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CONSULTANCY REPORT ON

CRYOPRESERVATION OF PLANT GERMPLASM

February 29 - March 11, 1988

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Cryopreservation of Plant Germplasm at the National Bureau of Plant Genetic Resources New Dehli, India

Review, Plans and Recommendations

March 11, 1988

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LIST OF TERMS

NBPGR National Bureau of Plant Genetic Resources, Indian Council of Agricultural Research, New Dehli, India

Cryopreservation
The use of ultra-cold storage temperatures using liquid nitrogen as the storage medium.

LN2 Liquid Nitrogen, -196 C.

Orthodox Seed/Pollen
Seed or pollen that can be easily dried to low moisture content levels, less than 10% fwb, without significant damage to viability. e.g. desiccation-tolerant. These materials are generally long-lived (month, years, decades).

Recalcitrant Seed/Pollen
Seed or pollen that cannot be dried, normally below 35-40% fwb, without loss of viability. e.g. Desiccation-sensitive. These materials are generally short-lived (hours, days, weeks).

fwb Fresh Weight Basis. Normally associated with the reporting of seed moisture content. e.g. 10% fwb.

$$\text{Moisture Content (\%) fwb} = \frac{\text{Fresh Wt.} - \text{Dry Wt.}}{\text{Fresh Wt.}} \times 100$$

Base Collection
A group of plant materials, seed, pollen, vegetative, being held for long-term preservation (greater that 20 years), normally restricted in distribution and acts as a back-up to a working collection.

Working Collection
A group of plant materials similar to a base collection that are held for short to medium periods of time (less than 20 years), for the primary purpose of regeneration, research, evaluation and distribution to end users.

Accession
"A sample of seeds (pollen and vegetative) representing a cultivar, a breeding line, or a collected field sample which is held in storage for conservation" (IBPGR, 1985).

IBPGR International Board for Plant Genetic Resources, Rome, Italy.

EXECUTIVE SUMMARY

Dr. Phillip Stanwood, Winrock consultant, reviewed and interacted with the staff and management of the NBPGR in New Dehli, India from February 29 to March 11, 1988. The two primary areas of interest were: 1) cryopreservation of seeds, pollen and vegetative materials; and 2) routine preservation of orthodox seeded species. Procedural and research direction are in place and appeared to be sound and progressive. There was considerable interaction with the staff and management concerning "fine tuning" details. Much of the time was spent developing cryopreservation research facilities, recommending equipment and discussing research lines of approach.

The following recommendations were provided:

1) Initiate a seed (orthodox) cryopreservation pilot project.

2) Complement scientific staff with permanent, full-time support technicians.

3) Provide laboratory computers for data acquisition, equipment interfacing, data analysis and reporting.

4) Relocate a planned cryotank room and build a LN2 generating and storage facility next to the cryotank room.

5) Evaluate on-site/off-site sources of LN2.

6) Formalize the recording of germination, moisture content, 100-seed weight, seed number and sample storage location.

7) Continue with the current line of cryopreservation research on plant materials, adding research directed at desiccation-sensitivity of recalcitrant materials.

8) Purchase selected pieces of equipment to complement existing and future preservation and research activities.

The time and effort spent by the consultant and NBPGR staff was very fruitful. A stronger NBPGR plant germplasm genebank and cryopreservation research program will in part be a result of this visit.

INTRODUCTION

The NBPGR is in the process of unifying and expanding their plant germplasm collection, evaluation, preservation and utilization capabilities. The Germplasm Conservation Division (GCD) and National Plant Tissue Culture Repository (NPTCR) specifically, are enhancing their preservation and research activities for seeds, pollen and vegetative materials.

A key element of this enhanced capability is the potential use of cryopreservation techniques to store these materials. Overcoming physiological deterioration of a stored plant sample is one of the most important and difficult tasks in the preservation process. Large-scale mechanical refrigeration systems, which hold materials at temperatures down to -20 C, have greatly increased the storage life of a sample compared to ambient storage conditions. However, deterioration and loss of viability can still occur at -20 C with increased time in storage, resulting in a loss of precious genetic material. Cryopreservation techniques (e.g. liquid nitrogen, -196 C) provides the potential for "indefinite" preservation by reducing metabolism to such a low level that all biochemical processes are significantly reduced and biological deterioration is virtually stopped.

The primary emphasis and assumption of this review is that the plant materials are being preserved as a "base" collection at the NBPGR, New Dehli facility. This determination is critical since cryopreservation by it's very nature, assumes that the preservation objective is for extended periods of time with a

limited need to routinely access a given accession.

OBJECTIVES OF CONSULTANT'S VISIT:

1. To review progress of cryopreservation work at NBPGR.

2. To assess conservation division and cryopreservation research facilities at NBPGR.

3. To develop plans, as needed, for comprehensive cryopreservation laboratory facilities.

4. To develop plans for experiments in cryopreservation of orthodox and recalcitrant seeds, pollen preservation, in vitro pollen preservation and stability analysis.

OBSERVATIONS:

Cryopreservation Activities

Cryopreservation research on plant materials (seeds, pollen and vegetative) at the NBPGR is at it's early stages of development. They have only recently obtained cryostorage tanks, which has been a major limitation of progress in this area. Research direction has been defined, but inhibited by a lack of

specialized equipment, further slowing progress. There are existing laboratory facilities that can facilitate limited cryopreservation research, with additional facilities (2 laboratories and 1 cryovat room) just now being occupied. Most of the basic laboratory equipment (balances, etc.) is available but may be limiting as cryopreservation research activities increase and they occupy their new laboratories. Tissue culture research and preservation capabilities are much further advanced and appears to be more than adequate to support cryopreservation research of plant tissue cultures.

The scientific personnel are well trained in their basic disciplines. They are enthusiastic about cryopreservation research and are strongly supported by their supervisors and management team. They are well poised to embark on plant cryopreservation research. I could not clearly determine if they had a scientist trained in seed physiology. If they do not, an addition of a scientist trained in that field would greatly enhance their capabilities.

The Division of Germplasm Conservation is in the process of completing new facilities to expand their -20 C storage capacity to roughly 200,000 samples, assuming appropriate sized accessions. They are drying their samples to the IBPGR recommended moisture content of 4-7% and storing in sealed aluminum foil packets. There is to be a backup power generator in the new facility. Refrigeration compressors and evaporators are not backed up. Existing support laboratories for the seed base collection are inadequate. However, new laboratories are

under construction and will be occupied soon. These new laboratories should be adequate for the next 3-5 years.

Seed germination and moisture content determinations are conducted on samples as they are added to the base collection. I could not determine the nature and extent of a program to monitor germination and moisture content of the stored accessions over time. They have only recently started their genebank and have not therefore, had samples in storage long enough to require a formalized monitoring program. A viability monitoring program policy should be developed as soon as possible. At the time of this report there were approximately 72,000 samples in the base collection.

The primary scientists in the Division of Germplasm Conservation appear to be well trained and motivated. However, the division appears to be understaffed in permanent full-time technical staff in relation to it's assigned duties and expected work loads.

Overall impressions of the two groups are:

1. They are well organized, adequately trained and strongly supported by management.

2. Current facilities are marginal, however new facilities are coming on-line and long-range facilities are planned.

3. Cryopreservation research has just begun. Facilities, equipment and research direction are needed.

4. The work loads for the routine preservation activities are greater than perceived by local management.

RECOMMENDATIONS

1. Initiate a seed (orthodox) cryopreservation pilot project.

Basic information and experience is needed with orthodox seeded species. A pilot project headed by the NPTCR in close cooperation with the Germplasm Conservation Division should be established with the goals of:

a) Developing basic information on orthodox seed cryopreservation.

b) Evaluate the feasibility of cryopreserving orthodox seeds on a routine basis at the NBPGR.

c) Transfer cryopreservation expertise to the Germplasm Conservation Division staff.

See "Pilot Project Implementation Guideline" for specific details beginning on page 17 of this report.

2. Complement scientific staff with permanent, full-time technicians.

Permanent, full-time professional technicians for each scientist should be added to the staff. Much of the scientific staff is burdened with routine laboratory work which inhibits creative scientific thought and creativity. This creative time is needed by the NBPGR if is going to develop and grow.

3. Provide laboratory computers for data acquisition, equipment interfacing, data analysis and reporting. See equipment list item 23.

4. Relocate the cryotank room and build a LN2 generating and storage facility next to the cryotank room.

An area specifically designated for LN2 production and bulk storage is needed. This facility should be as close to the cryovat storage room as possible. Piping LN2 for distances greater than 20-30' is very expensive and consumes a lot of LN2. Attachment IV provides a general diagram of the cryovat room and adjoining LN2 production facility. This recommendation has been discussed in detail with Dr. Arora and his staff.

5. Evaluate on-site/off-site sources of LN2.

There are two methods of obtaining LN2: 1) On-site production with a LN2 generator and off-site supplied LN2 coupled with large on-site storage tanks. Appendix V provides a cost comparison of the two methods. Currently, the reliability of commercially supplied sources of LN2 in the New Dehli area are questionable or unknown, which is an <u>overriding</u> factor in deciding which method to use. It is recommended that on-site facilities be developed at this

time. It is further recommended that experience be gained from commercial sources of LN2 by relying primarily on those sources and using the on-site production facilities only as an emergency back-up system.

