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No.

PRESERVATION OF PLANT GENETIC RESOURCES

TECHNICAL ASSISTANCE ACTIVITIES FOR GENETIC RESOURCES PROJECTS

JAPAN INTERNATIONAL COOPERATION AGENCY

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CONTENTS

I Preservation of Plant Genetic Resources in Japan, An Introduction

by

Hiroshi IKEHASHI

Chiba University

In the awareness both of endangered plant genetic resources in modern agriculture in one hand and of enormous potential value of genetic resources in other, it is for the first time in our history that the collection and preservation of genetic resources become a common global concern. For the past one or two decades, a number of international, regional or national projects have been initiated to preserve genetic resources with new equipments or facilities. Each of such genebanks have only been established after solving many problems of technical or administrative nature. But, even more difficult is to run such a project as routine daily works.

As a part of activities under the new JICA project which is implemented to provide assistance to local projects on plant genetic resources, a series of references are planned to exchange technical information. The first of this series is a collection of papers on the preservation technology of genetic resources. However, each of the papers should not be taken as an accomplished text book. And, the readers may not get quick answers from these papers to solve their problems. Yet, we hope that they may find any suggestion or idea in this booklet which states experiences in Japan. Also, we hope the readers may apply any idea, if there is, to their research or management of their project.

In the area of plant genetic resources, the development of each of local projects will be of mutual interest. If each country develops systematic collections as well as sound preservatories of genetic resources, we shall be able to access to useful materials through exchange between interested scientists. The development of systems on plant genetic resources benefits not only one country but also many other countries. We hope the papers here are informative to the colleagues abroad, who are in the same front as we are, namely, the collection and preservation of genetic resources for coming generations.

Preservation technologies

The method and magnitude of preservation of plant genetic resources largely depend on their propagation system. Viability of seed of most crop species can be preserved in dry and low temperature condition. The size of collection can be expanded without much difficulty. Thus, the strategy for collection is rather simple, if an adequate storage is available. The key is acquisition of storage facilities. Through recent monitoring of seed storage in some countries, we learned that valuable collections are still being lost in the lack of storage facilities. It is true that rigorous standards are necessary for long

term seed preservation as have been proposed. However, sound systems manageable at an allowable cost seem to be very much important for many centers serving as active collection. Aluminium foil package available for seed samples, for instance, seems to be reliable substitutes for expensive moisture control systems. For local, medium-term preservatories, mass-produced refrigerators would provide sound storage capacity.

Some areas in the preservation of genetic resources are yet to be solved. First, the long term storage of recalcitrant seed, like tea and citrus, which loose viability by drying and/or cooling, is not yet developed. But the handling of recalcitrant seed seems to be improved, though partly, by various ways of moist seed storage.

Second, most of vegetatively propagated crops or trees are still preserved tediously in fields, where virus infection is of incessant threat. Protective measures are essential against pests and diseases. A paper by Dr. I. KOZAKI and his colleagues covers in details the progress in the storage of fruit trees, providing innovative substitutes for laborious routine of fruit tree preservation. In place there is a prospect for cryopreservation of plant tissue instead of laborious plantation of these crops.

Such a technology would enable us to establish a genebank of vegetatively propagated plants or '*in vitro* genebank'. Sound systems based on such a technology are desired. A review paper by Dr. S. OKA on the progress of cryopreservation with liquid nitrogen is presented in this booklet. Tissue culture techniques developed for virus elimination seem to be an essential part of genebank both in handling introduction of plants and propagation of promising plants.

Third, reproduction of cross-pollinating crops needs isolation and adequate population size through successive growing in fields. Perhaps, the development of a technology for preservation of spore or pollen should be more encouraged in this area.

Collection strategy for those plant genetic resources which are not readily stored should be different from that for seed-crops. Perhaps this is an area where workers for plant genetic resources are challenged to develop workable concepts.

Status of preservation of plant genetic resources

The National Institute of Agrobiological Resources (NIAR) under the Ministry of Agriculture, Forestry and Fisheries (MAFF) has served as the 'Central Genebank' for seed crops in Japan. NIAR has preserved more than 40,000 accessions of 152 species in its long term storage facilities. Of 128,663 accessions which have been registered by 1987 under MAFF Genebank System, about 101,000 are stored in the form of seed. NIAR has just expanded its storage facilities by three times in a three year plan which started in 1985. In the present booklet, Dr. S. WATANABE who has played a key role as the head of the Seed Storage at NIAR presents a paper on their activities, including general managements and technical aspects of seed storage. In relation to seed technology, Dr. K. TAKAYANAGI presents a paper on testing seed viability. Although some methods shown in his paper are not routinely used in the seed storage, there may be suggestion for innovative approaches in dealing with seed viability.

By the end of 1987, National Fruit Tree Research Station (NFTRS) has preserved nearly 7,000 accessions in its field. National Research Institute of Vegetable, Ornamental Plants, and Tea has about 4,000 accessions of tea plants in its field. Besides, 1,675 accessions of mulberry, 5,377 of root crops, 1,525 of ornamental plants, 1,500 of vegetable crops and so on are preserved mainly by respective breeding stations of MAFF. For handling these increasing accessions, one solution was to expand resource allocation. In 1985, MAFF has started a comprehensive scheme through which National Seed Stock Production Farms have been integrated to support the evaluation and preservation of genetic resources, especially those of vegetatively propagated species.

National universities under the support of the Ministry of Education (MOE) have played also a significant role in preservation of their collections. Some of the collections of universities are designated by IBPGR as base collection. The size of each collection is indicated in the paper by Dr. S. WATANABE.

About 2,000 accessions of medicinal plants are preserved at the Medicinal Plant Research Station, National Institute of Hygienic Sciences, the Ministry of Health and Welfare (MHW). The station has started researches on preservation techniques. Some universities and pharmaceutic companies have developed their own collections, but the details are not known.

Wild plant species should be preserved in their native habitats. For research purpose, many of them are preserved in botanical gardens under the support of central or local governments. For instance, the Botanical Garden of the Tokyo University have preserved about 6,000 species besides herbarium specimens of 1.4 million. Edible sea weeds have been brought to preservatory in some institutes of MAFF. In view of potential utility of wild species as donor of chemicals or biomas resources, the preservation or collection of them becomes one of the important areas.

Information systems

Genetic resources should be preserved with respective descriptions of their characteristics, which can be called as 'character information or descriptors'. While, there is another kind of information which indicates sites or preservatories and the origin for each of genetic resources, which is called 'passport data'. Presently, all of the public institutions have published their lists of preserved germplasm. Most of the catalogue only presents accession names. And access to published lists is not easy.

So far, NIAR and FTRS periodically publish catalogues, but characteristics of the germ plasm are not yet listed. There is an urgent need to develop computerized, retrievable information systems which should include the characteristics as well as the site from which requested materials are available. Construction of data base for MAFF germplasm has been initiated in NIAR since 1983 with a specifically developed computer system, GRIMS/CGS.

Dr. S. SUZUKI, director of the Genetic Resources Center at NIAR, has played a leading role in information management. His paper which details the present status of information management in Japan is also included in this booklet. Like in other business in genebanks, the development of personal computers has provided easy access to information management. The paper by Dr. S. SUZUKI may provide general guidance to those who would like to develop an information system.

II Management of Plant Gentic Resources

by

Sinji WATANABE

National Institute of Agrobiological Resources
Ministry of Agriculture, Forestry & Fisheries

CONTENTS

1. Foreword

Recently genetic resources have generally been recognized as a source of new types of organisms and revolutionary strains or varieties. And there is a great expectation world-wide for its effective utilization today. In relation to this trend and from the viewpoint of preserving, managing and utilizing precious plant genetic resources, this article will center on Genetic Resources Storage Center, National Institute of Agrobiological Resources, Ministry of Agriculture, Forestry and Fisheries (Tsukuba).

2. Implication of Preservation and Management of Genetic Resources

Crop production should be supported by cultivating high quality and yielding varieties. For that purpose it is necessary to choose adaptable varieties. And these varieties are created by breeding, as generally known. In the course of breeding, genes locating in chromosomes determine varietal characteristics. Genes, stored in organisms, play a determining role in succession of life, are inherited to the next generation. And the genetic resources are collections of those genes.

Thus genetic resources should be always considered in association with life. Existing genes and related genetic resources have survived the innumerable changes and calamities since the beginning of the history. The number of species extinguished in a process of struggle for adaptation and survival for life, is estimated to be infinite. In that sense, existing genetic resources consist of champions of organisms which have succeeded to survive. Considering these background, existing genetic resources, especially wild species, species closely related to cultivated ones and old varieties are precious and they should not be lost.

However, many species of existing genetic resources are in danger of extinction, if they are left without cares. Therefore, the effort is necessary to keep present genetic resources for future using all the knowledge we have. There is an urgent need for preservation and management of plant genetic resources.

3. History of Plant Genetic Resources Preservation

1) History of plant genetic resources preservation in Japan

1893: Collection and evaluation of varieties, mainly in rice, wheat and barley started with the foundation of Central Agricultural Experiment Station, Ministry of Agriculture and Commerce in cooperation with many prefectural institutes.

1904: Total number of rice varieties collected at Kinai Branch of Central Agricultural Experiment Station reached 4,000.

1910: Rice breeding programme was started at Rikuu Branch of C.A.E.S. and so forth, and evaluation of varieties began at the same time.

