak et al., 1994; Vertucci et al., 1995; Ntuli et al., 1997) and the exact relationship can be predicted from water sorption isotherms that made specifically for each seed lot and maturity stage (Vertucci et al., 1994, 1995) If seeds are to be stored at sub-zero temperatures (as is necessary for long-term conservation in genebanks), the requirement for high water contents to prevent desiccation damage must be balanced with the increased likelihood of lethal ice formation. Stat-phase diagrams mapping the effect of temperature on critical water contents of desiccation damage (determined directly or predicted by critical water potentials) and moisture contents for lethal ice formation (detected using differential scanning calorimetry) provide insight into moisture content-temperature combinations that provide safe storage (i.e. Vertucci, 1993). Stage phase diagrams have been constructed for two varieties of cultivated *Z. palustris* at three maturity stages (Vertucci et al., 1994, 1995) and clearly show the potential for long term preservation of *Z. palustris* at sub-zero temperatures. These studies show that seeds, even immature ones, can be exposed to -10C with no loss of viability if their moisture contents are optimized. A 15-20% reduction in germinations was observed in fully mature (hard brow) grains exposed to -20C and higher moralities were observed in less mature (hard green and soft green) grains.

The advantage of storing seeds at temperatures of about -10C is that the equipment requirements are minimal (a single stage chest freezer and adequate packaging) and preparations of the material is simple (equilibrate to about 90% RH at 20C prior to cooling). Techniques must be developed to optimize the drying procedure to accommodate larger samples. The longevity of seeds stored under these conditions is unknown. If deterioration results from the same processes as orthodox seeds, which have a Q1- = 2 (Walkers, 1998a), we can expect longevity to increase about 3 fold by reducing the storage temperature from 5 C to -10C. However it is possible that ice eventual forms in embryos store with relatively high water contents at sub-zero temperatures. The kinetics of ice formation during storage at sub-zero temperatures can be determined using differential scanning calorimetry (i.e. Franks, 1985) and will be an essential part of the proposed experiments.

As predicted by state-phase diagrams (and confirmed by experimentation) optimum water contents (sufficiently wet to prevent desiccation damage and sufficiently dry to prevent freezing damage) cannot be achieved in *Z. palustris* embryos at the hard brown, hard green and soft t green stages (CV., Franklin harvested in Minnesota) that are exposed to temperatures less that -25C, -18C and -8C, respectively (Vertucci et al., 1994, 1995). If extreme longevities are required or if a sample contains a large portion of immature seeds cryopreservation in liquid nitrogen

is required (i.e. Vertucci, 1993) The technique is fairly straight forward but labor intensive. The water content of the embryo must be optimized to prevent desiccation damage (again, according to state-phase diagrams) and then the embryo is cooled so rapidly to -100C that ice crystals do not have time to form (Robards and Sleytry, 1985). This procedure is sometimes call "vitrification since the rapid cooling to low temperature induces formation of an aqueous glass. The viscosity of the cytoplasm is so extreme in the glassy phase that molecular motion leading to the eventual recrystalization of water or to chemical deterioration of macromolecular motion leading to the eventual recrystalization of water or to chemical deterioration of macromolecular constituents is limited (Karth, 1985). Theory and experience with other species suggest that the higher the critical water content, the more rapid cooling must be (Wesley, Smith, Eira, Crane, Touchell, & Walkers, unpublished). Seeds containing about 0.20 g H2)/g dw require cooling rates of about 50-100 c/min, while seeds containing 0.9-1.1 g H20/g must be cooled at /1000/sec. Cooling rates as fast as 100/ in can be easily accomplished by plunging whole seeds into liquid nitrogen (Vertucci, 1989). The most rapid cooling rates can be achieved by excising the embryonic axis from the seed (so a very small sample is used) and then injecting the sample into sub-cooled liquid nitrogen (Wesley-Smith et al., 1992). (The advantage of sub-cooling liquid nitrogen is 2-fold; the temperature is reduced to about 210C and the Lieden-Frost phenomenon is prevented (Wesley-Smith et al., 1992).) The techniques to preserve intermediate and recalcitrant embryos at -10C and in liquid nitrogen are well established and efforts are underway at the National Seed Storage Laboratory to initiate a pilot program to routinely store embryos of Coffea (Eira and Walters, unpublished) and citrus (Crane and Walker, unpublished). Because much of the preliminary work was conducted using Zizania embryos, its is appropriate that this genus be included in the pilot program as well.