6. Formalize the recording of germination, moisture content, 100-seed weight, seed number and sample storage location.

Record keeping procedures are crucial to a genebank operation. It is recommended that a "germination card" be developed that would be used for every accession. This card would be a permanent written record of sample handling procedures and be a source document for the developing NBPGR computerized database system. Cards should be kept at a centralized location except when being used with a sample. The following information should be on each card (I will send an example of a card from the NSSL when I return to the USA):

- Accession Identification
- Alternate Identification
- Genus
- Species
- Common Name
- Date Received
- Storage Location
- Total Sample Wt. (g)
- 100-Seed Wt. (g)
- Total Number of Seed (Calculated from above)
- General Comments
- Germination History
  - -Date Planted
  - -No. Seeds Planted
  - -Count Dates with No. of Normal Seedlings
  - -Total Normal
  - -Total Abnormal
  - -Total Hard
  - -Total Dead
  - -Average Germination
  - -Comments
- Moisture History (for each test)
  - -Date
  - -No. Seeds Used
  - -Moisture Content %
  - -Comments
  - -Method Used

7. Continue with the current line of cryopreservation research on plant materials adding research directed at desiccation-sensitivity of recalcitrant species.

The NPTCR has done an excellent job in identifying priority plant materials for cryopreservation research. There was considerable discussion with the research staff on research direction. It will not be reviewed here. It is recommended that support for their research direction be continued and remain as a high priority for the NBPGR. It is further

encouraged that research lines be developed in the area of seed and plant tissue desiccation-sensitivity. Knowledge and experience in this area is a prerequisite for cryopreservation research and technique development.

8. Purchase the equipment listed in the following table.

Equipment List for the Division of Germplasm Conservation and Tissue Culture Repository. Original list provided by the NBPGR via US-AID.

*** - Comments by the consultant.

Sl No.	Item Description	No.	Amount	Cost
1a.	Utility Van *** Acceptable - No comment	1	$9,000	$9,000
5.	Seed storage rooms to be operated at a temperature of -20 C (20'x100') *** These rooms are designated for the newly planned germplasm facilities. Approval of these room should occur later in the program since cryopreservation research and experience at the NBPGR may lead to a greater use of cryopreservation techniques thus requiring less need for -20 C mechanical refrigeration storage systems.	10	$50,000	$500,000
6.	Infrastructure for module shelves/ suitable shelving structure. *** See item 5 for comments.	10	$10,000	$100,000
7.	Emergency back-up generators *** Continuous, clean power is a serious problem at the NBPGR. Back-up generators should be purchased in concert with the purchase of other power consuming equipment. Included in the purchase should be power stabilizing units.	10		$133,000
8.	Controlled temperature (15-20 C) humidity (15-20%) equipment for seed drying and packaging. *** High priority. Should be purchased and installed as soon as possible. Units are available from Rotair, U.K.	2	$25,000	$50,000
9.	Temperature controlled walk-in seed germinator rooms (10' x 12') *** High priority. Should be purchased and installed as soon	3	$10,000	$30,000

as possible.

10. Seed germinators (Cabinet type) 21 $2,000 $42,000

*** Ten units are designated for the national laboratory and the remaining units for field sites. These units are critical for proper seed germination evaluation and should receive high priority. There are two basic types, dry chamber and water curtain. The dry chamber type would probably be preferred for NBPGR operations.

11. Microbalance (0.1 mg accuracy) 1 $7,500 $7,500

*** For seed evaluation work the microbalance should have ranges of accuracy, down to 1 microgram and have automatic taring (Cahn 26 or equivalent). A high priority piece of equipment since it is the primary balance used for seed moisture determinations in a genebank. Five more units should be purchased so that each of the remote sites have one each. In a genebank, seed has to be conserved. Consequently, only 3 replications of 3-5 seeds per replication are used for moisture determination. The total weight of 3 seeds can be relatively small, thus this balance works well with this kind of analysis.

12. Mettler Analytical Balance 5 $1,875 $9375

*** Acceptable unit. A high priority piece of equipment. No. of units should be increased so that field sites can have one each.

13. Top pan balance 0.1 g accuracy. 9 $1,500 $13,500

*** Acceptable unit. No comment.

14. Moisture determination balance. Infrared heater type. 2 $2,000 $4,000

*** Acceptable for general seed laboratory analysis. These units tend to consume a lot of seed. As an alternative to these units the oven drying method using small quantities of seed tends to be a more desireable method for seed moisture content determinations in a genebank operating environment. See item 11.

15. Soft X-ray machine, film reader and film processor. 1 $10,000 $10,000

*** Acceptable piece of equipment for a seed laboratory. The suggested cost may be a little low.

16. High temperature drying oven 8 $1,000 $8,000

*** A high priority piece of equipment necessary for the routine determination of seed moisture content.

17. Thermogradient plate with water baths 2 $11,000 $22,000

*** A good piece of equipment for a seed evaluation laboratory. There may not be any units commercially available, consequently component part may have to be purchased and assembled into a working unit. This piece of equipment has moderate priority.

19. Ultra-cold freezer (-75 C) 1 $7,000 $7,000
deep freezer (-20 C) 4 $1,000 $4,000

*** Useful for general seed physiology, tissue culture and cryopreservation research. These units have high priority.

20. Seed blower 10 $2,000 $20,000

*** These units have high priority in a seed genebank. Two types are generally needed. The South Dakota type is preferable for large seeded species. The analytical blower is preferable for the smaller seeded species.

21. Seedburo minivac small lot thresher and cleaner.

19 $750 $14,250

*** A necessary piece of equipment for general seed handling.

22. Hydrothermograph

10 $500 $5,000

*** A high priority piece of equipment. Units should be available to all field sites. These units should be "recording" hydrothermographs.

23. Microcomputer with printer, hard disc and software.

1 $6,000 $6,000

*** A high priority piece of equipment. Additional units should be considered for general laboratory research and specifically a unit for division heads. One unit per laboratory at a minimum, one unit per scientist preferred. These units are primarily for research and not the emerging NBPGR database system.

24. Bar code reader. 1 $1,000 $1,000

*** Not sure this would be a useful item at this stage of information handling and development.

25. Vitascope 1 $6,000 $6,000

*** Not familiar with this piece of equipment. No comment

26. Seed packet sealing machine 8 $900 $7,200

*** A necessary piece of equipment for genebank operation. These units have high priority.

27. Vacuum head seed planter 6 $1,500 $9,000

*** A high priority piece of equipment. There should be at least one unit per field station and 3 units at the main genebank. Consequently, 8 units should be ordered. The price per unit appears to be low. A wide variety of planting heads should be order with each unit.

28. Humidity guides. 20 $16 $320

*** These are low cost units that should be purchased with local funds. These units have low priority for this project.

29. PIX Box Image Processor - Analyzer. 1 $6,000 $6,000

*** This appears to be a nice unit and could open new avenues of research and evaluation of plant germplasm. The $6,000 cost is too low when a camera, disk storage units and output devices are added to the system. A more realistic number would be $14,000 to $18,000.

30. Accelerated aging cabinets 4 $4,000 $16,000

*** Useful for general seed physiology research experiments. Four units are too many. Two units should be sufficient.

31. Automatic seed analyzer 1 $10,000 $10,000

*** Not familiar with this piece of equipment. No comment.

32. Microwave oven 7 $500 $3,500

*** Assuming these ovens are for determinating seed moisture content. If so, the procedures are not worked out for most species. Consequently, I would suggest that only one unit be purchased for research on this technique.

62 Differential Thermal Analysis 1 $23,260 $23,260

*** This is a high priority piece of equipment for cryopreservation research. It would be preferable to build the DTA device rather than purchase a commercially made unit. Flexibility is gained by building their own unit. DTA can be done and development of cryopreservation freezing protocols can also be accomplished on the same unit. It is estimated that it would cost approximately $15,000 to $18,000. See Appendix II for detailed description and equipment needs. The building of this unit would make a good cooperative

project between the NBPGR and NSSL. All equipment could be purchased, assembled and software written at the NSSL by NBPGR and NSSL scientists, then shipped to the NBPGR for operating.

63 Cryenco Liquid Nitrogen Container			
Large	3	$6,000	$18,000
Portable Small	2	$1,975	$3,950

*** These are high priority pieces of equipment. I would suggest the following configuration:

Cryopreservation Research

MVE XLC 1830 Cryovat	2	$11,000	$22,000
SUC-1 Storage Boxes	990	$5.50	$5,445.
Voltrex Polyolefin Tubing			
3/4"	3000'	$1.10	$3,315.
1/2"	8000'	$0.70	$5,600.
3/8"	8000'	$0.73	$5,840.
Holding/Distribution LN2 Tanks			
MVE HLN800 (800 liters)	2	$10,000	$20,000.
Liquid Nitrogen Distribution Line			
MVE 3/4" Vacuum Insulated	22'	$90.	$1,980.