1953: Laboratories in charge of preservation and characterization of breeding materials were established in three research institutes of Ministry of Agriculture and Forestry as follows:

Crop	Institute
Rice	Nat. Inst. of Agricultural Sciences (Hiratsuka)
Wheat & Barley	Kanto-Tosan Agricultural Experiment Sta.(Konosu)
Legumes & Miscellaneous Cereals	Tohoku Agricultural Experiment Sta.(Morioka)

1962-66: Nat. Inst. of Agricultural Sciences and Faculty of Agriculture, Kyushu University participated in a joint programme to collect local varieties of rice in Honshu, Shikoku and Kyushu regions. 1,300 samples were collected and put into preservation.

1965: A section "Seeds and Seedlings Preservation and Introduction" was established in the Div. of Research Liason and Coordination, Agri.

Forest.Fisheries Research Council Secretariat, Ministry of Agriculture and Forestry. And "Consultative Committee for Seeds and Seedlings Preservation and Introduction" was also started.

1966: The Seed Storage Center (capacity: 20,000 accessions) was founded at Nat. Inst. of Agri. Sciences (Hiratsuka). A green-house for Isolated Cultivation and Net-house for Seed Production were established for phytosanitary inspection at Horticultural Res. Sta. (Hiratsuka).

1967: The Green-house for Isolated Cultivation of Rice was set up at NIAS.

1971: Tropical Agriculture Research Center started a collection of varieties of various crops in tropics and subtropics in cooperation with the countries in respective areas.

1975: Ministry of Agriculture and Forestry started "Exploration and Introduction Programme" to collect useful crop genetic resources from abroad.

1978: The Germplasm Storage Center (capacity: 50,000 accessions) was established at NIAS (Tsukuba) to replace the function of Seed Storage Center in Hiratsuka.

1981: Ministry of Agriculture, Forestry and Fisheries (MAFF) established "Consultant Committee for Seeds and Seedlings Introduction".

1981-84: MAFF initiated a four-year programme for the collection of rare genetic resources within Japan, and collected those in 19 crops such as buckwheat, garlic, etc.

1983: MAFF started "MAFF Genebank Project" aiming at integrated systems for collections, management and utilization of biological genetic resources.

1986: January 25, 1986, MAFF made and proclaimed "Rules for Distribution of Plant Genetic Resources of Nat. Inst. of Agrobiological Resources for Research Purpose" to make NIAR's plant genetic resources available to outsiders, including private sectors.

1988: The Genetic Resources Storage Center, Ministry of Agriculture, Forestry and Fisheries" (capacity: 150,000 accessions) was newly established at NIAR.

2) History of plant genetic resources preservation in the world

1946: A programme concerning plant genetic resources was started by the United Nations (UN).

1947: Food and Agriculture Organization (FAO) was established as a subsidiary organization of UN.

1948: "Subcommittee for Breeding Materials of Plants and Animals" of FAO reached to an agreement to enhance cooperation in information exchange, collection and introduction of genetic resources in a world-wide scale.

1955-57: The 8th, 9th and 10th Meeting of FAO emphasized the necessity to provide wild species or original types of cultivated varieties for breeders.

1961: FAO held "Technical Conference for Plant exploration and Introduction" and formed an advisory opinion on which later activities were based.

1963: Center for Crop Research and Introduction was opened in Izmir, Turkey. Various countries started to build germplasm centers around this year.

1964: International Biology Programme was started. FAO supported the internationally cooperated research of IBP, especially in the field of "International standardizaion, Unification and Mechanization of Documentation and Processing of Data Concerning Crops".

1974: International Board for Plant Genetic Resources was founded, supported by CGIAR.

1981: The 21st General Meeting of FAO resolved to make a concrete proposal for intensifying the international activity in exploration, collection, preservation and utilization of plant genetic resources.

1983: "International Agreement on Plant Genetic Resources" was proposed to the 22nd General Meeting of FAO. The main point of it is to preserve and maintain agricultural genetic resources possessed by current germplasm banks and insure free exchange of them.

The number of Base Collections (to preserve seeds for rejuvenation) was five in 1976 and 25 (five of them were international ones) in 1983. In addition, there have been founded many Active Collections (to preserve multiplied seeds for distribution).

Table 1 The status of plant genetic resources preservation in MAFF genebank project

Crop	No. of genetic resources		
	Seed	Vegetative Plant	Total
Rice	21,218	0	21,218
Wheat & Barley	25,263	41	25,304
Legume	8,714	0	8,714
Root Crop	671	4,706	5,377
Miscellaneous Cereals & Industrial Crops	5,544	1,509	7,053
Forage Crops	23,952	5,188	30,140
Fruit Tree	0	6,922	6,922
Vegetables	14,925	836	15,761
Ornamental Crops	0	1,525	1,525
Tea	0	4,062	4,062
Mulberry	0	1,675	1,675
Tropical & Subtropical Crops	705	207	912
Total	100,992	27,671	128,663

Note: Project record in the fiscal year of 1987 (estimated).

4. Present Status of Preservation and Management of Plant Genetic Resources

1) Number of samples under preservation

(1) Japan

Table 1 indicates the situation of plant genetic resources preservation in 1987 according to "MAFF Genebank Project".

In addition to these, universities and their affiliated organizations, 14 in total, preserve substantial numbers plant genetic resources. For example, accession number only in rice adds up to 20,430 (1985). If we look at each example, following numbers are obtained: Botanical Garden, Faculty of Science, the University of Tokyo has 2,500 accessions of wild species and 3,500 of cultivated species such as flowers. Plant Germplasm Institute, Faculty of Agriculture, Kyoto University has 7,600 accessions of wheat, maize and their related species. Tsukuba Medicinal Plant Research Station, National Institute of Hygienic Science, Ministry of Health and Welfare has 2,000 accessions of medicinal plants. Institute for Agricultural and Biological Sciences, Okayama University has 5,700 accessions of barley and related species.

(2) Other countries

Table 2 summarizes the contents of germplasm preservation in USA, USSR, England, China and some other countries where International Organizations are located.

The 1980 data were in Table 2. The present accession number in 1988 must have increased a great deal. Not only those countries cited in Table 2, but also other countries are actively preserving plant genetic resources (PGR). Therefore, the total number of PGR accessions on earth under preservation & management can easily exceed one million. For example, more than 40,000 accessions are preserved in Bulgaria and Hungary respectively, and more than 20,000 rice varieties are preserved in Thailand.

Table 2 Organizations for preservation of crop genetic resources and contents of their preservation

Country	Organization	Number (×1,000)	Major crop
U. S. A.	Nat. Seed Storage Lab.	170	Wheat, Barley, Rice
U. S. A.	Plant Germplasm Lab.	73	Wheat, Barley, Oat
U. S. S. R.	N. I. Vavilov All-Union Scientific Res. Inst. of Plant Industry	326	Wheat, Barley, Legume
England	Plant Breeding Institute	73	Wheat, Barley, Vegetable
China	Crop Germplasm Resources Storage Center	300	Rice, Wheat, Legume
Japan	Nat. Institute of Agrobiological Resources	35	Rice, Wheat, Soybean
Philippines	*The Int. Rice Res. Inst.(IRRI)	70	Rice
Mexico	*Centro Internacional de Mejoramiento de Maiz y Trigo(CIMMYT)	42	Wheat, Maiz
India	*Int. Crop Res. Inst. for Semi-Arid Tropics (ICRISAT)	35	Legume
Colombia	*Centro Internacional de Agricultura Tropical (CIAT)		
Nigeria	*Int. Inst. for Tropical Agriculture (IITA)		

Table 2 Continued: 2/2

Peru	*Centro Internacional de la Papa		Potato
Lebanon	*Int. Center for Agricultural Res. in Dry Areas (ICARDA)		
Taiwan	*Asian Vegetable Res. Development Center (AVRDC)		Vegetable
Liberia	*West African Rice Development Association (WARDA)		Rice

* International organizations

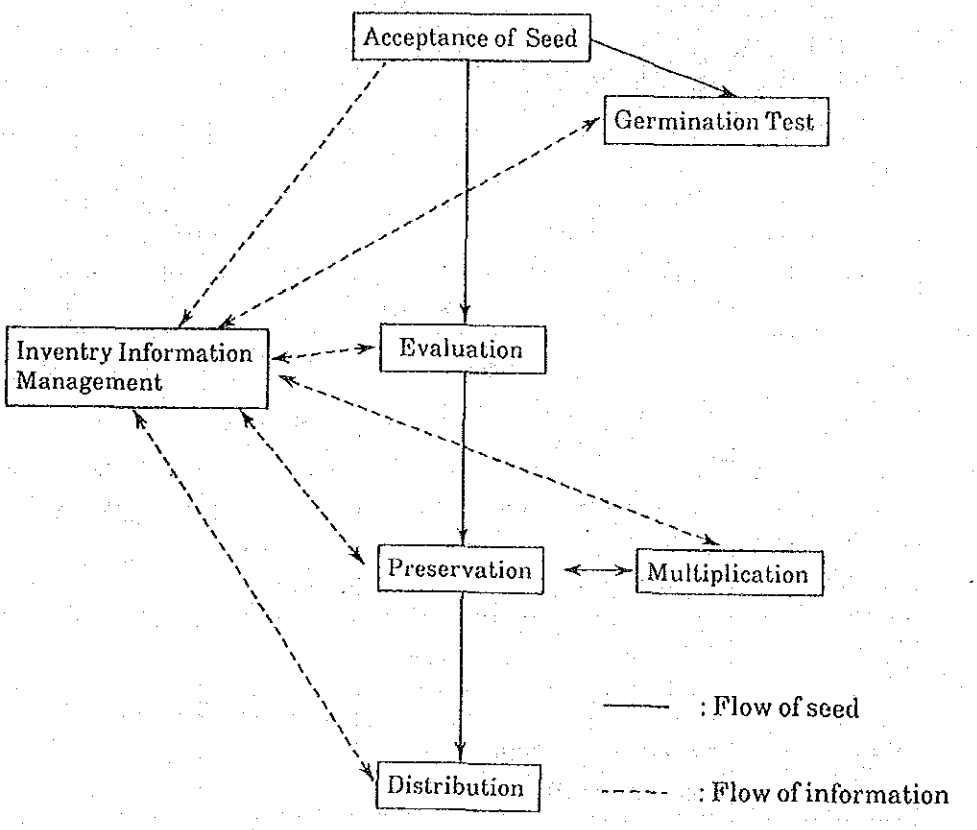

Fig. 1 Procedures for seed storage management

2) Outline of plant genetic resources management

The purpose of preservation and management is to accept various genetic resources in abundance, to evaluate and preserve them, rejuvenate them whenever necessary and distribute them on request. To realize these activities, the inventory information management is most necessary. The procedure for seed storage management is shown in Fig. 1.

