A Practicum Note for the
International Training Course on

Plant Genetic Resources and Genebank Management

Held at the Rural Development Administration, Suwon
Republic of Korea on 7–18 September 2009

Sponsored by:

Bioversity International

RDA

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Republic of Korea

September 2009

Foreword

During the "Global Seed Hub Declaration Ceremony" held by RDA in November 2008, delegates of partner countries adopted the Resolution on RDA's role-taking as "the Center of Excellence for International Cooperation and Training on Plant Genetic Resource" and "Cryo-preservation Center for vegetatively propagated crop"

In December 2008, RDA and BI agreed to sign an MOU on the Center of Excellence for International Cooperation and Training on Plant Genetic Resources. RDA and IPGRI signed an MOU on March 27, 2009.

In the new MOU, Bioversity recognizes the strong capacity of RDA as the Center of Excellence for International Cooperation and Training on Plant Genetic Resources. Bioversity also supports the establishment of an International Center for Cryopreservation R&D.

The international training course on plant genetic resources and genebank management will strengthen the capability of many countries to conserve and manage their genetic resources. Coupled with the safety-back-up in Korea, this will ensure the long term conservation of very important genetic materials of various countries. The cryo-preservation R&D and facilities will greatly improve our efforts to conserve our very fragile genetic materials that cannot be stored as seeds like coconut and other vegetatively propagated crops.

This practicum note was prepared for practical subsidiary of the International Training Course on Plant Genetic Resources and Genebank Management. We hope that this practical subsidiary will be useful for this training course.

Ki Hun Park
Director
National Agrobiodiversity Center, RDA

Contents

Time Table of this Training Course

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12:00-1:00PM	LUNCH BREAK					
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2:00-3:00PM						
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5:00-6:00PM						

Time Table of this Training Course

Date	Sept. 14 (Mon)	Sept. 15 (Tue)	Sept. 16 (Wed)	Sept. 17 (Thu)	Sept. 18 (Fri)	Sept. 19 (Sat)
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12:00-1:00PM	LUNCH BREAK					
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4:00-5:00PM						
5:00-6:00PM						

Vegetable Seed Regeneration and Quality Preservation- Andreas Ebert
Standardized protocol for seed regeneration and seed storage of eggplant -Andreas Ebert
Sept. 13(Sunday) : We(trainee) plan to visit Seoul city(capital of Korea) for cultural experience - Dong Suk Park

Part 1
Subsidiary Lectures

LECTURE 7 SUBSIDIARY. Utilization of Plant Genetic Resources in Pre-breeding/ Parental Rice Line Breeding for Genetic Enhancement

Woon-Goo Ha.

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Introduction

The low utilization of conserved plant genetic resources in most genebanks is due to lack of documentation and inadequate description of collections, lack of the information desired by breeders, and lack of evaluation of collections. In order to fully utilize available genetic diversity in gene banks, pre-breeding or parental line breeding of exotic/unadapted materials should be undertaken.

Pre-breeding//parental line breeding refers to all activities designed to identify desirable target characteristics and/or genes from unadapted (exotic or semi-exotic) materials, including those that, although adapted have been subjected to any kind of selection for improvement. Exotic materials include any germplasm that do not have immediate usefulness without selection for adaptation for a given area.

Pre-breeding//parental line breeding is a vital step to link conservation and use of plant genetic resources especially in breeding programs. It aims to reduce genetic uniformity in crops through the introduction of a wider base of diversity, as well as to increase yields, resistance to pests and diseases, and other quality traits.
Pre-breeding//parental line breeding programs can generate new base populations for breeding programs and also assist in identifying heterotic patterns for hybrid programs. Pre-breeding aims to provide breeders with enhanced germplasm materials which have specific traits of interest as well as a means to broaden the diversity of improved germplasm.
Pre-breeding//parental line breeding programs is one of the breeding program, most of breeders normally use this programs. So we can't identify with method or tools. When increased utilizations of wild genetic resource with all breeding method and program. Normally we talk as parental line breeding. Also, we evaluate some characteristics of new genetic resource for breeding, we talk pre-breeding.

Objectives

1. To discuss the value of pre-breeding or parental rice line breeding for genetic enhancement in the utilization of conserved germplasm in genebanks
2. To discuss methods of pre-breeding/parental rice line breeding

Lessons to learn

1. What is pre-breeding//parental line breeding?
2. Commonly used conventional breeding methods
   - Mass selection

- Pure line selection
- Pedigree breeding
- Bulk breeding method
- Single-seed descent
- Recurrent selection
- Back cross breeding
- Additional method

3. Modern tools : Role of biotechnology
   - Anther culture
   - Molecular marker
   - Genetic engineering
   - Wide hybridization
   - Mutation breeding
4. Major achievement of rice breeding in Korea
5. Breeding program in Korea
6. Rice breeding practices
7. Practices of cold-tolerant rice parental line breeding

Considerations in parental line breeding

- Basic study of target characteristics
- Development of screening method
- Screen of germplasm for target characteristics
- To make genetic variation and breeding population
- Screen of breeding population for target characteristics
- Identification gene of target characteristics
- To release as new variety and to use for parental line of target characteristics

LECTURE 8 SUBSIDIARY. Genebank Development and Management

Introduction

Virtually all countries in the world maintain germplasm collections, with varying facilities, budget, capabilities including staff, physical environment and policy framework. Maintaining plant genetic resources under optimum conditions of management is of prime importance if only to insure that the objectives of germplasm conservation and sustainable use will be met.

Genebank management begins with institutional legal and policy framework. In addition, the relationship of the genebank with national and global policy regimes should be clearly understood.

The effect of infrastructure and budgetary considerations on the management of germplasm collections, in addition to the constraints posed by the physical environment in which the genebank facilities are located, should also be considered.

The germplasm managers and curators should always keep abreast of and take into account in the management of genebanks and germplasm collections the good practices from acquisition to registration to regeneration to conservation to characterization to distribution to documentation.

The management of germplasm banks for ex situ conservation includes a sequential development of stages, that is collection, multiplication, regeneration, documentation, characterization, evaluation, and, lastly, distribution.

After 2 decades of intense concern to create germplasm banks, interest is shifting towards developing strategies to improve the composition and management of collections. The increasing global emphasis on short-term solutions has further increased the need to ensure that decisions are optimal for the long term.

When you have completed this course, you should be able to identify the most important aspects of managing plant germplasm banks.

Objectives

1. Discuss the options and considerations in developing effective genebank management strategies
2. Discuss the important elements of management of the genebank and the collections
3. Discuss options for efficient and cost effective management of seed collections in genebanks
4. Discuss risk identification and actions to minimize or manage risks at the various genebank activity and operations

Description of lessons covered

This lecture will discuss the biological factors that influence breeding system, seed storage characteristics, conservation and utilization concepts and maintenance of

seed quality; and the routine operations of genebank operations and practices and their implications for maintaining genetic integrity. This lecture will also cover the issues on the risk identification and actions to eliminate or manage the risks in the genebank management procedures including collection, multiplication, regeneration, documentation, characterization, evaluation and distribution.

Lessons to learn

The lessons learned from the previous modules will be integrated into an effective genebank strategy that takes into account policy, infrastructure, personnel, physical facilities and routine operations

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Part 2
Practicum Note

PRACTICUM NOTE 1. Seed Processing and Drying

Young-Wang Na, Yu-Mi Choi

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Introduction

Seed storage is the preferred method for 90% of the six million accessions conserved in ex situ collections worldwide because it is practical and economical. It is the principal conservation method for species producing orthodox seeds that withstand desiccation to low moisture contents and storage at very low temperatures. Techniques for conserving orthodox seeds have been perfected for several decades. These involve drying seeds to low moisture contents (3-7% gresh weight, depending on the species) and storing them in hermetically-sealed containers at low temperature, preferably -18℃ or cooler (FAO/IPGRI, 1994).

Seed cleaning is necessary to 1) reduce bulk during transportation by removing extraneous materials, 2) improve sample purity by removing damaged and immature seeds, 3) optimize storage space and reduce costs.