III. Description of Methodology:
A. Hypothesis: Critical water contents for maturing *Zizania palustris* remains constant at about -12 MPa once dry matter accumulation has been completed.

Grains of *Z. palustris* from the three study populations will be harvested at various times during the growing season and separated into age classes based on the color and texture of the glumes, pericarp and endosperm and the dry mass and water content of the embryo. Embryos from each age class will then be rapidly dried to water contents ranging from 1.5 to 0.1 g H2O/g dw (8 to 10 moisture treatment studies), exposed to temperature of 20, 20, 10z, 0, -10, -20 and -30C and evaluated for survival through assays of electrolyte leakage and germination. To circumvent the stratification requirement, embryos will be excised from the grain and placed into culture. Usually 25 embryos per treatment are used. We expect to study seed from 5 harvest times with 3-4 maturity classes per harvest time. Water sorption isotherms will be constructed for each harvest date and maturity class so that water content at harvest and critical water contents can be converted to water potential measurements. Isotherms are made by equilibrating embryos and/or grains over saturated salt solutions with known relative humidities or polyethylene glycol (MW=8000) solutions at known water potentials. Isotherms will be constructed at 0, 10, 20 and 30 C.

The experiments are routine at the National Seed Storage Laboratory and present no technical problems. Funding for a student has been requested to offset the labor-intensive aspects of this study. These experiments will provide necessary data for subsequent cryopreservation experiments and will provide the first documentation of water relations in developing grains of aquatic species. Results of these experiments will enable us to determine if critical water contents for desiccation damage at different storage temperatures are predictable from water sorption isotherms constructed for embryos at different maturity levels. If this is true, optimum moisture contents for storage for any Zizania accession can be easily predicted from a few simple measurements. If the hypothesis is not supported, then optimum water contents must be determined for each accession.

B. Hypothesis: The interaction between temperature, water content, enthalpy of melting transitions, glass formation and kinetics for recrystalization very in Zizania palustris embryos as a function of maturity.

The calorimetric properties of water in embryos of *Z. palustris* from one study population will be evaluated for different age classes using differential scanning calorimetry. Individual embryos with water contents adjusted between 2.0 and) g H2)/g dw will be hermetically sealed into DSC pans and subjected to calorimetric measurements. To determine basis water melting/freezing properties, embryos will be cooled and warmed at 10C/min at temperature ranges between 20 and -150C. The temperature and energy of freezing and melting transitions will be quantified by instrumentation software, and this will enable use to construct phase diagrams on the freezing temperature of water and the water content at which water is not available to freeze. Standard scanning

techniques do not detect glassy behavior in seeds at sub-zero temperatures and colleagues at NSSL have developed a more sensitive method where heat capacity is directly measured at a range of temperatures. This technique has not been applied to biological materials yet. It requires rapid cooling (1000/sec) of embryos and then systematic measurement of heat capacity in 10C intervals from -150 to 20C. The rate of recrystalization will be measured in a similar way, except heat capacities will be measured at temperatures between -150 and -10C after samples have been annealed for several days (annealing at -10C treatments at high moisture contents) to several months (temperatures -80C, all moisture treatments; or temperatures -8-, moisture contents moisture content where water does not freeze).