3) Practice of plant genetic resources management

The Genetic Resources Storage Center of NIAR (shown in Fig. 2) practices procedures for seed storage management as follows:

(1) Acceptance, drying and germination test of seeds

Seeds are sent into the Genetic Resources Storage Center domestically and from abroad. For introduced seeds from foreign countries, isolated cultivation is done if necessary. Code numbers are put to accepted varieties, characteristic information is put into the computer, in the Data Processing Room. Seeds are purified from contaminants, inmature and damaged seeds, then dried in the Seed Drying Room (at 20 °C, 10% RH for 15 days as standard) until the moisture content becomes 6 to 8%. Germination test is done when seeds are accepted and every 5 to 10 years after storage in the Germination Test Room. The samples with low germinability are sent to the institutes in charge for rejuvenation.

(2) Packing and multiplication of seeds

Dried seeds are divided into the lots for long storage (base collection) and those for distribution (active collection) in the Operation Room. Seeds for long-term storage are packed into tin cans with a capacity of 180 ml and sealed under vacuum condition and then brought into the Long-term Storage Room(at −10°C,30% RH; constructed in 1978).

On the other hand, seeds for distribution are packed into plastic bottles with a capacity of 400 ml and brought into the Active Seed Storage Room (at -1°C, 30% RH;constructed in 1988). When seeds for distribution become scarce or germinability decreases to less than 50%, a part of seed in long-term storage is taken out and sent to the institutes for multiplication.

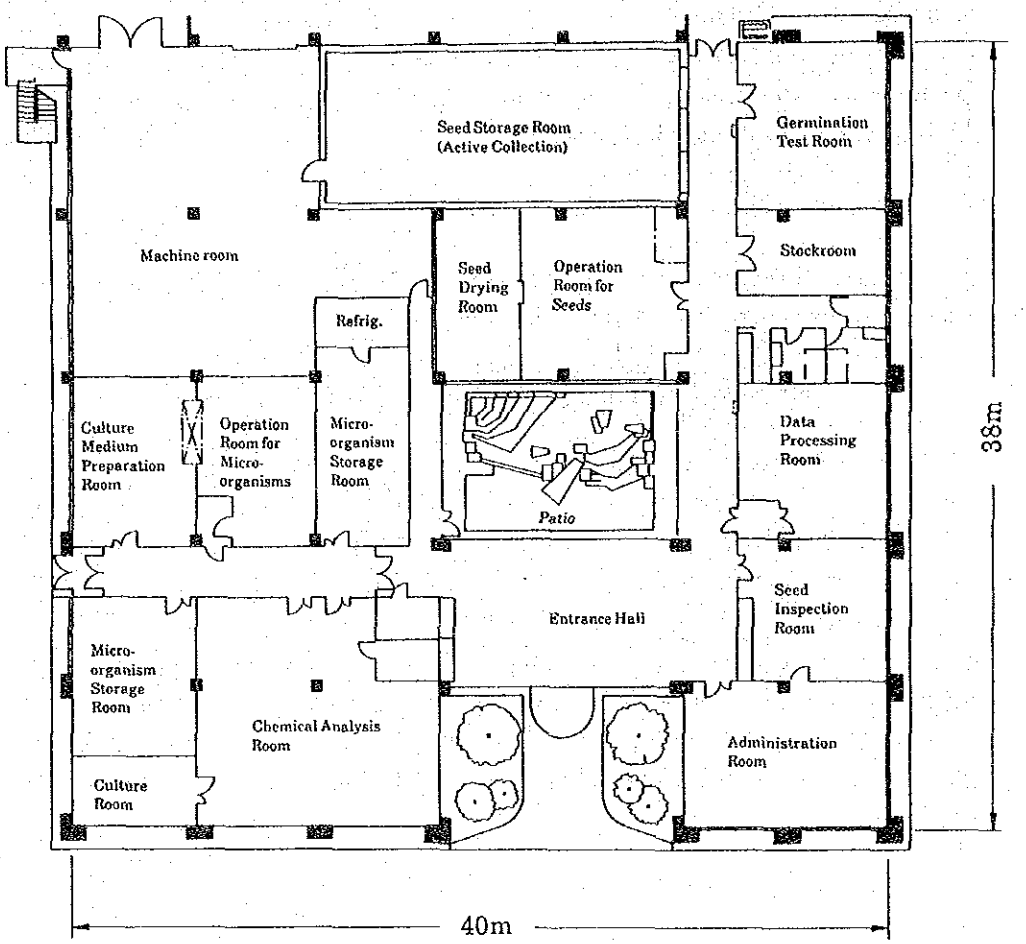

Fig. 2 Plan of Genetic Resources Storage Center, NIAR, MAFF

Housing and delivary of seeds in storage room is operated by automatic seed carrier controlled by a computer installed in the Operation Room. Therefore, there is no need for the operator to enter the cold storage room for bringing in and taking out seeds.

(3) Inventory information management of seeds

All the information concerning varieties under storage is stored in a computer in Data Processing Room. Therefore, inventory management, such as check of seed amount of each variety and picking up samples which need rejuvenation based on the germinability or other data, retrieval of samples based on the passport data or characteristics information, printing out of selected necessary data, etc. can be done as required.

(4) Distribution of seeds

a. Requirements for plant genetic resources utilization

Final aim of germplasm storage is an effective utilization of stored seeds. For that purpose, information of stored varieties, such as history or characteristics should be easy to know and available whenever demanded.

There are four important points:

a) Stored seeds of each sample are genetically pure without contaminant, and their viability should be well maintained.

b) Passport data should be fully supplied and filed as a data base.

c) Characterization or characteristics evaluation should be completed and the information be stored in a data base.

d) Information retrieval by users should be easy (i.e., search by computer or use of catalogues).

b. From a seed request to dispatch

Germplasm seeds stored as Active Collection of MAFF Genetic Resources Storage Center are distributed in response to domestic or international requests. In practice, the distribution of genetic resources is carried out according to "The

Rules for Distribution of Germplasm Seed of NIAR for Research Purpose" (Notification No.157 of MAFF) notified on January 25th, 1986. The applicants are requested to fill the "Application Form for the Distribution of Germplasm Seed for Research Purpose" and submit to the Director General of NIAR.

Requests from overseas are generally done in the form of letters. Distribution of germplasm seeds to other countries, is generally encouraged from the viewpoint of international cooperation. It is desirable to exchange and share stored genetic resources by the countries involved for the purpose of realizing more extensive utilization of them. Toward this purpose, the first prerequisites is to exchange catalogues of seeds for distribution.

4) Storage condition and longevity of seed

For the seeds stored as genetic resources, sustaining germinability and growth activity after germination and being free from genetic change during storage are primarily requested. The longevity of seed can be defined as the duration in which it is keeping good germinability and growth activity after germination. For lengthening the longevity, low temperature and low humidity are effective in general and a contact with the air should be avoided as far as possible. Addition of nitrogen or carbon dioxide gas sometimes tends to work positively.

HARRINGTON (1960) estimated that the germinability decreases to half per 5 °C raise of storage temperature or 1% increase of moisture content of seed within the range of 0-50 °C of temperature and 5-14% of seed moisture contents.

IBPGR recommends the following procedure as a desirable method for ensuring the long-term seed longevity: Dry seeds under the condition of 15°C of temperature and 10-15% of relative humidity and then store them at the low temperature of −10-−18°C. In this connection, the procedures practiced at Nat. Seed Storage Laboratory at Fort Collins, Colo. of USDA are shown below: Seeds are dried until the moisture content decreases to 3-5% and then packed into cans or paper bags internally laminated with aluminum foil. The storage temperature varies depending on the intended storage period; 4°C for 10-15 years, −12°C for 30 years, respectively with a relative humidity of 35%.Seeds under storage are periodically checked of their germinability. Moreover, conditions for lengthening seed longevity, genetic variations possibly caused by the artificial storage, changes of enzymes and components in seeds and so on are also surveyed.