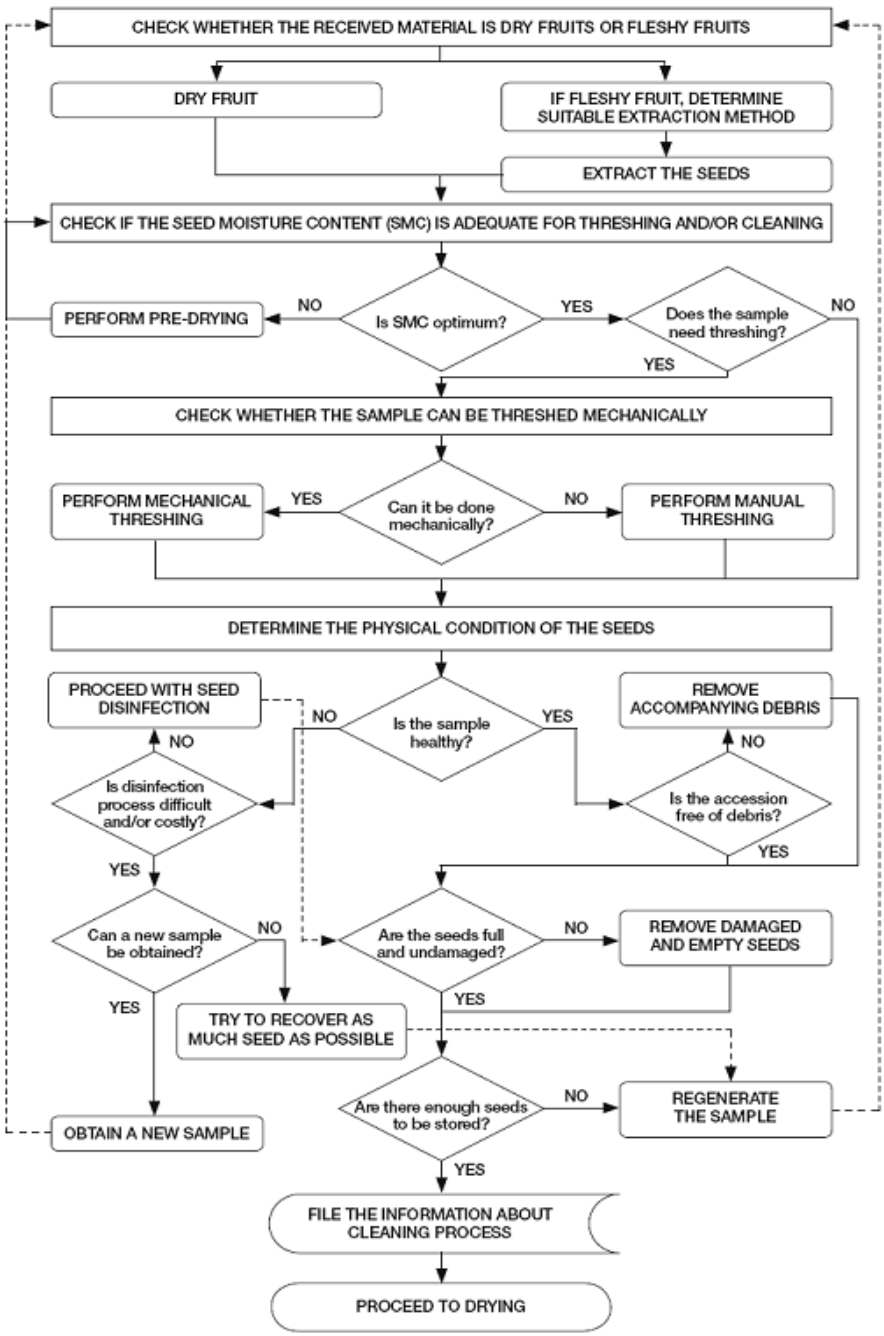
Objectives

- To gain practical experience on PGR management processing
- To practice on seed cleaning
- Purity analysis
- To take a measurement of seed quantity
- To determine the seed moisture content (SMC)

Materials

- Rice 2 accessions and perilla 2 accessions
- Equipment
    - Seed cleaning: Sieves, small trays, tweezers, seed blower
    - SMC determination: Drying oven, non-corrosive drying containers, grinder, balance (0.001~0.0001g), desiccators, gloves

Methods
- Seed cleaning



- Purity analysis

$$\text{Purity (\%)} = \frac{\text{Weight of pure seeds (g)}}{\text{Total weight of working sample (g)}} \times 100$$

- SMC determination

- **High constant temperature method for non-oily seeds**

Moisture content is determined in the following way;

1. Dry the containers at 130ºC for one hour and allow them to cool in the desiccator for one hour.
2. Label and weigh each container, including the lid, and record the weights on the data sheet shown in Table (column W1). For accuracy in moisture determination, the size and weight of the containers should be relative to the sample weight used.
3. Place two 0.5.1.0 g sub-samples, randomly selected from each sample (pre-dried and ground if necessary), into two separate containers, which will serve as two replicates. Replace the lids, weigh again and record the weights in Table (column W2).
4. Place the containers with the lids removed in an oven maintained at 130℃~133℃.
5. Dry the seeds for one to four hours depending on the species (four hours for *Zea mays,* two hours for other cereals and one hour for other species).
6. Replace the lid on each container at the end of the drying period.
7. Move the containers to a desiccator and allow them to cool for 45 minutes.
8. Record the weight of the containers, including the samples, in Table (column W3).
9. Calculate the moisture content on a wet-weight basis and express it as a percentage to one decimal place, using the following formula;

$$\text{Moisture content (\%)} = \frac{W2-W3}{W2-W1} \times 100$$ where,

W1 = weight of container with lid;
W2 = weight of container with lid and sample before drying; and
W3 = weight of container with lid and sample after drying.

10. Repeat the test if the moisture content between the two replicates differs by more than 0.2%.

- **Low constant temperature method for non-oily seeds**

Dry seeds at 103±2℃ for 17±1 hours

Table. Recording and calculation of seed moisture content

Accession No.	Rep./ container No.	Wt of empty container with lid(g)	Wt of container with lid+seed before drying (g)	Wt of container with lid+seed after drying (g)	Moisture content % (wb)	
		W1	W2	W3	(W2-W3)/ (W2-W1)*100	Average (R1+R2)/2
	R1					
	R2					
	R1					
	R2					
	R1					
	R2					
	R1					
	R2					

PRACTICUM NOTE 2-1. Detection of Plant Pathogenic Bacteria

Dong-Suk Park

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◇ **Target pathogen : *Xanthomonas oryzae***

◇ **Pathogenic Bacteria Extraction for Bio-PCR**

1. Slice the naturally infected rice seeds and leaves into the symptom site (1 × 1 cm^2) and then soak them in 0.5 ~ 1 ml sterile distilled water for 30 min ~ 1 hr.

2. Aliquots of 1 ㎕ of each sample-soaked water are directly used for PCR assays.

◇ **PCR (Polymerase Chain Reaction) assays**

1. All amplifications are carried out in a final volume of 20 ~ 50 ml containing 10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin, 0.2mM of each dNTP, 10pM of each primer, and 2 units of Taq polymerase.

2. The total amount of genomic DNA from various microorganisms added to the PCR mixture is approximately 25 ~ 50 ng.

3. Amplification conditions

Temperature & Min	Cycles	
94℃ / 5min	1 cycles	Initial denaturation
94℃ / 1min	25 ~ 35 cycles	Denaturation
60℃ / 1min	25 ~ 35 cycles	Annealing
72℃ / 2min	25 ~ 35 cycles	Extension
72℃ / 10min	1 cycles	Final extension
4℃ / 5min ~ ∞	1 cycles	Storage

4. Agarose Gel Loading

An 8~10ml aliquot of each amplified PCR product is electrophoresed on a 1.0 ~ 1.5% agarose gel, stained with ethidium bromide (EtBr), and visualized on a UV transilluminator.

PRACTICUM NOTE 2-2. Seed Cleaning and Health

Chang Ki Shim

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Foreword

Intensive collaboration characterizes the international exchange of plant germplasm. But any exchange of germplasm involves the risk of also exchanging pests and diseases.

Seed healthy refers primarily to the presence or absence of disease-causing organisms such as fungi, nematodes, bacteria, viruses, and insects. Also physical conditions deficiency, temperature, and humidity, may also affect seed health (ISTA, 1985)

Objectives

The following objectives are consistent with the mission of the genebank to collect, maintain, evaluate and distribute seed healthy and pathogens-free plant material.

Seed cleaning

This is done to improve the seed lot by separating weed seeds and inert matter, and eliminating poor quality seeds and off-types. In cleaning, care should be taken to minimize damage to the seed and to avoid loss of good seeds. Manual cleaning limits contamination and damage especially when the seeds are very dry. Control measures should also be implemented to check the degree of selection and to minimize errors. This is done in the seed processing room, maintained at 40-50% RH and 22 °C.

Seed cleaning procedure

1. Prepare generate data sheets.
2. Pre-clean the seeds by blowing in a ventilated column to separate unfilled grains and light density materials.
3. Verify again using the seedfile.
4. Determine the selection to be done based on the recommendation during the verification process and the current storage status.
5. Examine the seeds and hand sieve with graded mesh sizes (if mixtures/off-types vary in size) to separate slender and bold grains.
6. Remove discolored, deformed, infected, soiled, immature, damaged seeds and off-types.
7. Determine the actual action to be taken based on the quantity of clean seed.
8. Prepare and label all the necessary envelopes for use in seed testing, viability testing, temporary storage, and final drying to minimize labelling errors.
9. Submit the selected samples together with the seed file, pre-labelled envelopes and the original seed container for double checking and quality control.

10. Check the selected sample against the seed file and the pre-labeled envelopes against the original container.
11. Place the cleaned samples again in the drying room while waiting for the viability and seed health test results for the final drying.

* Encode all related information.

Seed health

Only seeds of the highest quality should be stored for long-term preservation. Seed health evaluation determines the extent to which seeds are infected with diseases. Since germplasm is distributed worldwide and every country has its own set of quarantine requirements, seed health evaluation provides information on whether the materials will be acceptable worldwide. Early determination of this information will enable the genebank staff to immediately replace infected samples.

Seed health testing requires trained staff. In this case, standard routine seed health testing (Mew & Misra, 1994), is done by the IRRI, and ISTA Seed Health Unit.

Routine seed health test procedure

1. Prepare generate data sheets.
2. Use pre-clean the seeds by blowing in a ventilated column to separate unfilled grains and light density materials.
3. Next following procedures according to the infection type, contamination of seed surface, and seedborne pathogens detection;

3.1 Contamination of seed surface

- **Observation Test:** A hand or optical equipments (magnifying lens and stereobinocular microscope) may detect plant debris, sclerotia, smuts, discolored and malformed seed, and indications of infection such as dried bacterial ooze, resting mycelia on seed surfaces, and fruiting bodies, and insects, weed of seeds, and soil, etc.