The results from these experiments will be used to construct the part of the phase-state diagram describing the physical properties of water temperatures of freezing and melting transitions and glass transitions and water contents at which water is unfreezable. In addition, the experiments on the kinetics of recrystalization will provide the first information on the stability of glasses in biological materials. This information is critical for predicting the longevity of preserved germplasm. Based on preliminary calorimetric studies of water within maturing embryos of several species, we are expecting the glass behavior of mature and immature *Z. palustris* embryos to be different (immature grains produce less stable glasses) This needs to be established and if it is so, we need to adjust cryopreservation procedures (faster cooling rates, storage in liquid phase rather than vapor or post harvest treatments to increase tolerance as described in C) to enhance survival of immature embryos.

C. Hypothesis: Grains and embryos of *Z. palustris* can survive exposure to liquid nitrogen if the water content is optimized and cooling rates are sufficiently rapid.

Seeds from the final harvest of the three study populations will be divided into 3 maturity classes (hard brown, hard green, soft green) All studies will use both intact grains and excised embryos. Based on state-phase diagrams constructed in A and B, water contents will be adjusted to levels that will prevent desiccation damage at -150C by drying for specific time periods. Embryos will then be cooled to liquid temperatures at 40C/min (seeds in cryovials exposed to vapor) 200C/min (seeds in cryovials exposed to liquid), 1000C (seeds plunged directly into liquid), 1000/sec (excised embryos injected into liquid). Survival will be valuated as described in A.

Elements of this experiment have already been accomplished with mature grains (see III). Results will define basic cryopreservation routines.

D. Hypothesis: There is an optimum rate of drying that increases the levels of desiccation tolerance but minimizes the amount of aging in grains of *Z. palustris*. This optimum rate may be different for grains at different maturities.

Initial experiments will use on population of *Z. palustris* grains divided into three age classes (hard brown, hard green and soft green). The effect rate of drying to different water contents on survival will be determined by drying seeds over 20,15, 10, 5, 2, 1 and .5 and .1 days and sampling viability periodically. Drying rates will be controlled by moving seeds to progressively drier environments. This experiment will give us the time frame for which deteriorative reactions occur. Based on the results of the above experiment, we will choose an appropriate time (say 5 days) and study the effect of holding seeds at water potentials of -0.5, -1.0, -1.5, -2 and -3 MP on the level desiccation tolerance (minimum water potential and water content survived at 20C 9described in A) and optimum water content at -10C and liquid nitrogen (described in A, B and C). We will use three methods to control seed water potential: the intact grains will be dried to appropriate water contents according to isotherms constructed in A, excised embryos will be placed in culture with Murashige and Skoog media adjusted to different water potentials with additions of sucrose.

We know that seeds held at intermediate moisture levels deteriorate more rapidly than seeds held at higher or lower moisture levels. This experiment is designed to tell us the range of water potentials and water contents that are intermediate for *Z. palustris* and the rate at which aging occurs. These experiments are essential because it will tell us what water levels and time periods to avoid. Experiments from other laboratories 9briefly alluded to in Literature review) have suggested that exposure of tissues t slightly higher water levels can induce protective compound and allow tissues to acclimate to harsher conditions. Identification of these compounds is not a focus of the experiments proposed here. Rather, these experiments will determine if greater tolerance can be achieved in maturing grains of *Z. palustris*.

E. Hypothesis: Longevity of *Z. palustris* grains currently achieved by wet storage at 5C can be improved by storage at -10 C and liquid nitrogen. Longevities will vary with maturity stage.

Final harvest from all three study populations will be used and seeds will be divided into 3 maturity classes (hard brown, hard green, soft green). Water contents of grains (-10C) or embryos and grains (liquid nitrogen) will be adjusted to optimum and slightly sub- and supra- optimum moisture contents as determined in A, C and D (i.e. 3 moisture treatments per maturity class per population). Seeds will then be placed in storage at -10C and liquid nitrogen and monitored for viability periodically. For comparison, seeds will also be packed in damp peat moss and stored at 5C. Viability will be sampled in 3 month intervals during the first year of storage and then yearly after that.

Experimentally determined longevities will be compared with projections based on DSC measurements of ice recrystalization kinetics (described in B).