ROBERTS (1972) classified causes which make seeds to lose their activity into two catergories, internal and external ones. He cited the accumulation of hazardous materials, the denaturation of high molecular compounds and the exhaustion of metabolites as internal causes, the degenerative effects of radiation and microorganisms as external causes. He also formulated the following equation for longevity estimation, based on the results which showed that the longevity of seeds was mainly controlled by their moisture content and the storage temperature.

$$\log\bar{p} = K_v - C_1 m - C_2 t$$

$\bar{p}$: longevity of seed m: moisture content of seed (%)

t: storage temperature(°C)

K_v, C_1, C_2 are constants calculated from experimental data.

Table 3 indicates estimated longevity of rice and wheat derived from the caliculation using ROBERTS' formula.

Although above-cited conditions are applicable to most of the cases, some species of crops have their own optimal temperature and relative humidity for storage. For example, in relation to optimal relative humidity, they can be classified to several groups as below:

Species lose germinability with dehydration – Tea, sugarcane,citrus etc.
Species whose optimal RH is 30% – Pea,eggplant, cucumber, tomato etc.
Species whose optimal RH is 10% – Lettuce, bunching onion, onion, edible burdock etc.

Table 3 The number of years in which crop seed can retain germinability of 90 %

Crop	Storage temperature (°C)	Moisture content of seed	
		5%	10%
Rice	−10	5,336	856 (years)
	0	1,000	175
Wheat	−10	161	46
	0	51	15

Note: Number of years are estimated from ROBERTS' formula (ROBERTS:1972).

5. Future of Plant Genetic Resources (PGR) Management

It is clear that we should intensify and extend our activity of plant genetic resources management for the future. The followings are the points which will play important roles in the future PGR management.

1) Conservation of plant genetic resources

It can be easily deduced that many species and varieties used to be grown in a long period of history from the initiation of agriculture to the present. Many of them are supposed to have vanished because there were no means to protect them. Today with the facility of environment control, we should not repeat this kind of loss. Hopefully in future with more sophisticated method for preservation, the conservation of PGR will become easier. For permanent preservation of various kinds of plant seeds, it will be necessary to clarify optimal preservation condition for each species. Especially it is important, for recalcitrant seeds which tend to lose viability under low temperature and low humidity condition, to define optimal temperature and relative humidity exactly.

2) Plant genetic resources information for users

For the users of plant genetic resources, information of preserved varieties such as history or characteristics is indispensable. History of a variety is necessary to understand how the variety has been derived. Therefore, collecting and storing of passport data come as first priority in PGR preservation.

Secondly, information on characteristics is especially valued by users as an indicator of usefulness of genetic resources. Reliable characteristics data are obtained from rational evaluation. It means that keeping a good condition for evaluation is looked upon such as a substantial evaluation system. The saying "Genetic resources without evaluation have no value for utilization", has much truth in it.

Thirdly, the establishment of information system with computer should be referred as a medium in which characteristics information obtained through evaluation are easily relayed to users. The Genetic Resources Storage Center of NIAR recognizes the establishment of information system as a key role of PGR utilization , and is trying to keep it in order in a long term perspective.

3) Enhancement of international cooperation in PGR preservation and management

For PGR preservation and management and their future development, international cooperation is indispensable because if the activity is limited within a country, the range of collection and utilization of PGR becomes restricted. Preservation and management system of PGR varies greatly from country to country. Funds should be provided for the construction of seed storage facilities in such an area where genetic resources face the danger of extinction due to the paucity of suitable facilities.
On the other hand, publication and supply of genetic resources by the countries where PGR preservation and management are well developed should be welcome from the stand point of extending the range of utilization of genetic resources.

As stated above, preservation and management of PGR should be advanced according to the situation of each country. In the process, coordination among various countries' activities is necessary. In that sense, the role of IBPGR which plays a key role in this field will continue to be great. At the same time, supports by various countries to IBPGR will also be important for the future development.

6. Concluding Remarks

All the persons who have something to do with genetic resources will agree to the point that genetic resources are the source of biological improvement and that they are precious. However, once it comes to practical points such as collection, evaluation, preservation and distribution, the policy may vary depending where they belong. For example, the manner of approach may be different among nations, between nations and prefectures, between nations and private enterprises, etc. The more widely the recognition that PGR are important is accepted, the higher a barrier for their exchange tends to rise. This is the cause of so called "Seed War".

Preservation and management of PGR has two faces, both of research and administration. Therefore, a person in the area is expected to have a sense of these two fields, or an organization should have personels from both fields.

Although various problems may arise in the actual site of PGR preservation and management, it is clearly shown that there is a growing

universal recognition of its importance. It is desirable that PGR preservation and management system will be established everywhere as soon as possible, and be effectively managed. And it is wished earnestly that the activity will be extended in the future beyond regional and country boundaries.

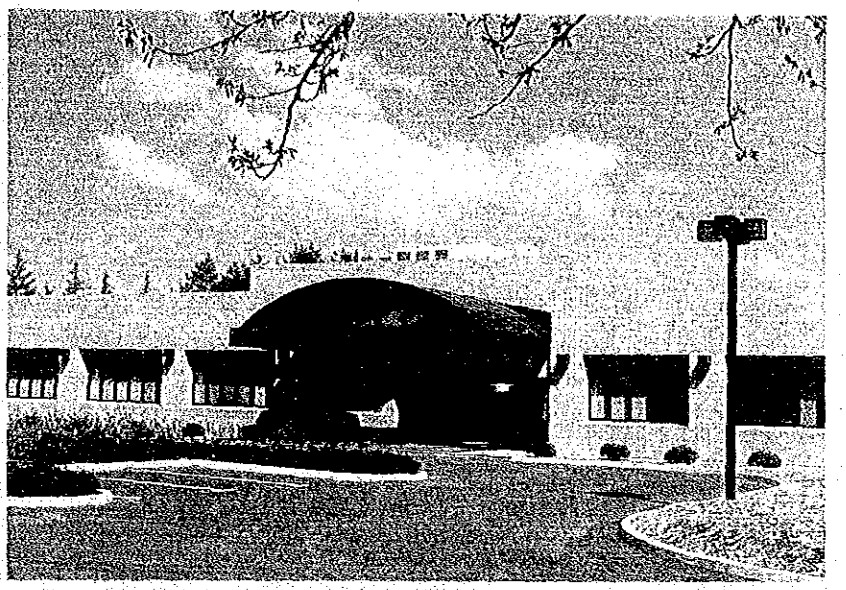

Photo. 1. Genetic Resources Storage Center of MAFF
(Completed in January, 1988)

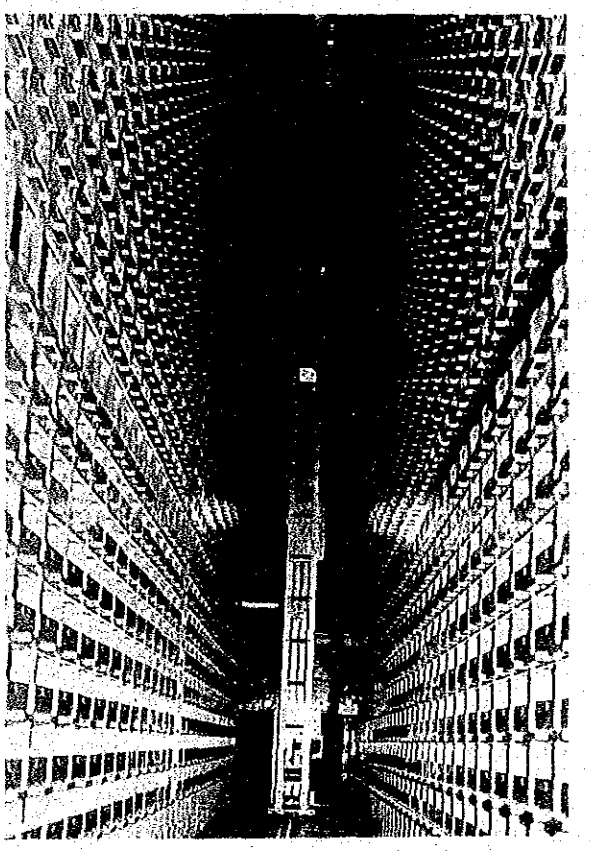

Photo. 2. Inside of Seed Storage Room
(The center is seed delivary apparatus.)

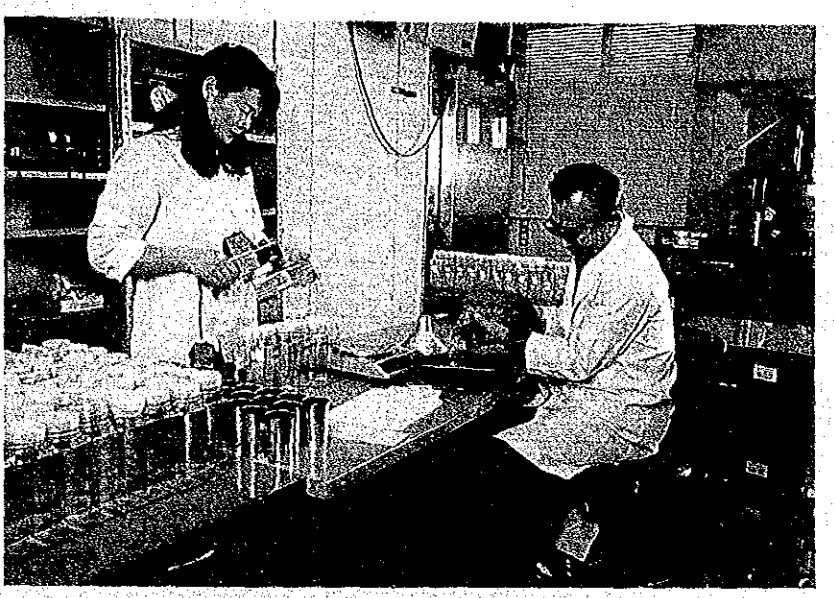

Photo. 3. Seed packing into cans (Seeds to be brought into storage room are packed into cans or bottles.)