- **Washing Test:** The washing test reveals identifiable spores or mycelia adhering to or growing on the seed surface. 1g of tested samples were washed with a 9ml of distilled water containing of 0.01% Tween 20 on the test tube. The sample was vigorously mixed with vortexes and concentrated with a low speed centrifugation at 3,500rpm for 5min. A compound microscope helps identify fungal spores, and a haemocytometer determine the concentration of spores.

3.2 Seedborne pathogens detection

- **Washing Test:** The washing test reveals identifiable spores or mycelia adhering to or growing on the seed surface.
- **Agagr plate Test:** The washing test reveals identifiable spores or mycelia adhering to or growing on the seed surface.

3.3 Detection and identification of seedborne pathogens

- **PCR Testing:** One such testing option is the use of the Polymerase Chain Reaction (PCR), a method that is able to extract, detect and identify pathogens based on fragments of their genetic information (ITS region). While highly sensitive, PCR testing can occasionally yield false positive results. It has also been noted to yield false negative results, which can be devastating for a seed company. House experts are able to accurately determine when PCR testing is most appropriate based on the pathogen in question, and the specificity of the primers and the sensitivity of protocols that have been developed.

- **ELISA Testing:** ELISA (Enzyme-Linked Immunosorbent Assay) testing, the variables come down to type of equipment, quality of reagents, and skills of the technicians. ELISA is a diagnostic test used to determine if proteins from a particular suspect plant pathogen (virus, bacteria, or fungus) are present in a sample. ELISA testing uses antibodies that detect specific proteins from the target pathogen. First, the wells of a microtiter plate are coated with the antibody. Then a sample is added to the wells and if the target proteins from the pathogen are present, they bind to the antibody. A second antibody with an attached enzyme is added and it binds to the pathogen protein. A chemical substrate is then added to the wells, which then reacts to the enzyme to produce a color change. A color change indicates that a sample is positive for the protein and therefore the pathogen of interest.

PRACTICUM NOTE 3. Viability Testing and Monitoring

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Introduction

Storage life of seeds will depend on initial viability, seed moisture content, relative humidity and temperature of storage facility. We have to ensure that the seeds should maintain its mc. It is also important that storage conditions specially temperature and relative humidity should be maintained at optimum levels.

The number of seeds to be kept in storage depends on the breeding system of the crop, heterogeneity of the accession and type of storage (short-, medium- or long-term). In active collections, an additional consideration is the frequency of distribution (frequently requested seeds).

Viability testing and monitoring are done to determine initial viability, to determine the trend of loss of viability and to provide data to predict when regeneration needs to be done.

Objectives

- To provide the participants with the good practices on viability testing
- To gain practical experience on viability testing and monitoring

Materials (for viability testing)

- Rice 2 accessions and perilla 2 accessions
- Equipment: Petri-dish, filter paper, germination paper, dispenser, tweezers, incubator, name pen, polyethylene bag, spray bottles, razor blade, 1% tetrazolium chloride solution

Methods

Germination Test: Top-of-paper method (TP)

Material: Perilla seed

1. For Petri dishes, round filter paper such as ADVANTEC No.2 of appropriate diameter can be used.
2. Place the paper substrate at the bottom of Petri dish.
3. Label containers with accession number, number of replicate and testing date; use a pencil or permanent marker (name pen) for labeling.
4. Add the required volume of distilled water. If distilled water is not available, boiled and cooled tap water can be used. The volume of distilled water depends on the thickness of the paper substrate and the size of container

5. Firm down the paper substrate in the container using an upside-down funnel or tweezers.
6. Spread the seeds uniformly on the surface of the paper so that they are not touching. It is recommended that the distance between seeds should be at least three to five times the seed diameter.
7. Cover the containers and ensure that there is no air lock resulting from excess moisture on the covers.
8. Place the containers in a germinator or incubator maintained at the recommended temperature for germination of the species.
9. Check the moisture level of the substrate regularly, especially when humidity inside the cabinets is not controlled or when the temperature is set at 25°–30°C. Blotters usually need to be watered several times during the test. Alternatively, keep the containers in a thin plastic bag (loosely folded at the open end, but not sealed to allow diffusion of oxygen) to prevent the substrate from drying.
10. Run the test for the recommended period and count the number of seeds that have germinated.
11. If some seeds have not germinated and appear to be dormant, treat with appropriate techniques to stimulate germination and continue the test until all seeds have germinated or until no further germination has occurred after two consecutive counts.
12. Make a note of the seeds that did not germinate but are firm and sound at the end of the first count, and those that failed to germinate and are presumed dead at the end of the germination test.

Germination Test: Between-paper method (BP)

Material: Rice seed

1. Prepare a convenient size of germination paper to hold one replicate of the seeds.
2. Label the paper at one end with the accession number, replicate number and the testing date. Use a pencil or permanent marker (name pen) for labeling.
3. Moisten the paper with water.
4. Arrange the seeds in rows at regular intervals—about 4 cm from the top edge, leaving a 3–4 cm gap on the sides. Ideally, the distance between seeds should be at least three to five times the seed diameter.
5. Cover the seeds with another sheet of moist paper towel.
6. Roll the paper loosely from opposite the label end.
7. Use a paper clip or rubber band to hold the rolled papers and prevent them from falling apart.
8. Keep the rolls upright in a deep-bottom plastic tray.
9. Add a sufficient quantity of water to the tray (covering the bottom 3 cm of rolls).
10. Place the tray in an incubator or germinator maintained at the recommended temperature and run the test for the recommended period.
11. Keep the towels moist by spraying with water (use spray bottles) if necessary, especially when temperatures are high (25°–30°C).
12. Count the germinated seeds by unrolling the paper carefully to avoid tearing it or damaging the roots of young seedlings.
13. If some seeds have not germinated and appear to be dormant, treat with an

appropriate technique to stimulate germination. Continue the test until all seeds have germinated or until no further germination has occurred after two consecutive counts.

14. Make a note of the seeds that did not germinate but are firm and sound at the end of first count, and those that failed to germinate and are presumed dead at the end of the germination test.

Tetrazolium test for seed viability

Material: Rice seed

Preconditioning

1. Remove the seed-covering structures (glumes, etc.).
2. Precondition the seeds by soaking in water or by placing them in a moist medium at 30°C. No preconditioning is necessary when un-germinated seeds are evaluated at the end of a germination test.

Staining

1. Bisect the seeds longitudinally through the embryo with a razor blade.
2. Discard half of each seed and place the other half in the staining solution at the recommended concentration in a glass vial.
3. Place the vials in an incubator in a dark area at the recommended temperature and duration for each species.
4. After staining, wash the seeds several times in distilled water to remove excess stain.
5. Immerse the seeds in lactophenol solution (1 litre of lactophenol prepared from 200 ml phenol, 200 ml lactic acid, 400 ml glycerine, and 200 ml water) for one to two hours before evaluating the seeds.
6. Evaluate the seeds for a staining pattern under a low-powered binocular microscope; viable tissues stain bright red. Pink and very dark red stains indicate dead tissue.
7. Classify the seeds into three categories depending on staining pattern:
   - completely stained seeds that are viable;
   - completely unstained seeds that are nonviable; and
   - partially stained seeds that will produce either normal or abnormal seedlings, depending on the intensity and pattern of staining.

PRACTICUM NOTE 4. Documentation and Information Management

Gyu-Taek Cho, Man-Jung Kang

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The mission of the GRIN-Global Project is to create a new, scalable version of the Germplasm Resource Information System (GRIN) to provide the world's crop genebanks with a powerful, flexible, easy-to-use plant genetic resource (PGR) information management system. The system will help safeguard PGR and information vital to global food security, and encourage PGR use. Developed jointly by the USDA Agricultural Research Service, Bioversity International and the Global Crop Diversity Trust, GRIN-Global will be deployed in selected plant genebanks worldwide in 2011.

The .NET Framework and Visual Studio development environment were chosen for the project. A core set of web services, enterprise services or other technologies will update data stored locally or on networks, distribute centralized data to off-site systems, and enable third party data sharing. The database and interface(s) will accommodate commercial and open-source programming tools, be database-flexible (MySQL, MS SQL Server, Oracle), and require no licensing fees. The database will be deployable on stand-alone computers or networked systems. Iterative programming strategies will support continuous product evaluation and refinement; advanced prototypes will be extensively beta-tested. Bioversity International will deploy GRIN-Global internationally, working cooperatively to document the new system in Arabic, English, French, Russian and Spanish, translate its interface, and implement it in developing countries. Implementation will be monitored and barriers to adoption identified. The impact of system use will be evaluated by users during and following database implementation.

References

GRIN-Global: An International Project to Develop a Global Plant Genebank and Information Management System, Poster Board # 333
G. Kinard, USDA-ARS-NGRL; P. Cyr, USDA-ARS-PIRU-NCRPIS; B. Weaver, Bioversity International; M. Millard, USDA-ARS-PIRU-NCRPIS; C. Gardner, USDA-ARS-PIRU-NCRPIS; M. Bohning, USDA-ARS-NGRL; G. Emberland, USDA-ARS-NGRL; Q. Sinnott, USDA-ARS-NGRL; J. Postman, USDA-ARS-NCGR; K. Hummer, USDA-ARS-NCGR; T. Franco, Bioversity International; M. Mackay, Bioversity International; L. Guarino, Global Crop Diversity Trust; P. Bretting, USDA-ARS-NPS

PRACTICUM NOTE 5-1. Packaging, Storage Conditions and Distribution

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Introduction

Seed should be packaged in waterproof containers and hermetically sealed without delay following removal from the drying room or cabinet.