The above equipment and supplies would complement and complete the cryovat room now being constructed and partially equipped by the NBPGR (See Recommendations Section).

Pilot Project (See Recommendations Section)

MVE XLC 1830 Cryovat	4	$11,000	$44,000.
SUC-1 Storage Boxes	1320	$5.50	$7,260.
Voltrex Polyolefin Tubing			
3/4"	6000'	$1.10	$6,630.
1/2"	16000'	$0.70	$11,200.
3/8"	16000'	$0.73	$11,680.

64 Cytophotometer w/accessories	1	$60,000	$60,000
65 Cold Room 4 C Small	1	$23,650	$23,650
66 Amino Acid Analyzer	1	$20,000	$20,000
67 Percival Incubator LVL Model	1	$12,500	$12,500
68 Edgegard Hood Laeminar Flow Cabinet	1	$9,842	$9,842
69 Tissue Culture Room (Small Unit)	1	$19,685	$19,685
70 Polytron Microgrinder	1	$10,000	$10,000

71 Buchi Waterbath Roat Evaporator	1	$11,810	$11,810
72 Gyrotary Shaker G-2 Table Top Model	1	$10,000	$10,000
73 Mettler Balance (0.001 mg)	1	$3,500	$3,500
74 Gallon Kemp Orbital Incubator	1	$15,000	$15,000
75 IEC Center N Refrigerated Centrifuge	1	$23,650	$23,650
76 Fischer Vers Bath	1	$10,000	$10,000
77 Stereo Zoom Microscope	1	$7,000	$7,000
78 Microtome (Low Temp.)	1	$10,000	$10,000
79 Portable UV Lamp Long/Short Wave	1	$10,000	$10,000
80 Media Dispensing Units	3	$3,000	$9,000
81 Lyophilizer	3	$6,000	$18,000
82 Small Mill Grinder	1	$10,000	$10,000
83 Chromatographic Chamber (All Glass)	6	$1,000	$6,000
84 Micropipets (All Sizes) 1 Set	1	$4,000	$4,000

*** Items 64 to 84 are beyond my scope of experience. No comment.

The following additional items are recommended to complement the above equipment.

Conductivity Meter Seed physiology and tissue culture	1	$1,500	$1,500
Karl Fischer Titrator Seed and Tissue Moisture Analyzer	1	$4,500	$4,500
Gilson Differential Respirometer w/Light. Seed and tissue culture respiration experiments.	1	$12,000	$12,000
Oxygen (gas) Monitor Servomix Model 1175 Servomis LTD, Crowborough Sussex, England	1	$4,500	$4,500

*** High priority piece of equipment needed to monitor oxygen levels in the cryotank storage room. The unit should be connected to visible and audible alarms (locally supplied). See Appendix III for specifications.

*** - General Note: Time did not permit a detailed listing of specifications of critical pieces of equipment. To develop such details would require further consulting time and better access to relevant equipment literature.

LIQUID NITROGEN PRESERVATION OF SEED GERMPLASM

PILOT PROJECT IMPLEMENTATION GUIDELINES

NATIONAL BUREAU OF PLANT GENETIC RESOURCES
NEW DEHLI, INDIA

BACKGROUND

Seed deterioration is a fundamental physiological concern of a long-term seed "base" collection such as the National BUREAU OF PLANT GENETIC RESOURCES (NBPGR). The rate of seed deterioration is a function of seed moisture content, seed storage temperature, the viability of the sample when placed in storage and genetic selectio. (species and selections within a species). Seed moisture content and seed storage temperature are the two primary factors that can be practically controlled. Currently, the suggested seed moisture content for maximum longevity of most species is between 4 and 7%. Storage temperature standards have been selected essentially as a function of mechanical equipment limitations and is currently -10 to -18 C.

Seed deterioration continues even when the above standards are applied. Figure 1. shows that the germination performance of a number of important species stored at the National Seed Storage Laboratory, Fort Collins, Colorado, USA for at least 20 years continues to decline. Storage conditions during that period were 5 C and 40% R.H. for the first 10 to 12 years. The storage conditions were then changed to -10 C to -20 C and 4 to 7% seed moisture content in sealed containers for the remaining storage time. Metabolic processes, controlled in part by seed moisture content and storage temperature, are believed to be the primary sources of the deterioration of these samples. It is evident that further steps are required to restrain seed deterioration and reduce

routine genetic maintenance processes such as germination monitoring and regrowing of deteriorated samples.

Cryopreservation using liquid nitrogen (LN2, -196 C), offers the opportunity to significantly reduce seed deterioration in storage by virtually halting metabolism. Research conducted on a large number of species indicates that LN2 preservation can be used successfully with seed and pollen germplasm.

A species being considered for LN2 preservation must fulfill several basic physiological and genetic criteria. These are:

1) Seed germination should not be significantly affected by short-term (less than 1 day) and medium-term (less than 30 days) exposure to LN2. Germination percentages should not be significantly different at the 1% level of probability from a suitable control sample.

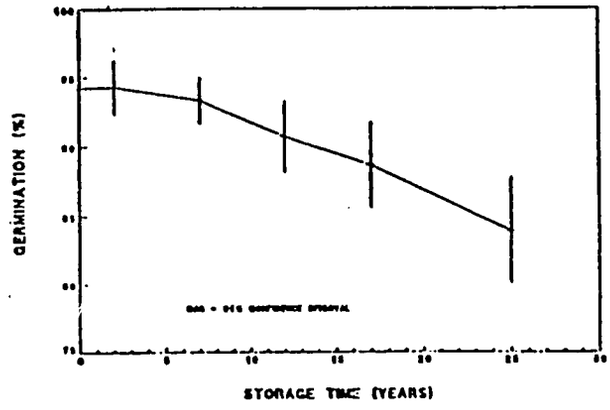
2) Seed moisture/LN2 exposure interactions. The relationship between seed moisture content and survival after LN2 exposure should be known in order to determine the optimum moisture content to ensure survival upon exposure to LN2. A safe seed moisture content region must exist, and seed must be easily adjusted to that moisture content. An example of this principle is illustrated in Figure 2. where the interaction of seed moisture and survival after exposure to LN2 are described for sesame seed. The decline in survival at high moisture contents occurs in all species and can range from 10 to 30% water. The decline in survival at low moistures occurs in a few species and at the time of this writing those species tend to be high in oil content such as in sesame seed.

3) Several selections should be tested to determine potential survival variation within a species due to genetic variation.

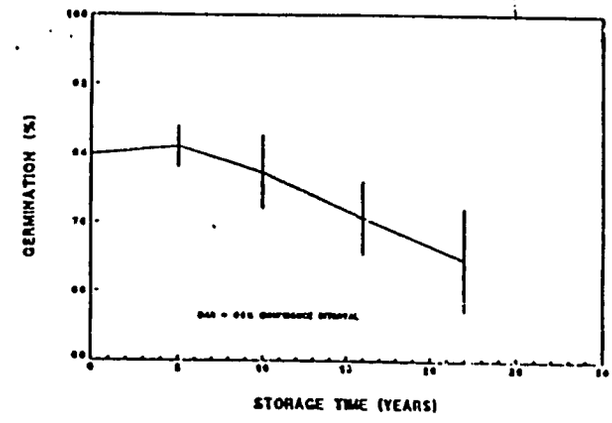
4) Seed cooling rates by either direct immersion into LN2 (approximately 200 C min-1) or into LN2 vapor (less than 30 C min-1) do not significantly differ in germination survival response. The exceptions are in high oil content crops where if the moisture content is excessively low and cooling rates are high, there may be some loss of germination. Figure 2.d illustrates this point with sesame seed. With a very high cooling rate of 200 C min-1 and seed moisture contents less than 6% there is a loss of up to 20 to 30% in germination. Therefore, to be conservative, the general recommendation is to cool the seed samples in the vapor phase. Seed warming rates from LN2 temperatures can vary significantly without damage to seed viability. The standard warming procedure is to remove the sample from cryogenic storage and let it warm under ambient laboratory condition to equilibrium. This would normally take 20 to 30 min. Once room temperatures are reached, the seed can be handled in a routine fashion. The LN2 cooling/rewarming cycle can be done a large number of times without risk of loss of seed viability.

Figure 1. Germination of seed samples stored at the National Seed Storage Laboratory for 20 to 25 years. Storage conditions were 5 C and 40% R.H. until 1978 when they were changed to standards of -10 to -20 C and 4 to 7% seed moisture content in sealed containers.