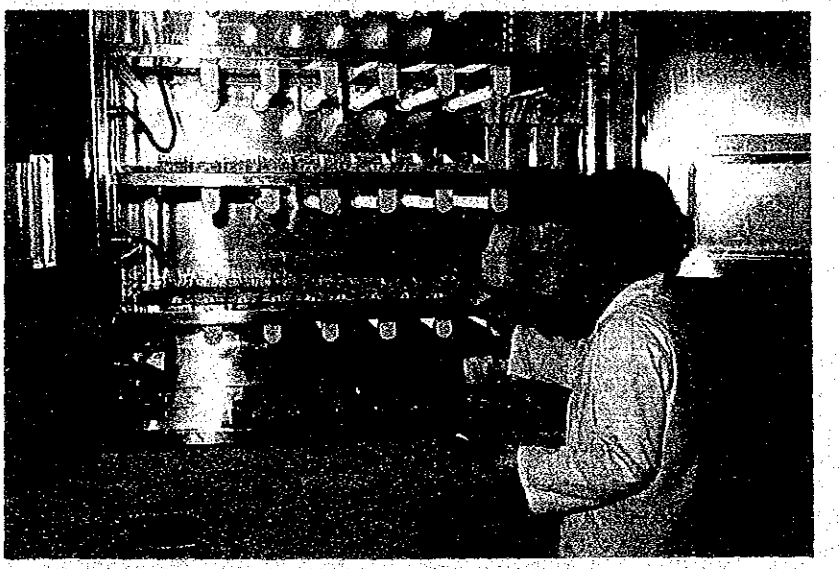

Photo. 4. Germination Test of Stored Seeds (Seeds are checked of their germinability before storage and every five years after that.)

III Viability of Seed and Testing Methods

by

Kenji TAKAYANAGI

National Research Institute of Vegetable,
Ornamental Plants and Tea (NIVOT)
Ministry of Agriculture, Forestry & Fisheries

CONTENTS

1. Introduction

Preservation of useful germplasm is a key step in plant breeding and conservation of natural resources. Seed preservation is one of the most effective methods for preserving the useful germplasm of seed plants. Recently there have been many attempts made in order to develop efficient methods of long-term storage of seeds, including pre- and post-harvesting managements and predicting seed storability or viability for seed storage. Seed viability is determined in several ways, such as seed germination tests, biochemical tests and others.

The seed germination test is generally conducted in compliance to the rules of the International Seed Testing Association (ISTA), the Association of Official Seed Analysts(AOSA)or the National Organization (Official Seed Testing Laboratory)."The ultimate object of testing for seed germination is to gain information with respect to the field planting value of the seed and to provide results which can be used to compare the value of different seed lots." However, it takes a rather long time to determine the seed germinability and it is a troublesome procedure to test germplasm seeds by the germination test.

As a biochemical test to determine slow germinative seed and/or dormant seeds, the tetrazolium method is recommended by the ISTA,AOSA or the National Rules. At the present time, a wide variety of seed vigor testing methods is required by seedmen and researchers. The AOSA published the progress report in the Seed Vigor Testing Handbook. "Seed vigor is the sum total of those properties of the seed which determine the potential level of performance and activity of a nondormant seed or seed lot during germination and seedling emergence." And, "A vigor test is a reproducible laboratory method which differentiates high vigor from low vigor seeds."

The AOSA seed vigor testing handbook (1976) includes the following ten methods for specified seed samples; a) accelerated aging, b) cold test, c) conductivity, d) cool germination test, e) seedling growth rate, f) slant board measure of root elongation, g) tetrazolium, h) HILTNER's brick-grit method, and i) simplified flower pot method.

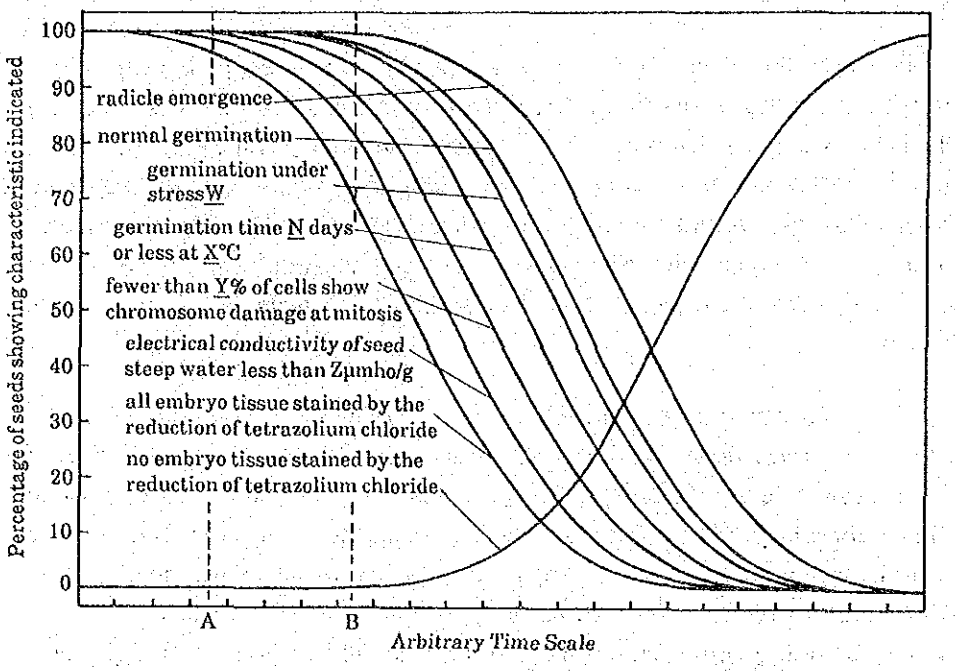

Fig.1. The pattern of deterioration in seed lots suggested from correlations determined between ageing symptoms and probit percentage germination which indicate the process of ageing in *individual seeds. Note that the distance in time between the curves* is a critical factor which, for some symptoms at least, differs between species (ELLIS and ROBERTS. 1980). Points A and B are referred to in the text. (ELLIS and ROBERTS, 1981)

2. Seed Deterioration with Aging

There are no rules for the seed viability testing of germplasm seeds. but the method should be reflective of the aging symptoms of the seed samples. Fig. 1 shows the symptoms of seed aging.

Upon harvesting at the proper stage of maturity, the seeds begin to deteriorate. The rate of deterioration varies with the species and with the storage conditions of the seeds. On the basis of the life span of seeds under optimum conditions, EWART (1908) divided seeds into three biological classes: (a)

microbiotic, whose life span does not exceed 3 years; (b) mesobiotic, whose life span ranges from 3 to 15 years; and (c) macrobiotic, whose life duration ranges from 15 to more than 100 years. However, we do not have decisive information on the optimum storage conditions for many kinds of seeds. Optimum storage conditions may differ with different species, but common important factors are the relative humidity and temperature of the ambient atmosphere.

The relative humidity of the air usually determines the moisture content of the seeds. According to HARRINGTON (1970), in a range of seed moisture between approximately 20 and 40 %, respiration of both the seeds and associated microorganisms results in heating during storage. If seed moisture is in the range between approximately 14 and 20%, the seed will deteriorate rapidly due to the destruction of its embryo by invasion of microorganisms.
Below 14% seed moisture for many species, a 1% loss in seed moisture doubles the life span of the seed. This rule of thumb applies down to about 4% seed moisture (HARRINGTON, 1963, 1970). Because of variations in chemical composition, each species has a different moisture equilibrium with a given relative humidity. Seeds of species with a high oil content such as genus *Linum* and *Brassica* equilibrated at lower moisture levels than cereal seeds.

NAKAMURA (1975) carried out storage experiments using vegetable, grass, legume and flower seeds for over 10 years. Based on the relation of seed moisture to life span, he divided the species into 3 groups including 2 subgroups each in the first and second groups:

Group I . Seeds whose viability is maintained in a humid atmosphere and at low temperature.

a. Seeds whose viability deteriorates rapidly in dry conditions. *Eutrema wasabi* MAXIM., *Quercus acutissima* CARRUTH., etc., 9 species.

b. Seeds whose viability deteriorates slowly in dry conditions. *Cheiranthus cheiri* L., *Salvia splendens* SELLO, etc,. 4 species.

Group II . Seeds whose viability is maintained best at 25-30% RH.

a. Seeds whose viability deteriorates rapidly below 10% RH. *Pisum sativum* L., *Phaseolus vulgaris* L., etc., 7 species.

b. Seeds whose viability deteriorates slowly below 10% RH. *Raphanus sativus* L., *Brassica pekinensis* RUPR., *B. rapa* L., *Daucus carota* L.,etc., 36 species.

Group III. Seeds whose viability is maintained best at 10 % RH. *Lactuca sativa* L., etc., 7 species.

More than half of the species tested were included in the subgroup b of the group II.