Seed storage is the preservation of seeds under controlled environmental conditions that maintain seed viability for long periods. Two types of seed stores are used for conservation of genetic resources: those holding seed samples for long term security-referred to as base collections - and those holding seed samples for immediate use- referred to as active collections. The temperature, RH, seed moisture content, containers and distribution arrangements of these stores vary.

Germplasm distribution is the supply of representative samples of seed accessions from a genebank in response to requests from germplasm users. In general, seeds are distributed only from active collections.

Objectives

To gain practical experience on packing, seed storage and distribution

Materials

1. Passport data (IT 221998~222029), 32 acc.
2. Rice sample seeds for packaging, active and base collection and distribution.
3. Storage containers(Plastic bottle, aluminum foil pack)
4. Computer-generated self-adhesive labels, Sealer for aluminum foli pack

Lessons to learn

Seed processing and drying should be done within the shortest possible time after harvest and upon arrival of collected materials in the genebanks

<u>Packaging</u>

1. Different types of containers could be used depending on the size, shape of the seeds and the purpose of conservation (i.e. whether for long-term or short-term). Decide on the most suitable container you will use for storing the seeds in your genebank.
2. Prepare and label your containers. Also prepare a label for inclusion within the seeds inside the container. The labels should contain at least the following minimum information:
    a. Accession number
    b. Genus and species

c. Container number
d. Weight of seeds
e. Date of storage

3. Weigh out each labeled container empty.
4. Fill the containers with the seeds and weigh again. Calculate actual weight of seeds.
5. Add the label prepared for the inside and seal the container immediately to prevent moisture from entering the seeds.
6. Check quality of the container after sealing by making visual examination of each container to make sure that there is no leakage.
7. Any container found below standard should be replaced immediately.

Table1. Model table for recording information on seed packaging.

Date of packaging:			**Name of staff:**		
Accession number	**Type of container**	**Container number**	**Weight of container empty**	**Weight of container and seeds**	**Weight of seeds**

Seed storage

Seed storage is the preservation of seeds under controlled environmental conditions that maintain seed viability for long periods.

Types of storage

Two types of seed stores are used for conservation of genetic resources : those holding seed samples for long term security-referred to as base collections - and those holding seed samples for immediate use- referred to as active collections. The temperature, RH, seed moisture content, containers and distribution arrangements of these stores vary.

1. Base collections

A base collection is a set of accessions in which each is distinct and as close as possible to the original sample in terms of genetic integrity. Normally, seeds are not distributed from base collections directly to users but are only used to regenerate

active collections. Base collections are stored for long periodsat below 0℃ -usually at -18℃ to -20℃- to maintain seed viability.

2. Active collections.

Active collections consist of accessions that are immediately available for distribution. These accessions are accessed frequently and maintained in conditions that ensure at least 65% viability for 10-20 years. It is more practical to use a lower moisture content and store at a higher temperature to save on refrigeration costs.

Germplasm Distribution

Step 1. Decide whether the accession can be distributed.
Step 2. Prepare the sample for distribution
If seeds are available for distribution

1. Resister the request by assigning a request number.
2. Prepare the list of accessions available for distribution.
3. Check the requirements for a material transfer agreement.
4. Prepare two sets of labels for the selected accessions and paste one of them on the envelopes that will be used for distributing seeds to the requester.
5. Check the inventory file and note the location of the containers in the genebank.
6. Move the containers from the genebank into a dehumidified room the evening before distribution to allow them to warm to room temperature before opening. Ensure ablsolute accuaracy in identification of accessions while drawing the seeds from the genebank.
7. Open the container and quickly draw the required amount of seeds into the labelled envelopes. It is suggested that 50-100 viable seed should be distributed to fill each request.
8. Close the container immediately after removing the seeds for distribution to prevent uptake of moisture from ambient air.
9. For extra security, a second label may be placed inside the envelopes before packets are sealed.
10. Compare the list of accessions drawn from the genebank with the labels on the envelopes.

Step 3. Prepare the information list to accompany the seeds
step 4. Dispatch the seeds

References

International Seed Testing Association. 1996a. International Rules for Seed Testing. Rules 1996. Seed Science and Technology 21, Supplement: 1-86.

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PRACTICUM NOTE 5-2. Introduction of Regeneration Program in National Agrobiodiversity Center, RDA

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Introduction

○ Timely regeneration must be a priority activity of all genebanks (FAO 1996)
○ More than 50,000 accessions should be immediately regenerated in Agrobiodiversity Center.

Regeneration target

○ Maximizing seed quality
○ Optimizing seed quantity
○ Maintaining genetic integrity
○ Minimizing the costs

Regeneration Procedure

○ Selection of accessions: Seed quantity, seed quality, origin
○ Selection of location and institute: Central bank, sub-banks etc
○ Selection of plots: Open field, glass house, plastic house etc.
○ Crop management
○ Harvesting, drying, threshing etc.

Regeneration examples of *Brassica* and *Raphanus* germplasm

○ Physiology of ***Brassica*** **and** ***Raphanus*** **germplasm**
○ Vernalization, pollination, isolation
○ No. of plants used for regeneration
○ Harvesting, drying, threshing
○ Information: bolting and flowering date, etc.
○ Image database: Plant, flower, silique, seed

Field Trip for *Capsicum* Germplasm Regeneration

PRACTICUM NOTE 6. Germplasm Exploration and Collecting

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Introduction

Germplasm can be acquired through donations, requests, exchange with another institution, and through field exploration and collecting in farmers' fields and natural habitats. The types of germplasm materials that can be acquired include farmers' varieties, landraces and primitive cultivars, wild and weedy related species, and products of plant breeding, e.g. improved cultivars, obsolete varieties, mutants, genetic stocks, breeding lines etc.

Careful planning is necessary in germplasm exploration and collecting in order to collect the maximum amount of genetic diversity in the target taxon. In addition to germplasm, information including indigenous and traditional knowledge associated with the germplasm should also be collected whenever practicable. When collecting germplasm, social and ethical considerations should also be given importance.

Changes in planned itinerary can occur. Flexibility therefore should be practiced to maximize the use of time and resources in collecting.

Objectives

- ○ To learn the good practices in collecting germplasm of crop wild relatives
- ○ To learn how to gather and document passport data and associated knowledge
- ○ To collect and prepare germplasm materials for herbarium collection

Materials

Practice 1. Collection of Crop wild relative

- ○ Target taxon : Vigna nakashimae

Practice 2. Make Specimens

- ○ Target taxon : Vigna sp., etc

Equipments

GPS(for GIS coordinate), Camera, Herbarium plant press(field press), Secateurs, Digger tool(for collecting underground organs), Paper bags(for seeds), Writing instruments, Note, Polythene bags, Colleting forms, Tags, Absorbent paper, Corrugates(corrugated cardboard), Electric heater, etc

Methods

Practice 1. Collection of Crop wild relative

- ○ Collection site(about 500m) : South area of Soe Ho Park, Suwon, Korea

○ Collection strategy

a. Mentally divide the area into a series of transects vertically and horizontally to form
b. Individul the samples : Collect every interval, every 100 steps, just 1 plant
c. Bulk the samples : Collect enough for minimum of 1,000 seeds, randomly in the collection area
d. If there are variants(plants with different appearance from the others) : Collect seeds from the plants and separate the seeds from the others
e. Do not collect from plants with diseases or pests
f. Fill up collecting labels(Collecting Number, Date, Site, Crop) : Always put two labels per variety collected. Put one label inside the bag or envelop ; put the other label outside the bag or envelope
g. Fill up the Collection Record Sheet : example 1 form
h. Extract seeds and air dry the collected material to maintain high viability of seeds

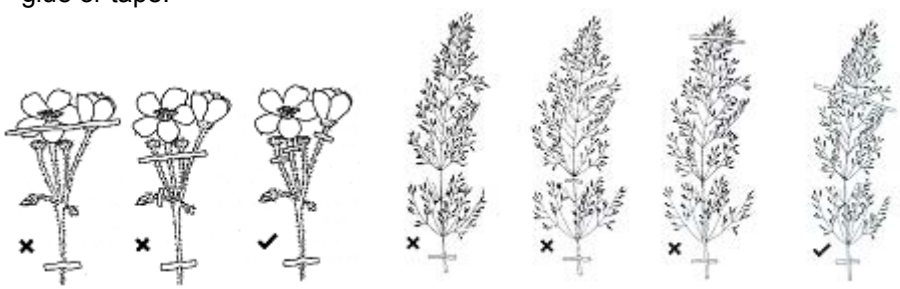
Practice 2. Make Specimens

a. Collect a suitalbe plant for specimens : the floral or fruiting season, label for each locality
b. Do not collect from plants with diseases or pests.
c. Clean the plants for specimens: remove a dust or a foreign substances
d. Press into a field press (absolute minimum : three). this consists of two sheets of stiff card held together by webbing straps and containing folded sheets of absorbent paper. The press is built up by alternating one or two

drying papers with each flimsy. Corrugates added between the drying papers allow circulation of air and will speed the drying process.

e. Drying: air-dried or gentle heat (a elective heater is very effective), if air-dried, drying papers must be changed daily.

f. Mounting: a dried plant is arranged on the sheet and attached by herbarium glue or tape.