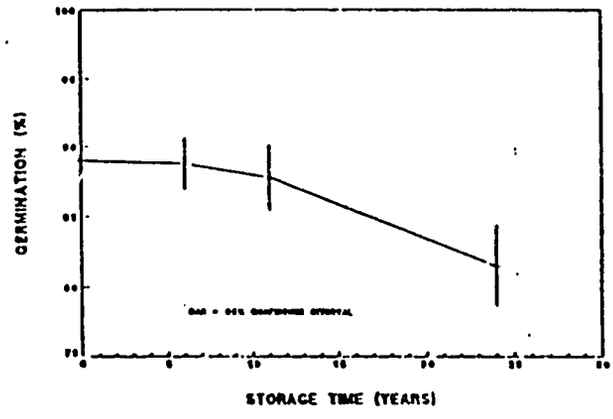
CORN SEED STORAGE
NSSL 1959-1984



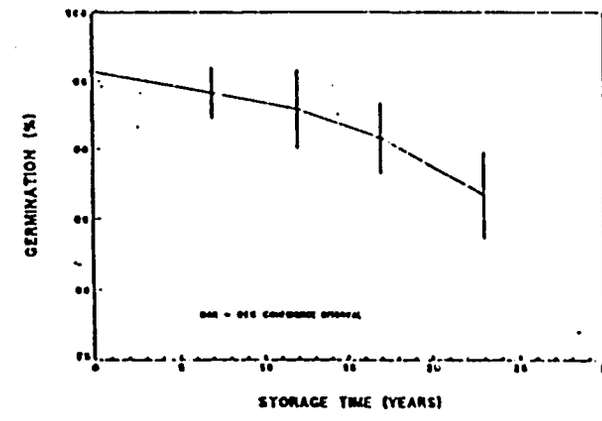
RICE SEED STORAGE
NSSL 1962-1984



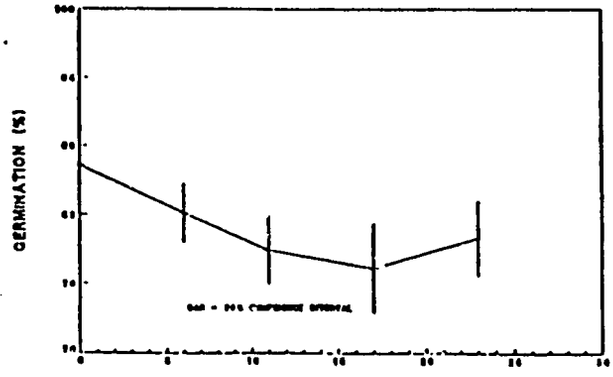
TOMATO SEED STORAGE
NSSL 1961-1985



WHEAT SEED STORAGE
NSSL 1961-1984



SORGHUM SEED STORAGE
NSSL 1961-1984



SOYBEAN SEED STORAGE
NSSL 1963-1984

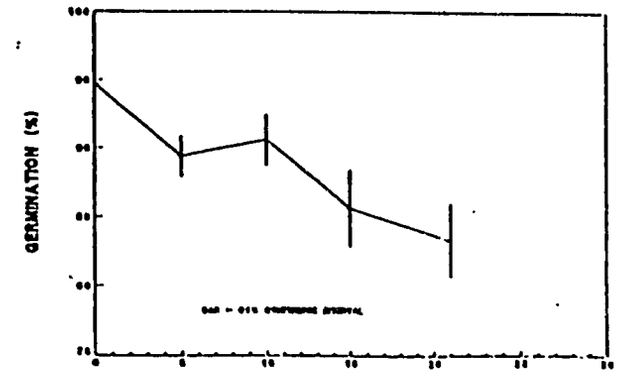
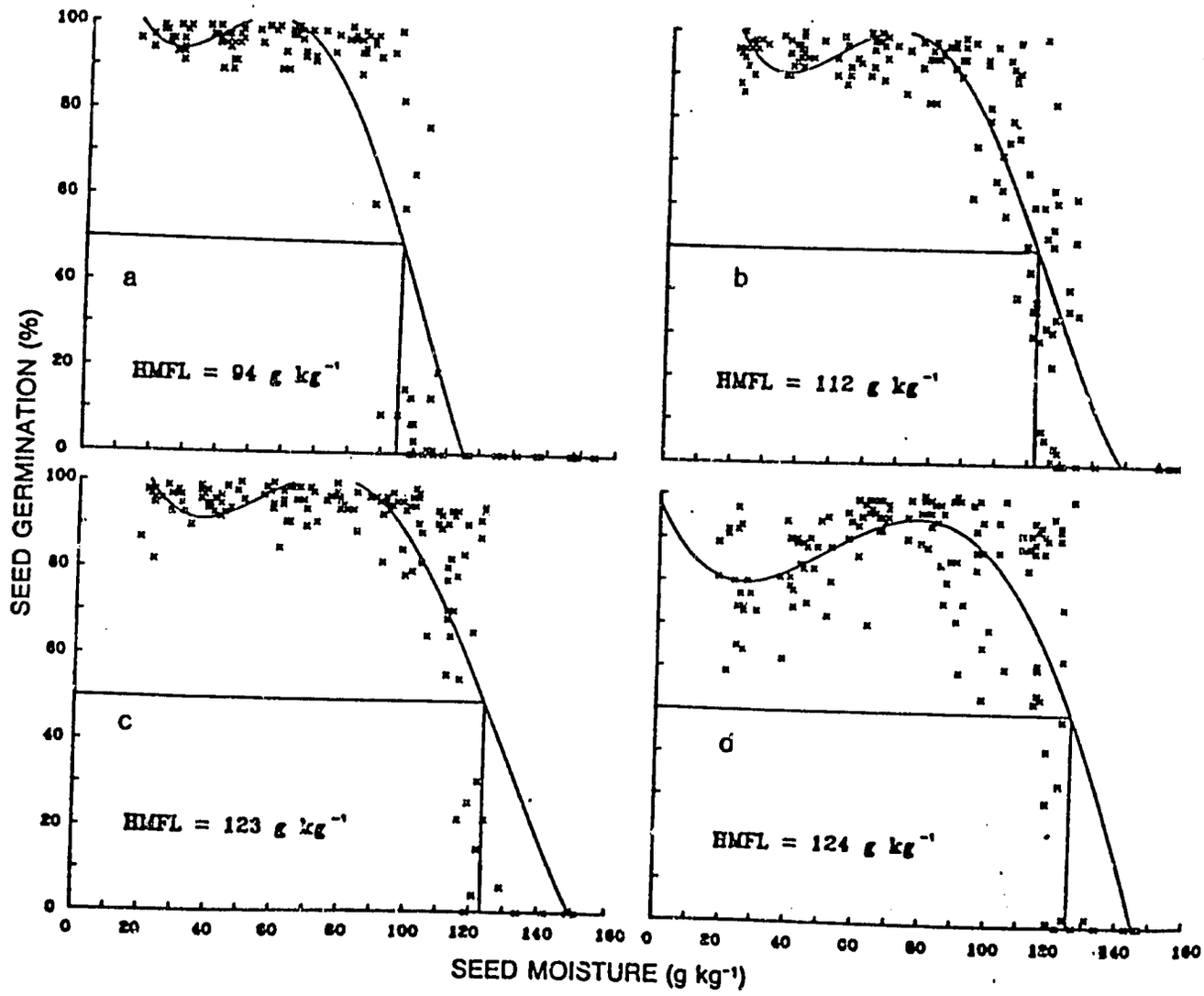


Figure 2. Survival (germination) of sesame seeds after exposure to LN2 at various seed moisture contents (fresh weight basis) and cooling rates: (a) 1 C min-1 cooling; (b) 10 C min-1 cooling; (c) 30 C min-1 cooling (d) 200 C min-1 cooling. Six selections were combined for each cooling rate. Data were fitted to a fourth order polynomial curve that were all significant at the 1% level of probability. High moisture freezing limits (HMFL) were determined by estimating seed moisture at 50% germination intersection on the curve (e.g., 94 g kg-1 for curve a.) (Note 94 g kg-1 = 9.4% seed moisture content).



GENERAL RECOMMENDATIONS

1) Sample size/container

The number of seeds per sample for LN2 preserved materials should be between 1,500 and 3,000 seeds. Clear plastic ("Voltrex" polyolefin) heat shrink tubes, "seed tubes", 3/8" to 3/4" in diameter and 12" long, sealed with end plugs will accommodate most medium sized seed such as sorghum and wheat. Smaller seeded species such as petunia can be stored in 1 to 5 ml plastic cryovials. NBPGR accession number, other accession identification number (e.g. cultivar name), genus, species and storage location should be on a label inside the storage container.

2) Cryotanks

MVE XLC 1830, 850 liter cryotanks or the equivalent should be used to store the seed samples. Samples should be stored in the vapor phase mode (Figure 3). Vapor phase storage mode is recommended based on the biological premise that a slower cooling rate for seed materials is more beneficial than rapid cooling (see previous section 4.) and that vapor phase storage provides a higher level of personnel safety. The vapor phase varies in temperature from -150 C to -196 C. From a cryopreservation stand point this temperature variance is relatively insignificant. Cryotank temperatures should not be allowed to be warmer than -120 C.

CRYOTANK CAPACITIES (MVE XLC 1830)

Sample Container	Number of Samples
1 ml plastic cryovial.....	49,000
5 ml plastic cryovial.....	20,000
3/8" seed tube.....	11,880
1/2" seed tube.....	5,940
3/4" seed tube.....	2,970

Figure 3. Seed tubes used for LN2 preservation of seed materials.