IV. Results and Products: These experiments are expected to provide generalized protocols for conservation of all wild populations of *Z. palustris* as well as provided a framework for the cryopreservation of recalcitrant seeds in general. It is our goal that preservation techniques are cost-effective, reliable, simple and applicable to a wide range of *Z. palustris* populations. This work will be useful to conservationists as well as breeders.

Protocols for long term storage of wild rice germplasm.

A. Refine protocols to cryopreserve of wild rice embryos efficiently.

Sept -Oct 1999 Experiments with whole seeds to determine the relationship between moisture content, temperature (5C to -20C) vx survival

Nov-Dec 1999 Experiments with isolated embryos to determine relationship between moisture content and cooling rate on survival in liquid nitrogen (-196C)

Jan-Mar 2000 Development of drying methods for whole seeds to routinely achieve optimumwater contents.

B. Determine post-harvest procedures that can be used to increase the desiccation tolerance of embryos to improve efficiency and survival of crypopreservation methods.

Sept-Dec 2000 Does exposure of whole seeds and isolated embryos to different plant growth regulators (ABA), lower water potentials (O to -1 MPa) and cold temperature (10C to -5C) enhance desiccation or freezing tolerance.

Jan-Apr 2001 Does loss of dormancy correlate with desiccation and freezing tolerance?

C. Initiate a pilot project to monitor the viability of stored germplasm.

Nov 99-2001 Monitor survival of whole seeds stored at temperature between (5C to -20C) and isolated embryos stored in liquid nitrogen. Repeat with same accessions each year. Goal: 10 accessions

D. Transfer technology developed during the study to the Minnesota Chippewa Tribes Water Quality laboratory.

Sept 2000 Site-visit of NSSL personnel to Chippewa Tribes Water Quality Laboratory (1-2 days)

Sept-Nov 2001 Site-visit of Tribes Waters Quality Laboratory personnel to NSSL (1-2 months).

V. Timetable: Experiments will start in the summer of 1999 with the first harvest of seeds 5-10 days after flowering. Assessments of desiccation tolerance, cryo-survivability and isotherms (A and C) of extremely immature seeds must be accomplished immediately. DSC measurements of extremely immature seeds (B) must also be accomplished immediately. Studies of more mature grains (hard brown, hard green and soft green) can be extended

into the fall and winter. Longevity experiments will be initiated in October 1999 and continue until grains show signs of deterioration. The experiments will be repeated in summer - winter 2000.

VI. Budget Requirements: The National Seed Storage Laboratory is willing and able to contribute all the necessary equipment and supplies to this project. In addition, a GS-11 research support scientist has recently been hired to initiate the pilot program on cryopreservation of recalcitrant seeds and can oversee much of the proposed research. The experiments in this proposal are extremely labor intensive, as they require seed sorting, excision of thousands of embryos, and multiple fresh and dry weight determinations. The NSSL is requesting $18,536.00 to finance hourly labor to perform these routine but necessary functions. The NSSL estimates an in-kind contribution of at least $39,000.00.

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Conservation of plan germplasm with special emphasis on seed genebanks, Embryo development and seed germination Mechanism(s) of freezing and desiccation damage/tolerance, Physiology of dry biological system, Calorimetry.

EDUCATION
Ph.D. August 1986, Botany Department, Cornell University
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PUBLICATIONS MOST RELATED TO PROJECT (from a list of 60 peer reviewed papers.

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RELATED HONORS AND AWARDS

1995 Awarded a grant from US Fish and Wildlife Service (Pacific Islands) to study the physiology seeds from endangered plant species in Hawaii.
1994 Awarded a grant from IPGRI to coordinate a global study of the optimum water contents for seed storage.
1992 Awarded a grant from the US Forest Service to study the feasibility of cryopreservation of seeds of the endanger species Taxus brevifolia.
1991 Winner, Northern plains Area Early Career Research Scientist of the Year.