g. The passport information on a label of herbarium voucher is attached at the lower right corner of mounting paper. : In paticular, name of collector, collecting date, taxonomic identification, locality, specific habitat and species abundance. In addition, notes and drawings of character of the plant

f. Storing: The vouchers store in condition of the regular habit and constant temperature

Result

Practice 1. Collection of Crop wild relative

1. Investigate the taxon(vigna spp.) : Taxon, Habitat, Distribution, Character, etc
2. Report on the filded collection data form(※ Refer to the example 1)

Practice 2. Make Specimens

1. Explain the important of specimens
2. Submit the herbarium vouchers

(Example 1)

Site No.	*I*	Collecting Date	*2009 . 10. 1.*
Collector(s)	*James Dean*	Type of Material	*√ Seed / Plant*
Scientific Name	*Vigna vexillata var. tsusimensis*	Status of Sample	*√ Wild / Weedy*
Common Name		Local Name	
Collection No.	*2*		
GIS coordinate	*Latitude 34˚25´09.40˝N, Longitude 126˚07´39.80˝E, Altitude 12m*		
Population Address	*Bongam reservoir, Oryuli Jisanmyon Jindogun Joennam*		
No. of Plants sampled	*No. of Plants sampled is 10 , covering 1000 m*	Sampling Method	*Bulk / √ Individual*
Area of Population (00 x 00 m)	*<1m^2 / √ 1-10m^2 / 10-100m^2 / 100m^2-0.1ha / 1-10ha / >10ha*	No. of plant found	*No. of plants in population is _____, covering _____m*
Population Variation	*Uniformity*		
Collecting Source	*Undisturbed natural habitat / √ Disturbed natural habitat / Weedy habit (road side, field margin) / Farmer' field / Farmer's backyard etc.*		
Dominant Species	*Vigna vexillata var. tsusimensis*		
Associate Species	*Dominant sp. : Humulus japonicus* *others : Amphicarpaea bracteata subsp. edgeworthii, Vigna angularis var. nipponensis, etc*		
Vegetation Type	*Forest / Bushes / Cultivated / Grassland / √ Other*		
Shading (%)	*Heavy / Medium / Light / √ Open (None)*		
Topography of region	*√ Flat / Undulating / Rolling / Hilly / Steeply dissected / Mountainous*		
Land form at the site	*Mountain / Hill / Upland / Plain / Plateau /√ Basin / Valley*		
Disease assessment	Leaf *no*	Pod	*no*
Pest assessment	Leaf *no*	Pod	*no*

Morphological Description	*Vine, Dark purple flower, The patterned leaflet*		
Leaf Pubescence	*less*		
Flower color	*dark purple*		
Local use	*edible root*		
Comments			
Viable seed/pod(10)	*160/10pod*		
Ovules/pod (10)			
Photo No.	Site	Habitat	Plants
	21	*22*	*23-25*
Herbarium specimens	√ *Yes* / No	(No. sheets : 2)	
Map 4			

PRACTICUM NOTE 7-1. Rice germplasm Assessment

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Introduction

'Descriptors for wild and cultivated rice (Oryza spp.)' developed by Bioversity International is a revision of the original IBPGR and IRRI publication 'Descriptors for rice (Oryza sativa L.)' (1980), expanded to include descriptors for wild rice species of the genus Oryza, and harmonized as far as possible with descriptors developed by the International Union for the Protection of New Varieties of Plants (UPOV 2004; www.upov.org) for new cultivars of cultivated rice. The 1980 list has been widely used and is considered to be the most valid descriptor system for rice. This revision has been developed in collaboration with Ruaraidh Sackville Hamilton, Renato Reaño, Socorro Almazan, Elizabeth Naredo, Maria Celeste Banaticla, Edwin Javier and Melissa Fitzgerald of IRRI, and subsequently sent to a number of experts for their comments. A full list of the names and addresses of those involved is given in 'Contributors'. Bioversity International (formerly known as IPGRI) encourages the collecting of data for all five types of descriptors (see Definitions and Use of Descriptors), whereby data from the first four categories - Passport, Management, Environment and Site, and Characterization - should be available for any accession. The number of descriptors selected in each of the categories will depend on the crop and their importance to the crop's description. Descriptors listed under Evaluation allow for a more extensive description of the accession, but generally require replicated trials over a period of time. Although the suggested coding should not be regarded as the definitive scheme, this format represents an important tool for a standardized characterization system and it is promoted by Bioversity throughout the world. This descriptor list provides an international format and thereby produces a universally understood 'language' for plant genetic resources data. The adoption of this scheme for data encoding, or at least the production of a transformation method to convert other schemes into the Bioversity format, will produce a rapid, reliable, and efficient means for information storage, retrieval and communication, and will assist with the use of germplasm. It is recommended, therefore, that information should be produced by closely following the descriptor list with regard to ordering and numbering descriptors, using the descriptors specified and using the descriptor states recommended. This descriptor list is intended to be comprehensive for the descriptors that it contains. This approach assists with the standardization of descriptor definitions. Bioversity does not, however, assume that curators will characterize accessions of their collection using all descriptors given. Descriptors should be used when they are useful to curators for the management and maintenance of the collection and/or to the users of plant genetic resources. However, highly discriminating descriptors are highlighted in the text to facilitate the selection of descriptors and are listed in Annex I. Multicrop passport descriptors were developed jointly by Bioversity and FAO to provide consistent coding schemes for common passport descriptors across crops. They are marked in the text as

[MCPD]. Owing to the generic nature of the multicrop passport descriptors, not all descriptor states for a particular descriptor will be relevant to a specific crop. A comparison table of standard colour charts is provided in Annex II for conversion of colour descriptors and Annex III has a table containing cross-references to other documentation systems and their recording stages. Any suggestions for improvement on the 'Descriptors for wild and cultivated rice' will be highly appreciated by Bioversity, IRRI and WARDA.

Objective:
To enhancing the expertise through experience using given descriptors

Materials:
Several rice germplasm at the Breeder's field

Used descriptors
- Bioversity International, UPOV, Japanese genebank

Scope of practice
- Investigate the traits by their given descriptors
- Organize and compare the data investigated between/among groups
- Discuss the gap between/among groups for further investigation

☞ **Time schedule for the practice**
13:00: Ride a mini bus in front of the Genebank building and move to the field
13:10: Reach at the National Academy of Crop Science rice Breeder's field
13:40: Short introduction on Rice breeder's field
14:40: Investigate the traits by their given descriptors, organize the data
15:00: Comeback to Genebank building
15:50: Discuss the gap between/among groups for further investigation

Appendix; Lists for investigation (examples of Japanese descriptors)

<Rice Primary essential character>
No, Characters, **No. of samples**, Methods, **Rank or measurement unit**, Remarks

1. **Culm length**, 5 plants, Measurement, cm (integer) Distance from ground level to the base of the longest culm
2. **Panicle length**, 5 plants Measurement, cm (round to the 1st decimal place), Distance from the base to the tip of panicle on the longest culm
3. **Number of panicles**, 5 plants, Measurement, Number per plant (round to the 1st decimal place), Number of productive panicles at ripening stage
4. **Apiculus color**, Block, Observation, 1:Straw 2:Tawny 3:Brown 4:Red brown 5:Light red 6:Red 7:Light purple 8:Purple, 9:Blackish purple, At three weeks after heading 5. Grain length, 5 grains, Measurement, mm (round to the 1st decimal place), Using a projector or dialgauge as the distance from the base of the sterile lemma to the tip of the fertile lemma or palea
5. **Grain width**, 5 grains, Measurement, mm (round to the 1st decimal place), Use a projector or dialgauge to measure the maximum distance across lemma and palea

8. **Brown rice length**, 5 grains, Measurement, mm (round to the 1st decimal place), Use a projector or dialgauge to measure the length of brown rice
9. **Brown rice width**, 5 grains, Measurement mm (round to the 1st decimal place), Use a projector or dialgauge to measure the maximum width of brown rice
10. **Endosperm type**, Block, Observation, 2:Non-glutinous 8:Glutinous, Reaction to potassium iodide solution or visually
11. **Heading date**, Block, Observation, date, Heading date corresponds to the day when 50% of the plants in an accession headed
12. **Lemma and palea color**, Block, Observation, 1:Straw 2:Yellow 3:Gold 4:Reddish yellow to orange 5:Brown 6:Reddish brown 7:Purple 8:Black 9:Other, At three weeks after heading
13. **Presence of awn**, Block, Observation, 0:Absent 1:Extremely scarce 2:Very scarce 3:Scarce 4:Slightly scarce 5:Intermediate 6:Slightly abundant 7:Abundant 8:Extremely abundant 9:Completely Scarce:10%, Intermediate:25%, Abundant:40%
14. **Awn length**, Block, Measurement, 2:Very short 3:Short 4:Slightly short 5:Intermediate 6:Slightly long 7:Long 8:Very long, Short:2 cm, intermediate:4 cm, long:6 cm

<Rice Primary optional character>

No. Characters, **No. of samples**, Methods, **Rank or measurement unit**, Remarks