Temperature is also important in affecting seed viability. The lower the storage temperature, the longer the life of the seed.
According to HARRINGTON (1963), between 0° and 50°C, every 5°C lowering of the storage temperature doubles the life of the seed.

ROBERTS (1961,1972) deduced the following equation showing the relationship between the life span of seeds and the environmental factors.

$$\log\bar{p} = Kv - C_1m - C_2t$$

where $\bar{p}$ = the mean viability period, m = moisture content, t = temperature (°C), and Kv, C_1 and C_2 are constants. This equation has been shown to be reasonably accurate for predicting the percentage viability of rice seeds from a few days to several years (ROBERTS, 1961, 1972; ROBERTS and ABDELLA, 1968). ITO (1965, 1970) found that the equation is satisfactory for predicting the viability of rice seeds for a period of at least 15 years. ROBERTS and ROBERTS (1972) constructed viability nomographs as a guide to prediction of seed viability under hermetic storage conditions for rice, wheat, barley, broad beans and peas.

The gaseous composition of the atmosphere is also another factor affecting seed viability. Seeds are favorably stored in hermetic containers at low temperature and low relative humidity for longterm-storage. In hermetic containers, for example a sealed ampule, the gaseous composition of the atmosphere alters with time because of the respiration of seeds and their associated microflora. ROBERTS and ABDELLA (1968) showed that when pea seeds at 18.4% moisture content were stored in a sealed ampule at 25°C, there was a more or less linear increase in carbon dioxide content and a decrease in oxygen content. If dried seeds are stored at lower temperature, seed respiration might be less.

ROBERTS (1961) described that anaerobic conditions do not appear to be deleterious to the viability of cereal seeds. Storage in nitrogen is said to be more favorable for most seeds than in air under similar conditions of temperature and moisture. High oxygen concentration tends to decrease the viability period. These facts suggest that aerobic conditions are not especially beneficial to the maintenance of viability. The effect of carbon dioxide in high concentrations is

not clear, but in any case it is certainly not markedly deleterious to viability. In our present state of knowledge, ROBERTS (1961) says that it would be profitable to concentrate on lowering the moisture and temperature of the seed.

3. Storage Experiments in *Brassica* Seeds

There have been several experiments on the seed longevity of *Brassica* and related genera under various conditions, i.e., in the soil, laboratory,refrigerator and so on.

In 1879, BEAL at Michigan State University began a classical experiment on buried seeds in the soil that lasted for over 90 years. In BEAL's experiment, 8% of *Brassica nigra* seeds still germinated after 50 years but did not after 60

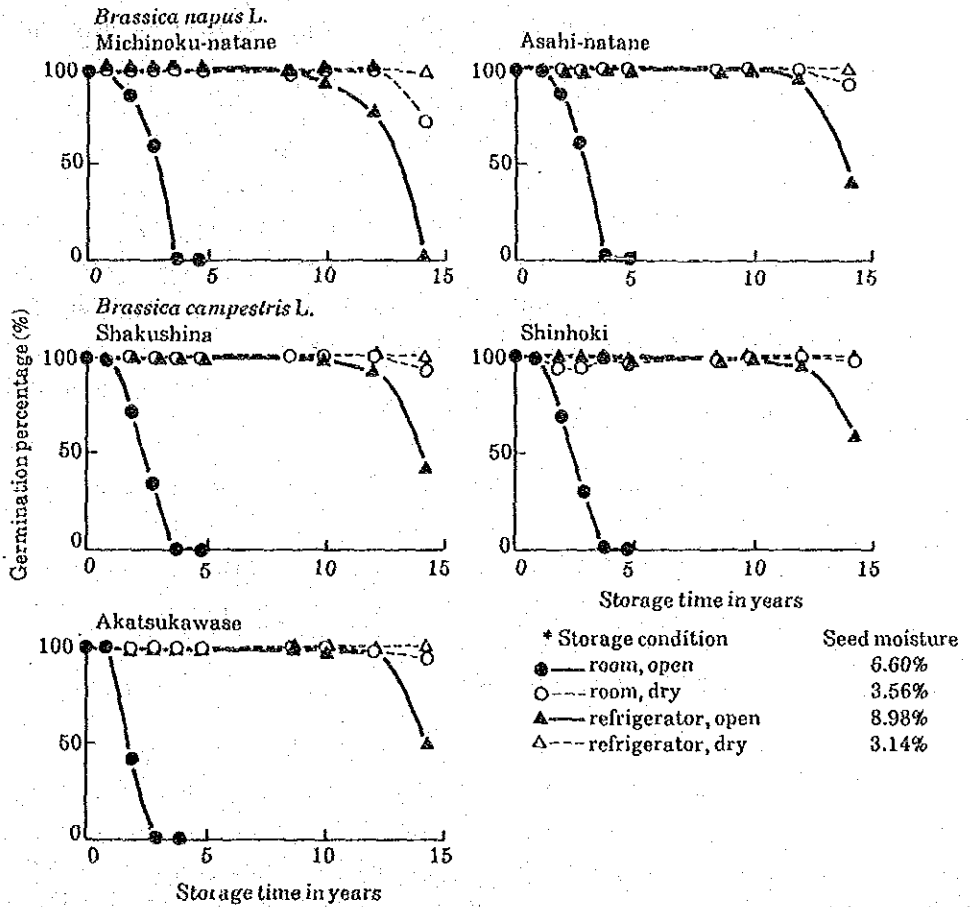

Fig. 2. Survival curves for rape seeds stored in different conditions for 15 years (TAKAYANAGI, MURAKAMI and YAMADA, unpublished data).

years.HARRINGTON (1972) summarized the records of species with seeds shown to have longevity of 10 years or more. Even in soil where the environment may be moist, dark, with less oxygen pressure and a relatively constant temperature, seeds can retain their viability for considerably long periods as shown by OHGA in the Indian lotus seed (*Nelumbo nucifera*). Similar experimental results are also given in the table prepared by OWEN (1956).

In our laboratory, the Division of Genetics, NIAS at Hiratsuka, Kanagawa Prefecture, storage experiments on rape seeds started in 1962. Seeds were sealed in a plastic box with silica gel(dry) or put into a gauze bag (open), and placed in the laboratory or in the refrigerator. Seed moisture in open storage in the laboratory may have fluctuated because summer is hot (30.3 °C average maximum daytime temperature in August) and humid (81% average relative humidity in August) and winter is cold (0°C average minimum daytime temperature in January) and dry (60% average relative humidity in January) in Kanagawa. Fig.2 shows the results obtained in the 15 years' storage experiment. The seeds stored open in the laboratory lost their viability within the 4th year, and those stored open in the refrigerator (2 to 4°C) began to deteriorate after 10 years of storage. Some of the seeds stored dry in the laboratory began to decline in the 15th year of storage, while the dried and cooled seeds were still viable in the same storage period. A combination of low moisture content and low temperature was best for extending the life span of seeds.

4. Seed Viability Test without Sprouting

Seed viability is ordinarily determined by a standard germination test. The International Seed Testing Association, the Association of Official Seed Analysis and other National Seed Testing Laboratories have their own rules for agricultural, horticultural and tree seeds. However, germination tests are time consuming. It takes 1 or 2 weeks for *Brassica* seeds to reveal their viability. Accordingly, it is desirable to know the seed viability as quick as possible by an easy method when there are many seed samples to be tested.

The prerequisite for the methods of testing seed viability are quickness and easiness, because there are restrictions on the quantity of germplasm seeds, such as small sample size and invaluable materials.

There are several methods developed so far for testing seed viability without sprouting, i.e., measurement of seed respiration (WOODSTOCK, 1965; WOODSTOCK and GRABE, 1967), measurement of enzyme activity (DAVIS, 1925;

LINKO and SOGN, 1960), embryo staining with dyes (NELJUBOW, 1925; EFFMAN and SPECHT, 1967), the tetrazolium or tellulite method (LAKON, 1942; HASEGAWA, 1936; GRABE, 1970), the electrical conductivity method (MATTHEWS and BRADNOCK, 1967, 1968), the seed exudate method (TAKAYANAGI and MURAKAMI, 1968; TAKAYANAGI, 1977), the x-ray contrast method (SIMAK, 1957; KAMRA, 1964; NAKAMURA, 1971) and so on. BARTON (1961), HEYDECKER (1969), ABDUL-BAKI and ANDERSON (1972), JUSTICE (1972), MACKAY (1972), WOODSTOCK (1973) and MCDONALD,JR. (1975) reviewed seed viability and vigor tests.

Among these methods, the following four will be explained in this chapter.

1) Tetrazolium method

(1) Original

HASEGAWA, K. (1936) ..... Tellulate for forest seeds
LAKON,G.(1942) ..... Tetrazolium salts for cereal seeds

(2) Principle

In a biochemical test, evidence of the reduction processes which take place in living cells is provided by the reduction of an indicator. The indicator used is a colorless solution of a tetrazolium salt absorbed by the seed. Within the seed tissues, the solution interferes with the reduction processes of living cells and accepts hydrogen from the dehydrogenases. By hydrogenation of the 2,3,5-triphenyl-tetrazolium chloride or bromide, a red, stable and non-diffusible substance, triphenyl-formazane, is produced in the living cells. This procedure makes it possible to distinguish the red-colored living parts of the seeds from the colorless dead ones. In addition to the completely stained viable seeds and completely unstained non-viable seeds, partially stained seeds may occur. Varying proportions of necrotic tissue are found in different zones of these partially stained seeds. The position and size of the necrotic areas in the embryo and/or endosperm determine whether such seeds are classified as viable or non-viable. Color differentiation is considered decisive mainly to the extent that it permits recognition and location of sound, weak and dead tissue. For this reason, and the indicator used, the test is designated as the Topographical Tetrazolium Test (from the International Rules for Seed Testing, 1976).