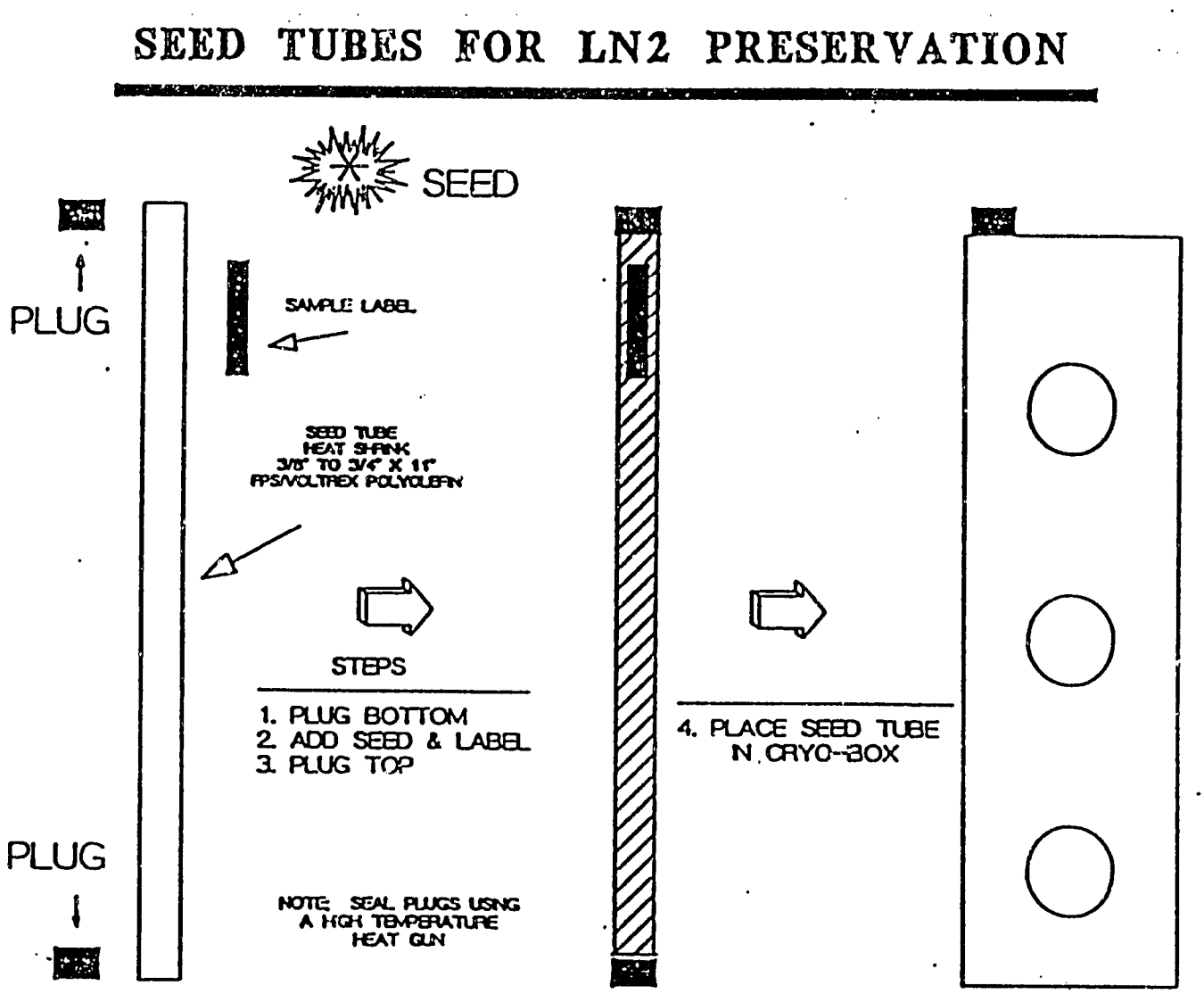


Figure 4. Liquid Nitrogen storage tank (cryotank): A) Cryotank operating in liquid phase (seed samples in LN2), liquid phase not recommended; B) cryotanl: operating in vapor phase (seed samples in vapor over LN2 reservoir), vapor phase recommended for seed cryopreservation.

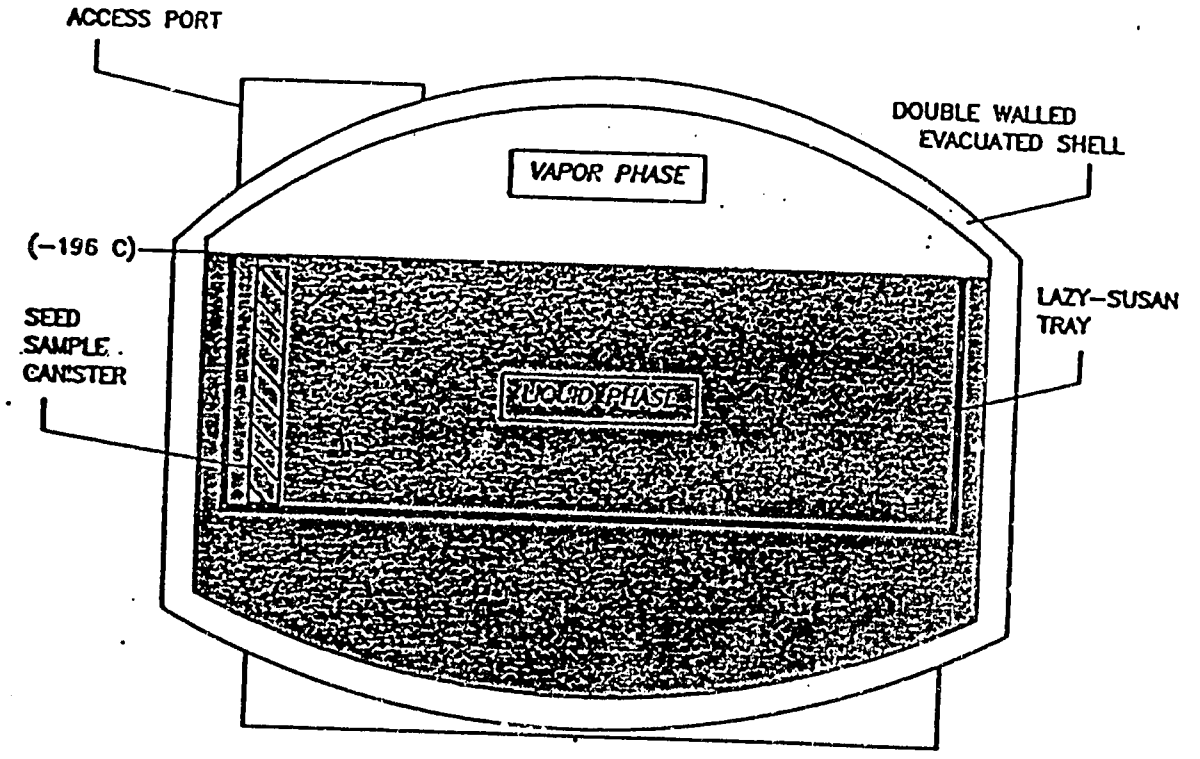


FIGURE 4A. Liquid nitrogen storage tank — liquid phase storage of seed.

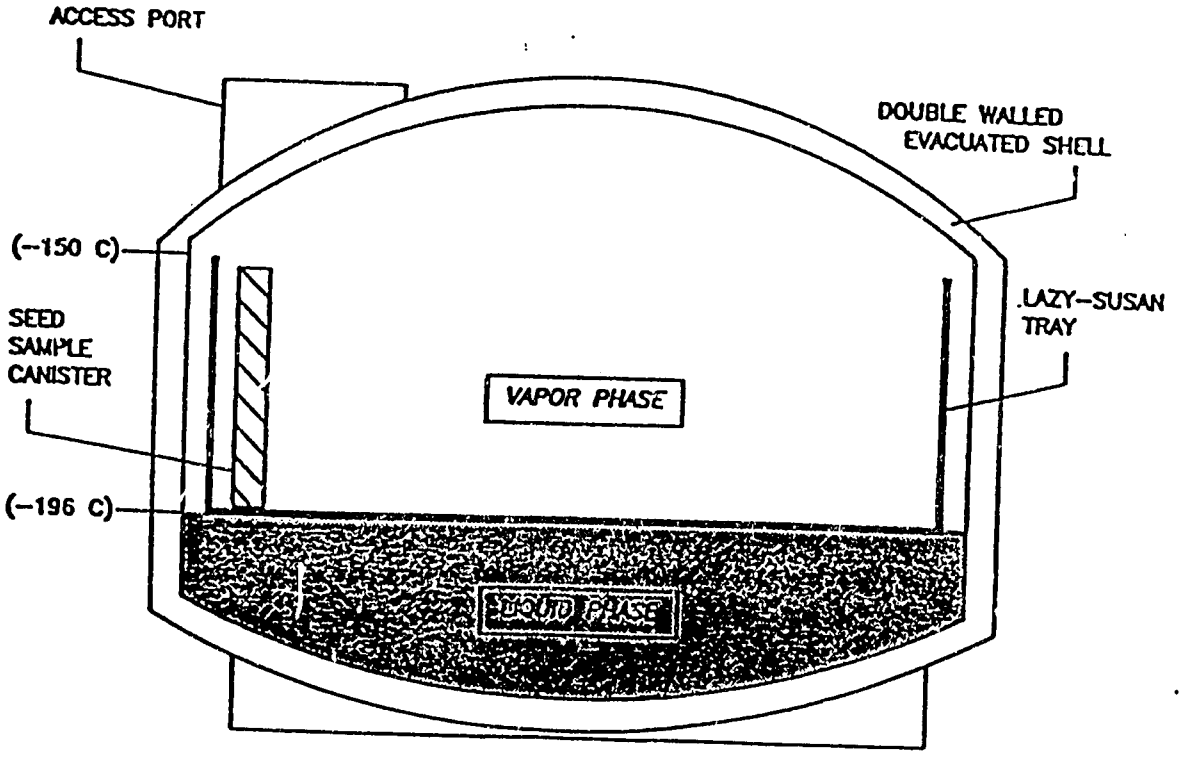


FIGURE 4B. Liquid nitrogen storage tank — vapor phase storage of seed.