1. **Plant type**, Block, Observation, 2:Super panicle weight type 3:Panicle weight type 4:Rather panicle weight type 5:Intermediate type 6:Rather panicle number type 7:Panicle number type 8:Super panicle number type
2. **Culm thickness**, Block, Observation, 2:Very thin 3:Thin 4:Slightly thin 5:Intermediate 6:Slightly thick 7:Thick 8:Very thick, At ripening stage
3. **Culm hardness**, Block, Observation, 2:Very hard 3:Hard 4:Slightly hard 5:Intermediate 6:Slightly soft 7:Soft 8:Very soft, At ripening stage 4. Leaf blade pubescence, Block, Observation, 0:Glabrous 1:Very scarce 2:Scarce 3:Little 4:Slightly little 5:Intermediate 6:Slightly abundant 7:Abundant 8:Very abundant 9:Extremely abundant, At tillering stage
4. **Flag leaf angle**, Block, Observation, 2:Erect 3:Semi-erect 4:Slightly semi-erect 5:Intermediate 6:Slightly descending 7:Semidescending 8:Descending, At dough-ripening stage
5. **Leaf blade color**, Block, Observation, 1:Yellow 2:Yellowish blotched 3:Light green 4:Green 5:Dark green 6:Purple blotched 7:Purple margin 8:Purple 9:Other, At tillering stage
6. **Basal leaf sheath color**, Block, Observation, 1:Yellow 2:Yellowish blotched 3:Light green 4:Green 5:Dark green 6:Purple blotched 7:Purple margin 8:Purple 9:Other, At tillering stage
7. **Spikelet density**, 5 plants, Measurement, (round to the 1st decimal place), Number of spikelets per 10 cm of panicle axis using a panicle on the longest culm
8. **Panicle exsertion**, Block, Observation, 2:Very short 3:Short 4:Slightly short 5:Intermediate 6:Slightly long 7:Long 8:Very long, The distance from the top of the flag leaf sheath to the panicle base
9. **Panicle type**, Block, Observation, 1:Lanceolate 3:Spindle 5:Clavated 7:Broom 9:Open, Based on the type of branching, angle of primary branches and spikelet

density

10. **Pubescence of lemma and palea**, Block, Observation, 0:None 1:Rare 2:Scarce 3:Little 4:Slightly little 5:Intermediate 6:Slightly abundant 7:Abundant 8:Very abundant 9:Extremely abundant
11. **Sterile lemma color**, Block, Observation, 0:White 1:Light yellow 3:Orange 5:Yellowish brown 7:Red 9:Purple, At ripening stage
12. **Phenol color reaction**, 5 grains, Observation, 0:Negative 9:Positive, Dip grains into 1.5% phenol solution for 6 hours and dry slowly
13. **Awn color**, Block, Observation, 1:Straw 2:Yellowish brown 3:Brown 4:Reddish brown 5:Light red 6:Red 7:Light purple 8:Purple 9:Blackish purple, At ripening stage
14. **Seed coat color**, Block, Observation, 0:White 1:Light brown 2:Brown 3:Reddish brown 4:Red 5:Brownish purple 6:Purple 7:Dark purple 8:Blackish purple 9:Other
15. **Hue of brown rice**, Block, Observation, 2:Very light 3:Light 4:Slightly light 5:Intermediate 6:Slightly dark 7:Dark 8:Very dark
16. **Maturity date**, Block, Observation, date, the date when more than 90% of grains on panicles become ripe
17. **Days from the first heading to the full heading**, Calculation, Block, (integer), Number of days calculated by subtracting date of head emergence from the date of full heading

PRACTICUM NOTE 7-2. Application of molecular markers to assess genetic diversity

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Introduction

Molecular markers play as essential role today in both plants breeding and assessing of germplasm diversity as providing information about allelic variation at a given locus. The increasing availability of molecular markers in crop allows the detailed analyses and evaluation of genetic diversity and also, the detection of genes influencing specific agronomic traits.

Plant genetic resources include the reproductive or vegetative propagated material of (i) cultivar in current use and newly developed varieties, (ii) traditional cultivars and landraces, (iii) wild relatives of cultivated species and (iv) elite breeding materials, anneuploids and mutant. General application of molecular markers in germplasm collection are genetic purity and genetic diversity analysis, construction of core collection, selection of interesting gene resources, monitoring of viability and health and genetic changes due to long term storage at low temperature.

A number of markers are now available to detected polymorphisms in nuclear DNA including variable number of tandem repeats (VNTRs), random amplified polymorphic DNA (RAPD), single strand conformation polymorphisms (SSCPs), restriction fragment length polymorphisms (RFLPs) and amplified fragment length polymorphisms (AFLPs). In genetic diversity studies, the most frequently used markers are microsatellites that can be applied high-though put system.

Many computer programs exist for analyzing molecular data for genetic diversity and most programs perform similar tasks and their main differences should be evaluated, depending on resources available and/or individual preferences. Nowadays, in addition to freely available computer programs, plenty of resources are also found on Internet to help us obtain both basic and more specialized information on method. Generally used software with which to analyze intra-specific genetic variation within the framework of evolutionary hypothesis were TFPGA(Miller, 1997), Arlequin (Schneider et al., 1997), GDA(Lewis and Zaykin. 1999), GENEPOP(Raymond and Rousset, 1995), GeneStrut(Constantine et al., 1994), POPGene(Yeh and Boyle, 1997) and PowerMarker(Lie et al., 2003)

Crop: Rice (Oryza sativa)
Part 1: DNA extraction practice (2 accessions per person)
Part 2: Diversity analysis with genotyping data of diverse rice accessions

Part 1: DNA extraction by CTAB Method

DNA extraction procedure

1. Add 80 ul 2-mercaptoethanol to 40 ml CTAB buffer (see recipe below) right before use (0.2%) and mix.

2. Preheat CTAB DNA isolation buffer to 65°C→ in a water bath.
3. Grind freeze-dried leaf tissue (~100mg) in a Mixer Mill for 3-5 min at 30 time/sec with one 3mm steel bead in a 1.1 ml tube strip from Qiagen
4. Carefully pour beads out of tubes and add 300 ul CTAB buffer into each tube with a multi-channel pipette (if a plate is used, you can leave beads in until the end of work)
5. Cap the tube strips and label each strip, caps and rack, incubate the samples in 65 °C water bath for 60-80 min, use shipping tape to secure the rack and lid to prevent caps from opening up while inverting the rack to mix samples.
6. Mix samples every 15 min by inverting rack 5-10 times to make sure plant tissue mixes well with the buffer. Watch for sample leaking from caps.
7. Take samples out of water bath, briefly spin sample box in a centrifuge for 1 min at 1500 rpm (balance box weight before spinning), separate caps from tube and put the cap strips on clean paper and arrange them in order so that they can be reused.
8. Add 300 Chl/IAA (25:1) into each tube with a multi-channel pipette, cap tube with original cap strips, put a plastic board between tube cap and rack lid to make sure every strip cap gets the same pressure from the lid, cover the box with lid, secure the lid with shipping tape. Mix the samples by inverting the box slowly for 15 min (Chloroform can create pressure and cause leaks of chloroform, so the tubes have to be tightly capped).
9. Centrifuge at 5,700 rpm for 10 min.
10. Transfer the aqueous phase using a wide-bore pipette tip (about 200ul) into a labeled clean plate (650 ul plate), add 150 ul of cold isopropanol (-20 °C), securely cover the plate with a plate cover (or cap strips) and mix gently by inverting the plate several times to precipitate DNA.
11. Recover the DNA by centrifuging at 5,700 rpm (6100g) for 10 min. Discard the supernatant immediately by suddenly inverting the plate and dry it briefly on several layers of paper towels by briefly touching the plate on paper towels two to three times on different positions of towel to remove excess liquid. This step should be very quick (a few seconds). Then turn the plate back upright and air-dry samples to remove excess liquid for ~10 min.
12. Add 500 ul of 70% Ethanol directly to the pellet, cover the plate, and invert it gently several times to wash for 5 min.
13. Centrifuge at 5,700 rpm for 8 min, and quickly discard wash buffer as described previously (check DNA pellet in each well to make sure DNA stayed in the bottoms of wells
14. Air-dry DNA pellet at room temperature for ~20 mins to remove excess liquid.
15. Re-suspend the DNA pellet in 200 u1 of ddH2O and leave it at 4°C overnight
16. DNA concentration will be ~100ng /ul and can be determined using either an agarose gel or a spectrophotometer.

CTAB DNA Isolation Buffer (500 ml)

2% (w/v) CTAB (sigma)	10.0 g
1.4M NaCl	40.91 g
20 mM EDTA	20 m1 of 0.5 M EDTA
100 mM Tris. HCl (pH 8.0)	50 ml of 1.0 M
Add ddH_2O to make a final volume of 500 ml	

Part 2: Analysis of diversity by PowerMarker
(Handout of protocol and explanation)

Summary of using PowerMarker software

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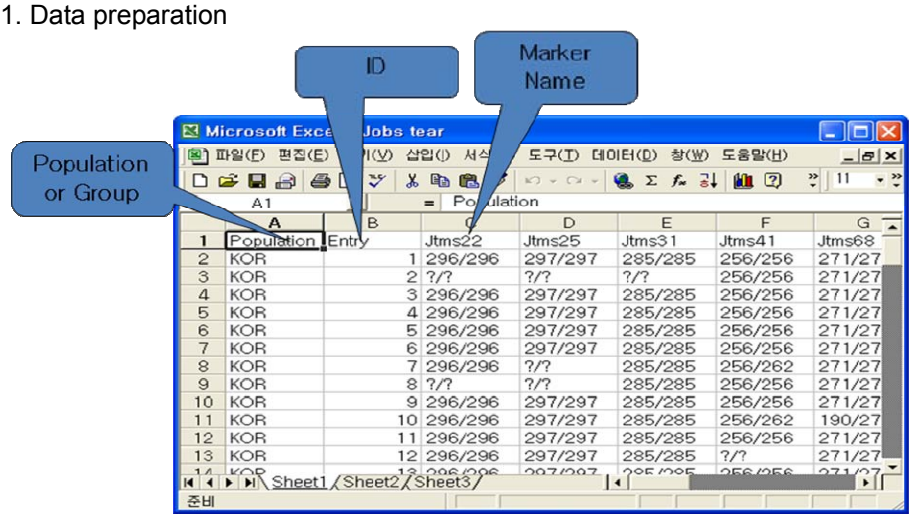