(3) Procedure

a. Reagent: 0.1 per cent (for bisected embryo) to 1.0 per cent (for non-bisected) 2,3,5-triphenyl tetrazolium chloride (or bromide), pH 6.0-8.0 (distilled water or phosphate buffer).

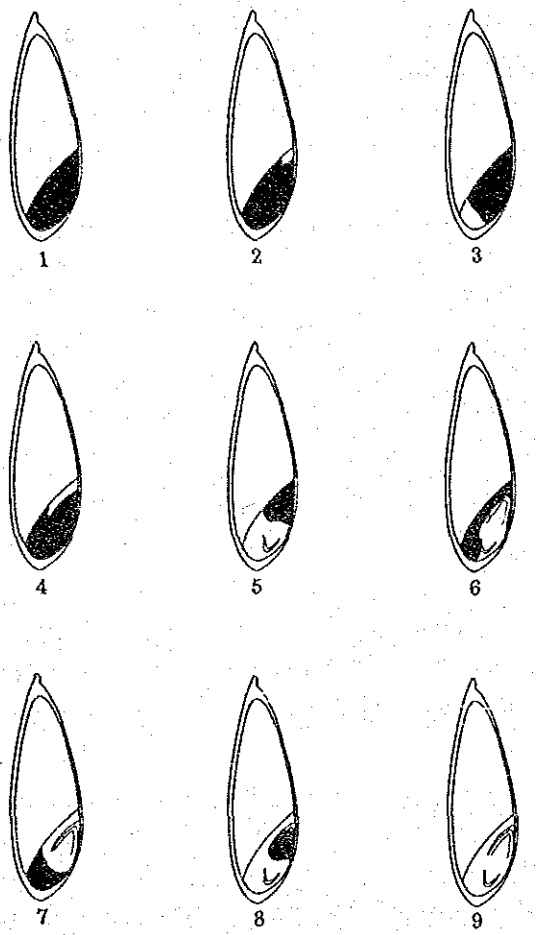

Fig. 3 Criteria for interpreting tetrazolium test results on Pensacola Bahiagrass seed. Black areas indicate stained, living tissue; white areas represent unstained, dead tissue.
No. 1 GERMINABLE. Embryo completely stained.
No. 2-4 GERMINABLE. Extremities of scutellum unstained.
No. 5 NON-GERMINABLE. Lower half of embryo unstained.
No. 6 NON-GERMINABLE. Embryonic axis unstained.
No. 7 NON-GERMINABLE. Upper half of embryo unstained.
No. 8 NON-GERMINABLE. Scutellum and radicle unstained.
No. 9 NON-GERMINABLE. Embryo completely unstained or only stained very light pink.

(after GRABE, 1970)

b. Seed sample: Place seeds on top of, or between, moist blotters or paper towels overnight. Bisect longitudinally (for cereals) or remove seed coat (for dicot).

c. Staining and evaluation : Immerse seed samples in the tetrazolium solution. Incubate for 1 to 6 hours at 35 °C in the dark. Evaluate the seed samples according to the rules (Fig.3 and 4).

(4) References

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GRABE, D. F. (ed.) (1970) Tetrazolium Testing Handbook for Agricultural Seeds. Contribution No. 29, Ass. Offic. Seed Analysts.

2) X-ray contrast method

(1) Original

SIMAK, M. (1957) ..... $BaCl_2$ and X-ray for forest seeds

(2) Principle

Loss of selective absorption or loss of membrane integrity of the seed results in the non-selective absorption of heavy metal salts by the seeds. Examine the seed transparency with soft X-ray. Examples of metal salts or umbragenous agents used: $BaCl_2$, LiCl, NaI, KI, NaBr, KBr, $CaCl_2$, $FeCl_2$, $Ca(NO_3)_2$, $AgNO_3$, $CoCl_2$, $PbCl_2$, $ZnCl_2$, Umbradil or Urografin.

(3) Procedure

a. Reagent: 25% solution of $BaCl_2$ as an umbragenous agent.

b. Procedure: Soak seed samples in 25% $BaCl_2$ solution for 16 hours at 25 °C. Wash and dry seed samples. Examine the seeds with the Softex EMB.

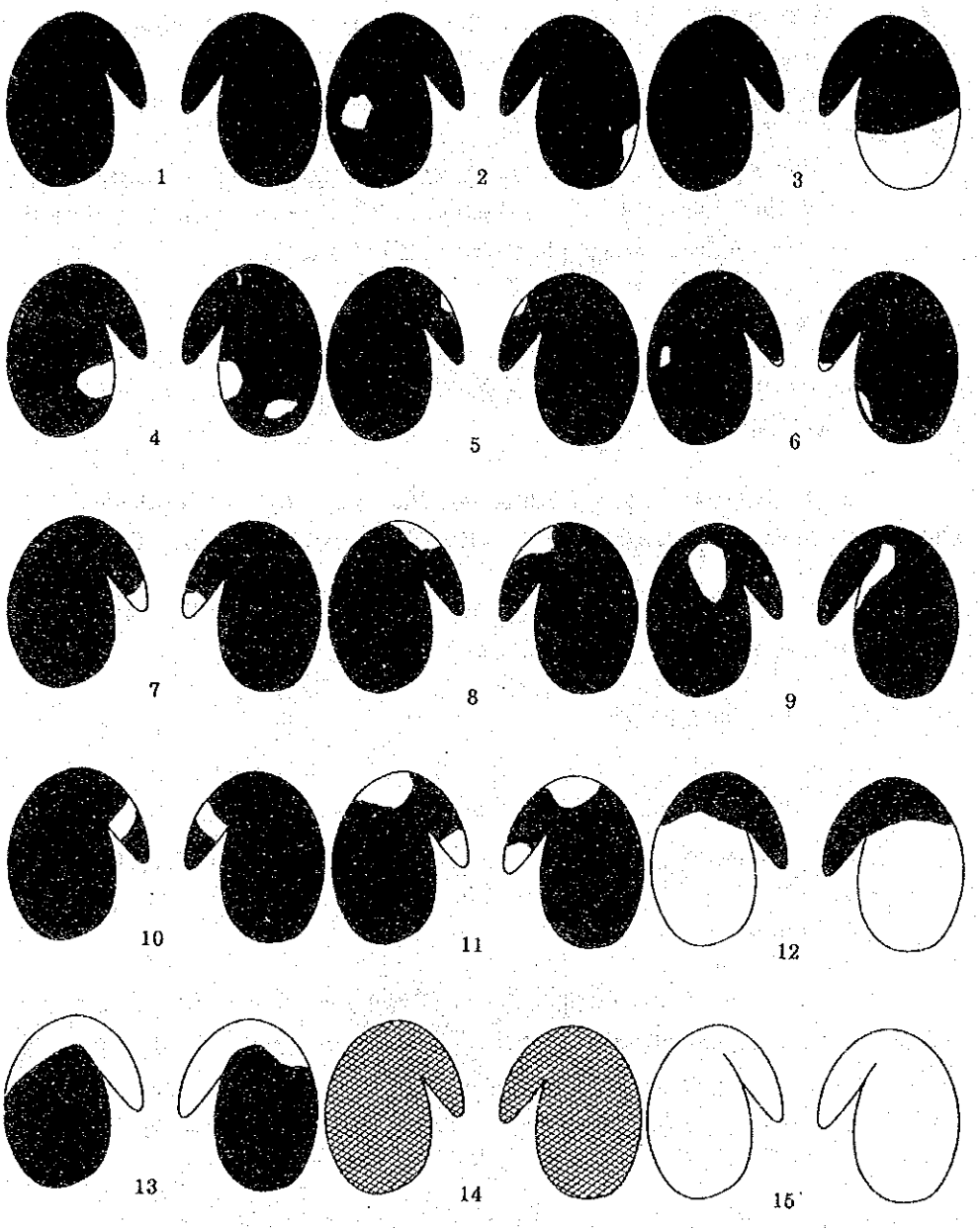

Fig.4. Criteria for interpreting tetrazolium test results on crimson clover seed. Illustrations are paired and depict both sides of the seed. Black areas indicate stained, living tissue; white areas represent unstained and dead tissue.

No.1 .... GERMINABLE. Seed completely stained.
Nos.2-4 . GERMINABLE. Minor unstained areas on cotyledons.

No.5 .... GERMINABLE. Minor unstained area on upper portion of radicle-hypocotyl axis.
No.6 .... GERMINABLE. Extreme tip of radicle unstained; minor unstained spots on cotyledons.
No.7 .... NON-GERMINABLE. More than extreme tip of radicle unstained.
No.8 .... NON-GERMINABLE. Unstained area at juncture of cotyledons and radicle-hypocotyl axis.
No.9 .... NON-GERMINABLE. Unstained area near point of attachment of cotyledons and radicle -hypocotyl axis over location where plumule develops.
No.10 ... NON-GERMINABLE. Radicle-hypocotyl axis bisected by unstained area.
No.11 ... NON-GERMINABLE. Unstained areas on radicle-hypocotyl axis and at point of attachment of cotyledons to axis.
No.12 ... NON-GERMINABLE. More than one-half of cotyledonary tissue unstained.
No.13 ... NON-GERMINABLE. Radicle-hypocotyl axis unstained.
No.14 ... NON-GERMINABLE Seed stained off color, grayish-red, orange-red or glassy or transparent red color.
No.15 ... NON-GERMINABLE Seed completely unstained.