3) PRIORITY SPECIES

Table 1. represents seed types (species), which are primary candidates for LN2 preservation. The type of seed container recommended; the estimated number of seeds that can be placed in that container; and the number of samples that can be stored in a cryotank are also presented. Small grains, most vegetable and grass seeds as a group have demonstrated the least amount of LN2 exposure problems and would therefore be highest on the recommendation list. High oil seeded species and large seeded species would have less priority as a group because of the need for more expansive storage facilities and a tendency for the oil in the seeds to have a negative impact on LN2 survival, especially if the seed is excessively dried and cooled to LN2 at a rate exceeding 30 C min-1.

Table 1. Seed Types Suggested for LN2 Long-Term Preservation.

Seed Type	Seed Container	Estimated No. Seeds/ Container
Triticum	3/4" Tube	1,760
Avena	3/4" Tube	1,500
Sorghum	3/4" Tube	2,100
Hordeum	3/4" Tube	1,300
Oryza	3/4" Tube	1,600
Lycopersicon *	1/2" Tube	2,560
Cucumis	3/4" Tube	1,200
Festuca	3/8" Tube	7,020
Secale	3/4" Tube	1,400
Nicotiana *	1ml Vial	11,148
Trifolium	3/8" Tube	15,000
Cucurbita	3/4" Tube 2ea	1,200
Dactylis *	1/2" Tube	7,000
Capsicum	3/4" Tube	7,000
Agropyron *	1/2" Tube	1,300
Lactuca	3/8" Tube	6,000
Beta	3/4" Tube 2ea	2,500
Eragrostis	3/8" Tube	1,200
Poa *	3/8" Tube	9,720
Allium	1/2" Tube	3,430
Lotus *	3/8" Tube	5,500
Raphanus	1/2" Tube	1,800
Phleum *	3/8" Tube	6,840
Spinacia *	3/4" Tube	3,300
Onobrychis *	3/4" Tube	2,700
Elymus *	1/2" Tube	2,800
Daucus	3/8" Tube	8,820
Petunia *	1ml Vial	10,000
Saccharum	1ml Vial	5,000

* Seed moisture content/LN2 exposure survival curves needed.

Table 2. is a listing of seed types which are not recommended for LN2 preservation at this time. Of particular concern are Linum and Medicago which have excessive problems of seed and cotyledon cracking. The other species in the list are either very large seeded which generates problems of efficient use of storage space or more information needs to be collected.

Table 2. Seed Types Not Recommended for Liquid Nitrogen Long-Term Preservation

Seed Type	Reason for Not Recommending
Aegilops	More exposure and moisture data needed
Apium	More exposure and moisture data needed
Arachis	Large seeded, more exposure data needed
Datura	More exposure and moisture data needed
Eleusine	More exposure and moisture data needed
Glycine	Large seeded, some seed cracking
Linum	Excessive seed cracking
Lolium	More exposure and moisture data needed
Medicago	Some cotyledon cracking
Oenothera	More exposure and moisture data needed
Panicum	More exposure and moisture data needed
Phalaris	More exposure and moisture data needed
Phaseolus	Large seeded, some seed cracking
Solanum	More exposure and moisture data needed
Zea	Large seeded, more exposure data needed

NOTES:

LARGE SEEDED - seed size is so large that LN2 preservation may be currently impractical.

MORE EXPOSURE DATA - minimal LN2 exposure studies indicate seed type survives but more selections need to be tested.

MOISTURE DATA - high and low seed moisture content/LN2 exposure response data needed.

4) PROCESSING PROCEDURES - GENERAL

Table 3. outlines the generalized in-processing procedure for samples scheduled for LN2 preservation. An important procedure is testing seed viability after exposing the germination sample to LN2. The objective is to determine the viability of each accession after exposure to LN2 to and provide an initial germination result for record keeping. The test is conducted by taking a sub-sample of the main sample and exposing it to LN2 for at least 2 hours, rewarming the sample, then evaluating seed germination using standard evaluation procedures. Germination of the LN2 exposed sub-sample must not be significantly lower than the minimum acceptable germination for that species. If the variance is not acceptable, a retest will be conducted to confirm the result. Seed samples will not be placed into LN2 storage until the germination evaluation has been completed and minimum standards have been met. Significant variations can occur in different species. Consequently, final disposition of LN2 preservation of an individual seed sample will be made by the base collection curator.

Table 3: CRYOPRESERVATION OF SEED GERMPLASM
GENERALIZED PROCEDURES

NEW SAMPLES	EXISTING SAMPLES
1. Check-in Sample	1. Re-weigh seed sample, conduct moisture and LN2 germination test. Place seed sample in temporary storage until tests are completed.
a. Place sample in temporary storage at 5 to 15 C with a relative humidity of less than 40% (15-20% preferred) for drying to optimum seed moisture content for LN2 storage.	
b. Create master computer record and generate appropriate labels and hard copy documents.	2. Place sample in permanent LN2 or conventional storage as appropriate.
2. Clean, weigh, and repackage seed sample.	
3. Conduct moisture and LN2 exposure germination test. Place seed sample in temporary storage until tests are completed.	
4. Place sample in permanent LN2 or conventional storage as appropriate.	

SUMMARY

The preservation of seed germplasm using LN2 is practical for a large number of species. There are species differences however, so the decision to use LN2 as a storage medium has to occur on a species by species basis, taking into account biological adaptability, risk factors relating to other storage techniques and cost. This paper provides a general background to LN2 seed preservation. Specific recommendations for a genus or species will be provided as information becomes available.

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ADDENDUM 1.

SEED CRYOPRESERVATION RECOMMENDATION
NATIONAL SEED STORAGE LABORATORY
FORT COLLINS, COLORADO

Wheat (Triticum sp.) seed has been identified as a species that tolerates exposure to LN_2 and is recommended for long-term cryopreservation at the National Seed Storage Laboratory.

GENERA: Triticum; all species

COMMON NAME: WHEAT

CRYOGENIC METHOD: LN_2 vapor phase.

AMOUNT TO BE PRESERVED: 1,500 to 3,000 seeds per accession

STORAGE CONTAINER: 3/4" polyolefin seed tubes.

CRYOVAT: Cryenco MX-50, 2,970 seed tubes/cryovat or equivalent.

SEED MOISTURE CONTENT: 4% to 7% (f.w.b.)

LN_2 EXPOSURE TEST: Yes, sub-sample of each accession. (Minimum of a 2 hour exposure).

MINIMUM REQUIREMENTS: 85% germination

1,000 seeds with a requirement that replenishment seed is forthcoming.

EXCESS SEED DISPOSITION: 1) Return to donor
2) Distribute to other users

VIABILITY MONITORING: Technique: Representative accession monitoring. A minimum of one accession in every 500 accessions will have 2 extra seed tube samples placed into LN_2 storage. During initial implementation a minimum of 500 accessions will be needed to represent the expected number of wheat accessions to be cryopreserved. A higher rate of sampling can be done as deemed appropriate by the base collection curator. These duplicate samples will be used to monitor viability and moisture content of the stored accession. Viability and moisture content will be conducted on the monitoring samples once every 10 years.

ADDENDUM 2.