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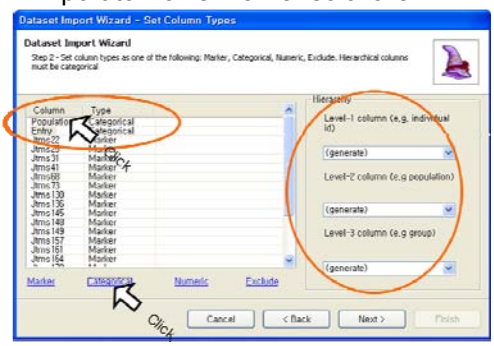
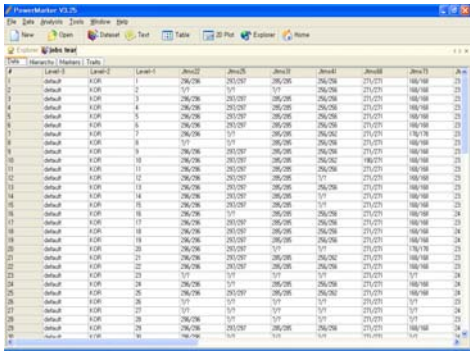
3	KOR	2	?/?	?/?	?/?	256/256	271/27
4	KOR	3	296/296	297/297	285/285	256/256	271/27
5	KOR	4	296/296	297/297	285/285	256/256	271/27
6	KOR	5	296/296	297/297	285/285	256/256	271/27
7	KOR	6	296/296	297/297	285/285	256/256	271/27
8	KOR	7	296/296	?/?	285/285	256/262	271/27
9	KOR	8	?/?	?/?	285/285	256/256	271/27
10	KOR	9	296/296	297/297	285/285	256/256	271/27
11	KOR	10	296/296	297/297	285/285	256/262	190/27
12	KOR	11	296/296	297/297	285/285	256/256	271/27
13	KOR	12	296/296	297/297	285/285	?/?	271/27

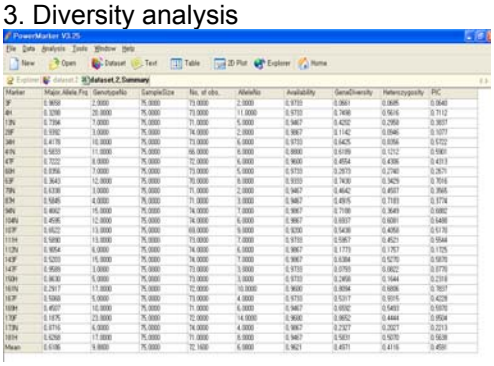
Haploid input: x Diploid input: x/x
Missing data: ? (ex. in diploid : ?/?)
Copy to Clipboard

2. Import to PowerMarker software





3. Diversity analysis



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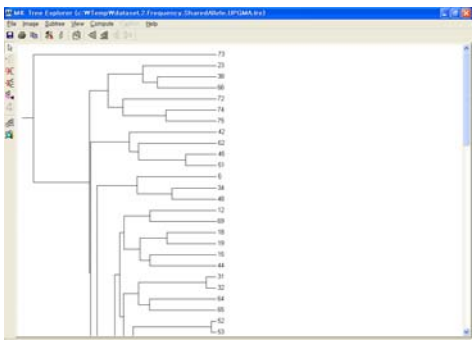

<Dendrogram>

PRACTICUM NOTE 8-1. Cryopreservation of Vegetatively Propagated Plants

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Introduction

Cryopreservation method has been considered as an alternative option for long-term conservation of recalcitrant samples which cannot be stored in cold storage room with lower moisture content. It includes vegetatively propagated species and recalcitrant seeds.

Recently large scale implementation of cryopreservation for long-term germplasm conservation is increasing with a development of new technology, such as droplet-vitrification procedures. Droplet-vitrification, a combination of droplet-freezing and solution-based vitrification, produced higher recovery than droplet-freezing or vitrification, since it ensures direct contact of explants with liquid nitrogen and thus facilitating cooling and warming of samples.

Objectives

To provide the participants with the good practices on cryopreservation of vegetatively propagated In Vitro plants by using droplet-vitrification protocol.
To gain practical experience on tissue culture and cryopreservation procedures

Lessons to learn

The three-hour practicum will consist of one hour introduction and two-hour practice.

Introduction (1 hour)
The participants shall get ideas on basis of cryopreservation, cryopreservation techniques, and cryopreservation activities at the National Agrobiodiversity Center, including large scale implementation of garlic germplasm cryopreservation.

Practice (2 hours)
The participants shall be divided into two groups for practice.
One group will practice overall procedures of plant tissue culture and preparation of samples for cryopreservation.
The other part will practice the droplet-vitrification procedure which includes loading of cryoprotectants solutions, droplet-freezing by using aluminium foil strip, thawing, unloading and postculture.

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Kim, HH, JW Yoon, YE Park, EG Cho, JK Sohn, Taesan Kim and F Engelmann. Cryopreservation of potato cultivated varieties and wild species: critical factors in droplet vitrification. CryoLetters 27(4): 223-234.

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DROPLET-VITRIFICATION OF POTATO SHOOT TIPS

Droplet-vitrification procedure is a combination of solution-based vitrification and droplet freezing method (27). (Kim et al., 2006)

Checklist for Droplet-Vitrification of *In Vitro* Potato Shoot Tips

Items needed to prepare plant material

1. Scissors, scalpel, forceps for subculture inoculation
2. Sterile Petri dishes with sterile filter papers
3. 100 ml Magenta bottles
4. Air-ventilated culture vessels (13~15 cm in height)
5. Subculture medium (hormone free MS medium + 3% (0.09 M) sucrose and 2.2 g/L phytagel, pH 5.8)

Items needed to cryopreserve

1. Small bench top dewar
2. Sterile containers for cryoprotectants and liquid media
3. Stools to change solutions
4. 100 ml Magenta bottle
5. Sterile dispense pipets for exchange solutions
6. Cryovials (2 ml) and markers
7. Canes, Canisters and long-term storage dewar with cane inventory system
8. Accession numbers and names of accessions
9. Sterile aluminum foil strips (7 x 20 mm)
10. Tube holder frozen in a block of ice
11. A water bath set at 40 °C
12. Preculture medium (MS medium + 0.3 M sucrose, pH 5.8)
13. Recovery medium (MS medium + 0.05 mg/L IAA + 0.3 mg/L zeatin + 0.05 mg/L GA_3 + 3 % sucrose + 1.8 mg/L phytagel)

14. PVS2 (glycerol 30 % + EG 15 % + DMSO 15% in MS basal medium with 0.4 M sucrose)
15. Unloading solution (0.8M sucrose in MS medium)
16. Sterile Petri dishes with sterile filter papers for draining unloaded explants

The procedure:

This is a two days procedure. In the morning isolate shoot tips and preculture liquid MS medium + 0.3 M sucrose for 6-8 hours. In the evening, transfer shoot-tips to liquid MS medium with 0.7 M sucrose and incubate for 16-18 hours.
Next morning, precede remaining procedures (*i.e.* loading, dehydration, freezing, etc.).

Step 1. Plant material

This protocol uses axillary shoot tips of *In Vitro* grown cultivated potato species (*Solanum tuberosum* L.).

1. Transfer nodal segments consisting of a piece of stem to MS basal medium containing 30 g/l sucrose, 2.2 g/l phytagel without growth regulators.
2. Incubate shoot tips at 24 ± 1 °C, under a photoperiod of 16 h light/8 h dark, with a light intensity of 100-130 µmol/m^2/s for around 6 weeks. Subculture duration may different between species depends on growth rate. *i.e.* 6-7 weeks for cultivated species and 3-5 weeks for wild species. The last subculture duration before shoot tips isolation is critical.
3. Dissect axillary shoot tips (1.5-2.0 mm in length) from upper and middle part of the mother plantlets after 7 weeks of subculture.

Step 2. Cryopreservation

Preculture:

1. Preculture shoot tips in liquid MS medium with 0.3 M sucrose for 7~8 hours at 23 °C, under a photoperiod of 16 h light/8 h dark, with a light intensity of 100-130 µmol m^{-2} s^{1}.
2. Transfer shoot tips in liquid MS medium with 0.7 M sucrose and further preculture for 17~18 hours (overnight) under the same conditions.

Freezing procedure:

3. Transfer shoot tips in 10 ml PVS2 solution (24; glycerol 30 % + EG 15 % + DMSO 15% in MS basal medium with 0.4 M sucrose) and incubate for 20 min with continuous shaking (60 rpm).
4. A few minutes before plunging in liquid nitrogen (LN), place seven drops (2.5 $\mu\ell$ each) of PVS2 solution on an aluminum foil strip (7 x 20 mm).
5. Fill small dewar with liquid nitrogen.
6. Put one shoot tip in each of the seven PVS2 drops and then immediately plunge foil strip in LN. After a few minutes, transfer two foil strips to a 2 ml cryovial by forceps.
7. Keep samples in LN for a few minutes for cooling and transfer two foil strips to a pre-LN filled 2 ml cryovial.
8. Store for a desired period.