Date: Sept. 2. 1986

Common Name: Wheat
Genus/species: Triticum sp.
No. Seeds/gram: 25

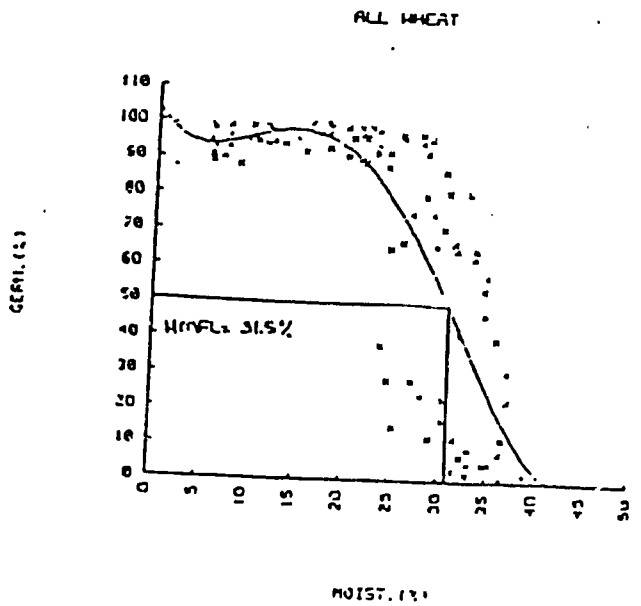
I LN2 Exposure Results - Short Term (Less than 1 month)

No. of Selections	Storage Time	Seed Moist.	Germination Before LN_2 Exposure	Germination After LN_2 Exposure	Reference
3	1 mnth	8.8%	87.7%	83.3%	3
1	1 hr	5-16%	80.0%	80.0%	6
1	6 hr	NR	96.0%	100.0%	9
1	2 hr	<17.0%	90.0%	90.0%	10
1	2 min	10.6%	92.0%	92.0%	11

II LN2 Exposure Results - Long Term (more than 1 month)

No. of Selections	Storage Time	Seed Moist.	Germination Initial	Germination After LN_2 Exposed	Reference
1	1 yr	8.3%	95.0%	96.0%	2
3	5 yr	8.8%	87.7%	88.3%	4
3	3 yr	8.8%	87.0%	83.0%	5
1	180 dy	9.0%	94.0%	90.0%	7,8
129	2 yr	8.2%	97.0%	97.0%	12

III High Moisture Freezing Limit Results



HMFL = 31.5%

(Reference on reverse side)

ADDENDUM 3.

SEED GERMINATION AND MOISTURE CONTENT
STORED FOR 5 YEARS AT 5, -18 AND -196°C

Selection	Original Germination	Germination 5C	Germination -18C	Germination -196C	Moisture (fwb) 5C	Moisture (fwb) -18C	Moisture (fwb) -196C
	%						
Barley(3)	97a	94a	97a	96a	12.6a	11.7a	9.0b
Corn(1)	99a	99a	98a	96a	8.6a	8.6a	8.8a
Pearl Millet(1)	85a	73b	87a	79ab	13.9a	13.3a	7.5b
Rice(2)	96a	90a	93a	94a	14.5a	12.1b	9.8c
Sorghum(7)	96a	94a	95a	94a	14.4a	12.1b	9.5c
Wheat(3)	84ab	76b	89a	85a	12.7a	11.7a	9.3b

() Number of selections

NOTE: Germination and moisture content values within a given selection with the same subscripts (a,b or c) are not significantly different at the 1% level of probability as evaluated by the LSD test.

ADDENDUM 4.

GERMINATION COMPARISON OF NSSL SAMPLE VS. LN2 SAMPLE
AFTER TWO YEARS IN STORAGE

Triticum aestivum L.
STORAGE TEMPERATURE (2yrs)

NSSL No.	-18 C DATE	-18 C GERM	-196 C DATE	-196 C GERM	GERMINATION *DIFFERENCE (%) -196C MINUS -18C
123485.01	9/81	96	11/86	96	0
28690.01	8/84	96	11/86	96	0
125143.02	3/83	96	11/86	96	0
52597.01	7/84	96	11/86	95	-1
140419.02	3/83	97	11/86	100	3
177150.01	3/83	97	11/86	98	1
124937.02	2/83	97	11/86	100	3
124967.02	2/83	97	11/86	95	-2
116202.01	4/81	97	11/86	98	1
124868.02	12/82	97	11/86	99	2
124855.02	1/83	97	11/86	99	2
124944.02	2/83	97	11/86	95	-2
124930.02	2/83	97	11/86	97	0
124864.02	12/82	97	11/86	95	-2
124918.02	2/83	97	11/86	97	0
177128.01	1/83	97	11/86	100	3
144269.02	12/82	98	11/86	98	0
177134.01	1/83	98	11/86	99	1
125140.02	2/83	98	11/86	98	0
124933.02	2/83	98	11/86	97	-1
124406.02	2/83	98	11/86	97	-1
124988.02	2/83	98	11/86	97	-1
125010.02	2/83	98	11/86	97	-1
124945.02	2/83	98	11/86	100	2
124821.02	12/82	98	11/86	99	1
124928.02	1/83	98	11/86	99	1
124926.02	2/83	98	11/86	98	0
28691.01	8/84	98	11/86	94	-4
124938.02	2/83	98	11/86	97	-1
124991.02	2/83	98	11/86	92	-6
28692.01	8/84	98	11/86	100	2
20603.01	7/84	98	11/86	91	-7
177077.01	1/83	98	11/86	99	1
125239.02	3/83	98	11/86	98	0
124890.02	2/83	98	11/86	99	1
177125.01	1/83	98	11/86	100	2
124942.02	2/83	98	11/86	98	0
125278.02	3/83	98	11/86	99	1
54006.01	5/85	98	11/86	93	-5
124965.02	2/83	98	11/86	94	-4
177073.01	2/83	98	11/86	99	1
124881.02	2/83	98	11/86	98	0
177081.01	1/83	98	11/86	96	-2
124794.02	12/82	98	11/86	100	2
177074.01	2/83	98	11/86	96	-2
125020.02	2/83	99	11/86	96	-3

APPENDIX I.

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Dr. K. Chaudhury
Ms. S. Lakhampaul

Division of Germplasm Conservation

Dr. P.P. Khanna (Head)
Dr. S.K. Jain

Documentation Unit

Dr. R.L. Sapra

USAID - Dr. Mark Smith (India)

Winrock International

Dr. Flyod Williams (USA)
Dr. Guy Baird (India)
Ms. Bhatt (USA)

APPENDIX II

Items for a DTA Device

Major Items

1. Microcomputer AT Compatible 1 $12,000

1 meg internal memory
360 kbyte floppy drive
20 to 30 mbyte hard disk
Parallel & Serial Ports
Internal Battery Operated Time/Clock Calendar
EGA or VGA Color Graphics Board and Monitor
with Basic/Lotus 123

2. Text/Graphics Printer 1 $750.

Minimum of 18 pins
132 Characters Wide
Parallel Interface

3. Data Acquisition Board - Burr Brown or Equiv. 1
$3,000.

16 paired analog inputs A/D Conversion 14 or 16 bits.
On-board zero temperature compensation for thermocouples.
4 Digital Outputs TTC with optically isolated relays
IBM AT Compatible
4 Digital Inputs TTC

4. Thermocouple Wire Type "T" 22 gauge. 1000' $200.

5. Cryomed LN2 cooled sample chamber.
w/Solenoid, fan, heaters and LN2 hose & connections.
1 $1,500.

6. Misc connectors and relays. $500.

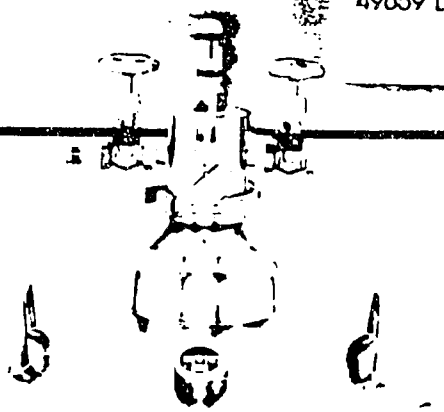
7. MVE Mini Pressure Vessel 1 $1,200
Model LW-60 w/stand (See next page)

Appendix II

SOLD and SERVICED BY:

CRYOMED

49659 Leona Dr. • Mt. Clemens, MI 48045
(313) 949-4507



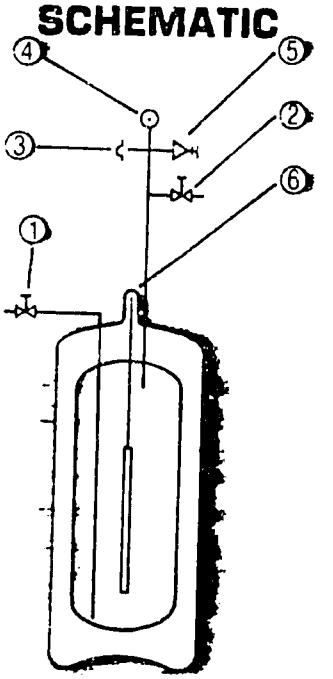
MINI PRESSURE VESSEL

Cryogenics

MODEL LW-60

The Model LW-60 is designed for users of liquid nitrogen who require less capacity than commonly provided by 110 or 160 liter cylinders. This includes many research and medical laboratories as well as industrial applications. Designed to operate at 22 PSI the LW-60 has a 60 liter liquid nitrogen capacity and is completely fabricated from *stainless steel.* Ease of operation is provided by a ⅜ inch fill valve, ⅜ inch vent valve, and float type liquid level sight gauge. A roller base is also available. The LW-60 has two convenient carrying handles.