Thawing and unloading and recover procedure:

9. Preheat liquid MS medium with 0.8 M sucrose (unloading solution) at 40°C water bath before warming.
10. On warming, taken off foil strips from cryovials and immediately plunge in 6-7 ml of pre-heated (40 °C) unloading solution for 15 seconds.
11. Once ice has melted add the equal volume of the same pre-chilled unloading solution. (If the temperature of the preheated unloading solution is cooled enough, you need not add.)
12. Incubate shoot tips (explants) further in this medium at room temperature for a total of 30 min to facilitate unloading.
13. Retrieve explants from liquid medium and transfer to recover medium (MS medium + 0.05 mg/L IAA + 0.3 mg/L zeatin + 0.05 mg/L GA_3 + 3 % sucrose + 1.8 mg/L phytagel) and culture at 24±1 °C in a culture room equipped with fluorescent lamps, under a photoperiod of 16 h light/8 h dark and a light intensity of 25 $\mu E.cm^{-2}.s^{-1}$.
14. Evaluate survival 14 days after cryopreservation by counting the number of shoot tips that were green and swollen (≥ 3 mm in length).
15. Transfer explants to standard culture condition.

Possible Problems and comments:

Some of the followings are critical for successful cryopreservation.

1) Subculture condition

In *In Vitro* conservation system, plantlets usually grow weakly in airtight culture vessel under low light intensity at ambient or lower temperature. You need to product healthy and vigorous plantlet during subculture procedure for successful cryopreservation.

Subculture condition for acclimation and multiplication is recommended to be (1) air ventilation of culture vessels (Gaooze containers (height 13 cm, diameter 9 cm; KSTI Co, Korea, sealing 1 round with cling film), (2) high light intensity (100~130μmol/m^2/s), (3) low planting density (7 node cuttings per Gaooze culture vessel).

2) Subculture duration

The optimum subculture duration may different between species, depend on growth rate and maturity of axillary shoot tips, *i.e.* 6~7 weeks for cultivated species, 5 weeks for STN13 (*S. stenotomum*), 3~4 weeks for *S. goniocalyx*, *S. chacoense*.

3) Maturity of shoot-tips

Axillary shoot tips are formed, elongated, matured and sprouted. The optimum stage for cryopreservation is fully mature stage. Survival of cryopreserved shoot tips increased as the shoot tips size increased, before they sprouted. Suitable size of shoot tips at this optimum stage is 1.5-2.0 mm for cultivated species and 1.0-1.5 mm for STN13.

In general, apical shoot tips become degenerated after reach on top of the culture vessel and lower parts (1~2 shoot tips) are not fully matured at this stage.

4) Dissect shoot tips
You may need scalpel to dissect potato axillary shoot tips, instead of hypodermic needles, since you do not need to trim larger expanded leaves from the shoot tips. Dissect shoot tips with some node tissues attached by scalpel blade (No. 11).
5) Optional loading treatment
No loading treatment was performed in this protocol.
If the two-day preculture procedure is not profitable, you may constitute preculture with 0.7 M sucrose to loading treatment (0.5 M sucrose + 2 M glycerol in MS medium for 60 min). In such a case preculture duration at 0.3 M sucrose need to be increased to no less than 16~17 hours.

6) Unloading solution and duration
Unloading duration in this protocol is relatively longer than others. Prolonged incubation in unloading solution eliminates the necessity of transferring explants after one day of postculture. And the optimum sucrose concentration of the solution is 0.8 M sucrose, which is lower than ordinary one, *i.e.* 1.2 M sucrose.

7) Recovery culture
You may not need to transfer explants to a new medium after one day of recovery culture, since cryoprotectants are expelled enough during extended unloading with 0.8 M sucrose.
In many literatures, explants were post-cultured in dark condition for around one week. Explants do not need whole dark treatment, but a lower light intensity is enough.

8) Application to diverse genotypes including wild species
Growth rate and maturity of axillary shoot tips may different between species. Therefore you may need to identify optimum subculture duration and location of shoot tips in mother plants. You may apply this protocol for all *Solanum* species with some adaptation in subculture duration and maturity of axillary shoot tips.

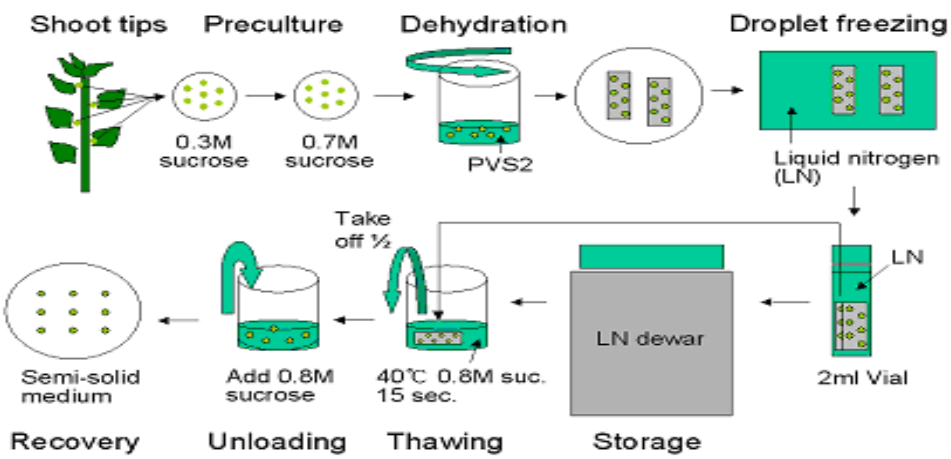

Figure 1. Diagram of droplet-vitrification procedure for *In Vitro* grown potato shoot tips.

PRACTICUM NOTE 8-2. Focusing on Liquid Nitrogen Storage of Living Fungi Using Polypropylene Straw

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There are many methods available for preservation of living fungi. These methods can be divided mainly into two groups, continuous growth and suspension of metabolism. The former includes periodic transfer, mineral oil storage and water storage, and the latter can be split into two methods, freezing and drying. The freezing techniques include liquid nitrogen storage and storage in deep freeze. The drying method includes freeze-drying, liquid drying and soil storage. The choice of method of fungal preservation depends upon the users' condition. Range of fungi, facilities available, level of stability and longevity required etc. must be taken into consideration. Recently, Korean Agricultural Culture Collection (KACC) established the preservation method for living fungi in liquid nitrogen using polypropylene drinking straws. We explain the method in detail and following is the protocol of KACC.

Liquid nitrogen storage using polypropylene straws

1. Preparation of cryo-protectant
   1.1 Composition of suspension media for freezing
   10% glycerol
   Glycerol (Merck 1.04093) 10 ㎖
   Distilled water 90 ㎖
   1.2 Sterilized at 121℃ for 15 mins.
   1.3 The cryo-protectant is dispensed in 5 ㎖ amounts in Nutrient Agar (NA) and Malt Extract Agar (MEA) plate and incubated at 25, 55℃ for 3 days as sterility check.
   1.4. Preserved in 4℃ until use.
   2. Preparation of straws and equipments for handling straws
   2.1 Polypropylene medical straws (diam. 4mm) (Hongik Chemical co., Korea) are cut into pieces of 50 mm, heat-sealed at one side with an Automaster sealer (SK-310/5.0, Tamsteck Co, Korea) and autoclaved at 121°C for 20 minutes (fig. 1).
   2.2 To handle conveniently the straws, straw holder and adjusting frame (Daeil Biotech co., Korea) were made as Stalpers et al.(1987) recommended. Cork borer with 2.8 mm diameter were imported from Centraalbureau voor Schimmelcultures (CBS), the Netherlands. Straw holders, adjusting frame and cork borer were autoclaved at 121°C for 20 minutes and dried in dry oven (fig. 2).
3. Filling of straws
   3.1 The fungi are grown on the adequate media on a 9 cm petridish. After incubation, the identity is checked by the specialist.

3.2 When the identity is correct and the culture is actively growing, 4-6 plugs of 2.8 mm diam. are punched out from the agar culture with a cork borer with pin, and transferred into the straw. At this time, 2 plugs are taken from central part of agar media and the other plugs are taken from marginal part of the culture. Ca. 0.2 ㎖ sterilized 10% glycerol is added to the straws with a sterile syringe and the straws are heat-sealed to close them (fig. 3). Straws are checked for leakage and if necessary resealed.

3.3 Labels (Brady, USA) with the KACC accession number and date written by label printer (300X-Plus, Brady, USA) are glued around the straw.

3.4 The straws are put in icebox, stored in 4°C refrigerator overnight and cooled at a rate of ca. -1°C/min to -80°C in deep freezer. And then they are stored in the gas phase above liquid nitrogen in the tank (Cryo Preservation LN_2 Container System, MVE 1520 HE-190, USA). Straws are stored in aluminium racks, containing 10 drawers divided into 64 squares. For each strain, 10 straws are prepared; one is opened for a viability check 1 week after storage, and 9 are stored in an aluminium rack.

4. Revival and viability check

4.1 Organisms are revived one week after storage and after 5 years. For revival, straws are thawed in a waterbath for five min at 30°C. (Oomycota are thawed at 25°C). The straws are rinsed in ethanol 70%, opened with a sterilized pair of scissors and placed on the suitable agar medium. Growth and identity of the 4 to 6 plugs per dish is checked by the specialists.

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