SCHEMATIC



Specifications

Liquid Capacity	60 Liters
Loss Rate	1.4 Liters Per Day Max.
Operating Pressure	22 PSI
Weight Empty	62 Pounds
Weight Full	165 Pounds
Outside Diameter	18 Inches
Overall Height (To top of liquid level sight gauge)	32½ Inches
Overall Height (To top of flange)	27½ Inches
Connections	
Fill and Withdraw	⅜" Female NPT
Vent	⅜" Female NPT

NOMENCLATURE

1. Liquid Valve
2. Vent Valve
3. Burst Disc (45 PSI)
4. Pressure Gauge
5. Relief Valve, 22 PSI
6. Liquid Level Gauge

Minnesota Valley Engineering

407 7TH ST. N.W., NEW PRAGUE, MN 56071 U.S.A. TELEPHONE 612-758-8291 TELEX NO. 29-0571

Appendix III

Servomex

7982-2570
Issue 1
May 1986

Instruction Manual

SERVOMEX 1175

Oxygen Monitor

Servomex Ltd
Crowborough
Sussex
England

SYBRON

1.3 Sampling System

The sampling system of the monitor includes a combination filter/automatic flow control device, designed to keep a constant flow of sample gas through the measuring cell for varying input pressures and to prevent the entrance of particulate matter into the measuring cell. Excess flow is vented to the by-pass.

1.4 Specification

Performance Specification (typical)

Repeatability:	Better than +/-0.2% O2 under constant conditions.
Alarm accuracy:	Better than 1% O2 of the absolute oxygen value for ambient temperature changes within +/-10 deg C of calibration temperature.
Drift:	Less than 0.2% O2 per week under constant conditions. (Excluding variation due to barometric pressure changes; reading is proportional to barometric pressure.)

Outputs

Display:	3 1/2 digit LCD reading 0.0 to 25.0% oxygen with overrange capability.
Output:	4-20mA (isolated) or 0-1V for 0-25% oxygen available on internal connector block.

Alarm output:

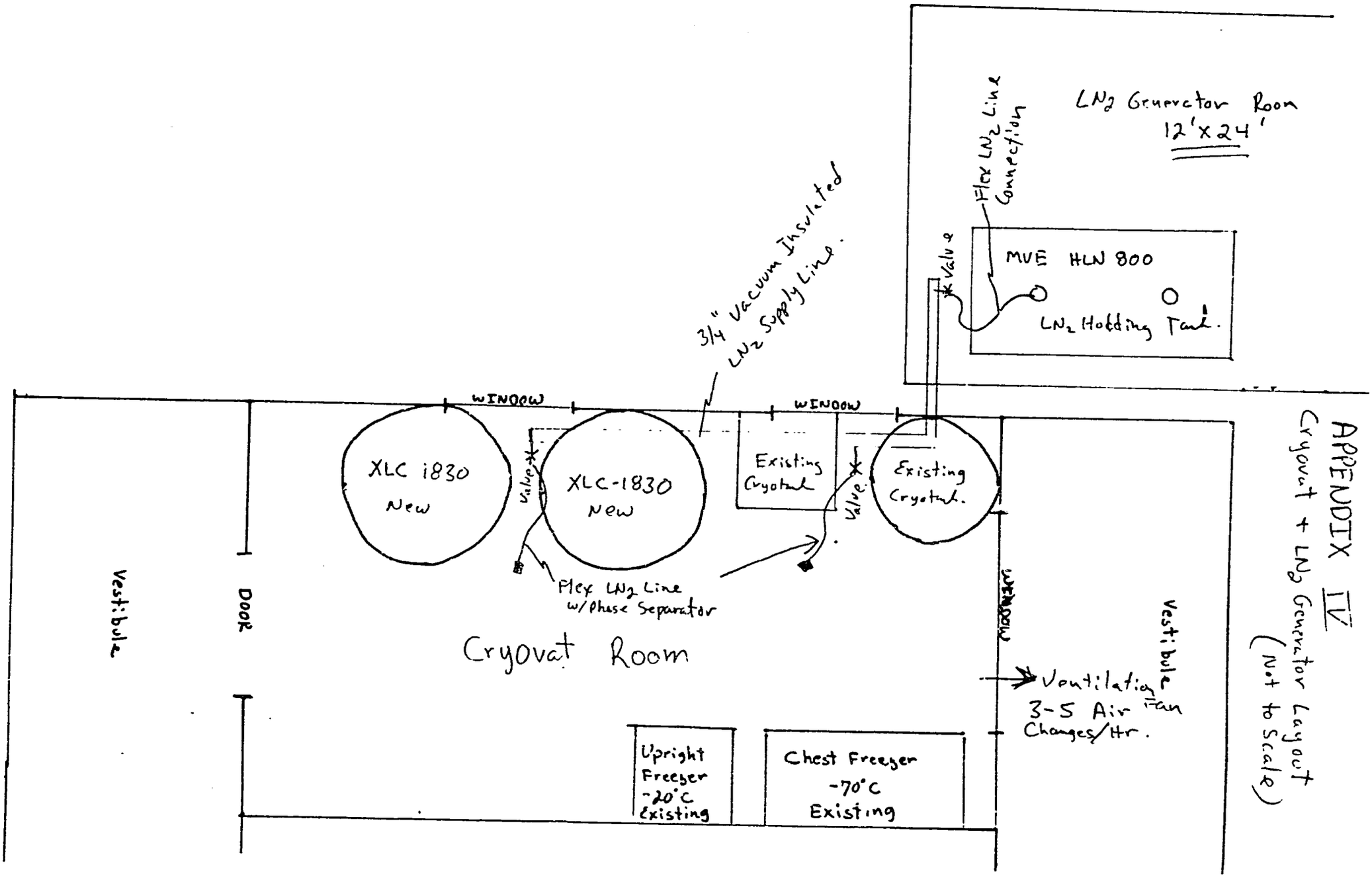
Oxygen level:	Change over relay contacts rated at 3A/115V ac, 1A/240V ac or 1A/28V dc. 4 contacts available, 2 single pole change over, 2 normally closed.
Flow fail:	Separate alarm contact for sample flow fail, SPCO.
Local alarm:	Red LED lamps flash when alarm active.

Sample requirements

Condition:	Clean, dry gas with dew point 5 deg C below ambient temperature.
Response time:	Less than 15 secs. to 90%

APPENDIX IV

Cryovat + LN_2 Generator Layout
(Not to Scale)



APPENDIX V.

LIQUID NITROGEN SUPPLY SYSTEM

COST AND OPERATING COST ANALYSIS

SYSTEM A - On-Site Production and Storage of LN2
(10 day operating reserve)

EQUIPMENT COST

1)	LN2 Generator 10 LPH Sulzer Model LIN1T 10	$ 153,800.
2)	Holding/Distribution LN2 Tanks MVE Model HLN600 2ea @ $10,000 ea	$ 20,000.
3)	LN2 Distribution Line - Vacuum 22 feet @ $90/ft	$ 1,980.
	Total Equipment Cost	$175,578.
	Expected Lifespan 10 yr. Cost/yr	$ 17,578./yr

MAINTENANCE COST	2.4% purchase price/year	$ 3,715./yr
ELECTRICITY COST	Approx. $.35/yr 15,000 l/yr	$ 5,250./yr
	TOTAL COST/YEAR	$ 26,543./yr
	COST LN2/LITER	$ 1.77/l

(LN2 Analysis - Continued)

SYSTEM B - Purchase LN2 Off-Site, Storage On-Site
(50 day operating reserve)

EQUIPMENT

1)	LN2 Storage Tank MVE VCS-900SS-250 3,400 liters	$ 32,000.
2)	LN2 Distribution Vacuum Line 22 feet @ $90/ft.	$ 1,980.
	TOTAL EQUIPMENT COST	$ 33,980.
	EXPECTED LIFESAPN 20 YRS	$ 1,699./yr
LN2 COST	15,000 l/yr ($0.75 to $1.00 / liter)	$ 15,000./yr
	TOTAL COST/yr	$ 16,699./yr
	COST LN2/liter	$ 1.09/l

APPENDIX VI.

LN2 Consumption Estimates - Full Operation

LN2 Storage Container			Capacity liters	LN2 Consumption (liters/day)
1)	LA RCB 1000S	On Order	933	8.0
2)	CMS-86	On Order	86	3.0
3)	CMS-328	On Order	328	7.0
4)	IBP60 2each	On Hand	60	0.2
5)	IBP40 4each	On Hand	40	0.4
6)	IBP20	On Hand	20	0.1
7)	MVE XLC 1830	Recommended	860	7.7
8)	MVE XLC 1830	Recommended	860	7.7
9)	MVE HLN600	Recommended	600	5.5
10)	MVE HLN600	Recommended	600	5.5
11)	General Research Activities			10.0
	SUB-TOTAL LN2 CONSUMPTION			55.1
	MISC. LOSS 10%			5.5
	TOTAL EXPECTED LN2 CONSUMPTION/DAY			60.6 l/day
	YEARLY CONSUMPTION			22,220 l/yr