(after GRABE, 1970)

c. Evaluation: Transparent seed ··· good and healthy
Shadowed seed ····· poor germinable or dead

(4) References

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SWAMINATHAN, M. S. and S. K. KAMRA (1961) X-ray analysis of the anatomy and viability of seeds of some economic plants. Ind.J.Genet. and Plant Breed. 21, 129-135

NAKAMURA, S. (1971) Determination of germinability of egg plant and pepper seeds with X-ray contrast method. Proc. Int. Seed Test. Ass. 36, 99-104

3) Electrical conductivity method

(1) Original

FICK, G. L. and R. P. HIBBARD (1925) .... forage seeds

(2) Principle

Deteriorated seeds leach electrolytes into the water in which they are soaked.

(3) Procedure

Seed samples (x g) are soaked in deionized water (50x ml) and incubated at 20° or 25°C for 24 hours. Measure the electrical conductivity of the resulting steep water with a conductivity meter. Results are expressed as μS (siemens)/cm/g of dry seeds. An example is shown in Fig.5.

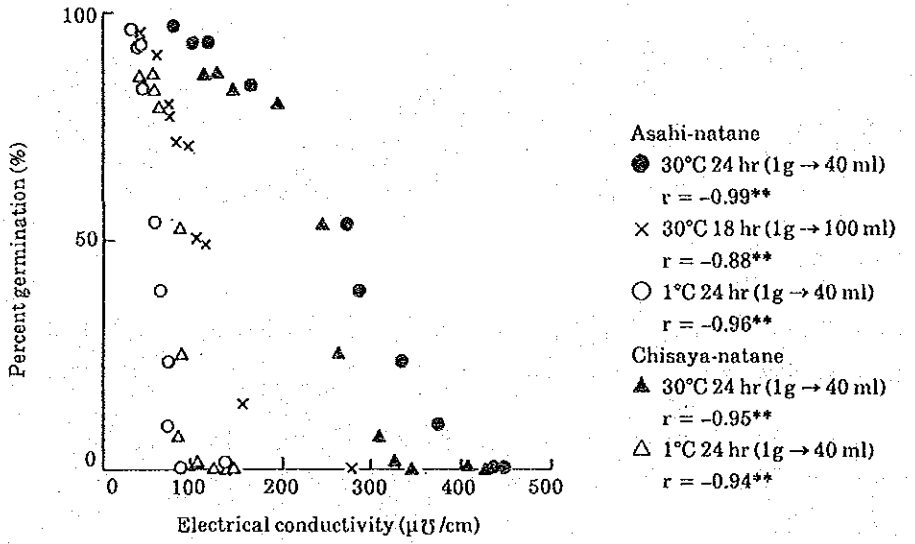

Fig 5. Relationships between electrical conductivity of the water in which the seeds were soaked under various conditions and seed germinability. Electrical conductivity was measured by a conduct meter, Toa Electronics Ltd., Model CM 2A (TAKAYANAGI, 1977)

(4) References

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4) Seed exudate method

(1) Original

TAKAYANAGI, K. and K. MURAKAMI (1968) ..... rapeseed

(2) Principle

Deteriorated seed leaches sugars into the water in which they are soaked, especially monosaccharide, glucose exudation, revealing the earliest symptom of the seed deterioration (Fig.6).

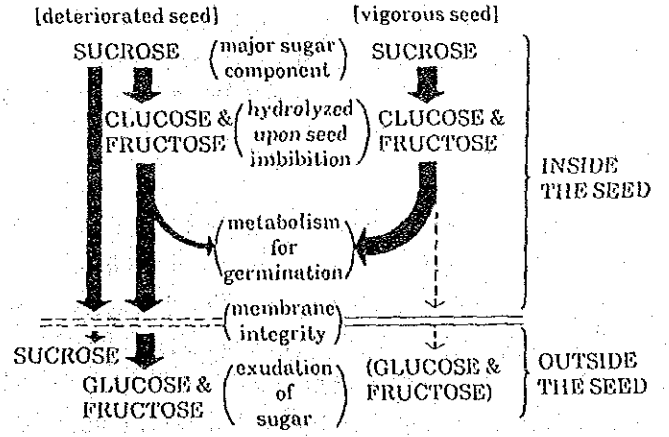


Fig 6. An expected sugar flow upon imbibition of deteriorated and vigorous seed of *Brassica napus* L. (TAKAYANAGI, 1977)

(3) Procedure

a. Mass test

One hundred to 200 mg of seed samples are soaked in 0.5 ml of sterile water and incubated at 30 °C for 6 hours. After incubation, exuded sugar (glucose) is determined either with a sugar analysis paper such as Tes-Tape (a urine sugar analysis paper,Ili Lilly Co. Ltd., Illinois) or other analytical procedure (anthrone-sulfuric acid method etc.). If the Tes-Tape reaction is negative the seed will be viable. If the Tes-Tape color (yellow) changes to light green or green, the seed will be weak or non-germinable or dead, respectively (Fig.7 and Photo.1).

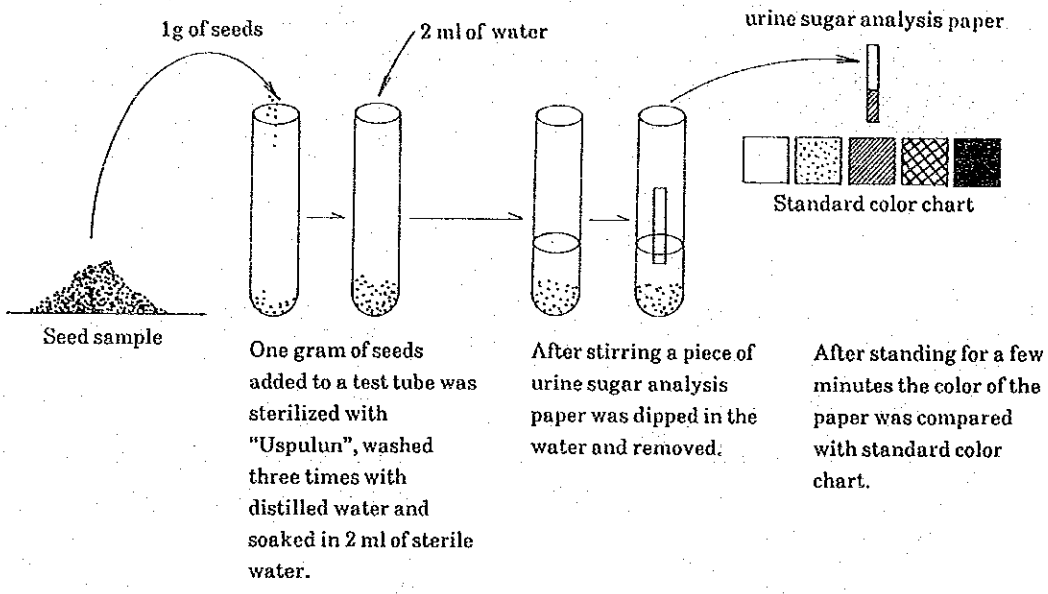
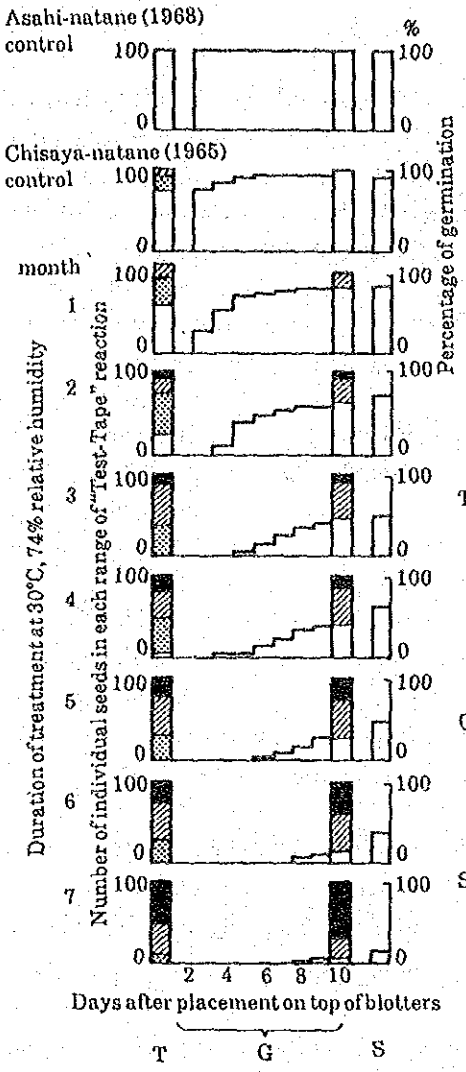


Fig. 7 Procedure of testing seed viability by using urine sugar analysis paper mass test (TAKAYANAGI and MURAKAMI, 1969).

b. A single seed test

A single seed of rape was soaked in 0.02 ml of sterile water in a small hole (5 mm of diameter, 3 mm of depth) in the plastic plate (50 holes on each) and incubated at 30°C for 24 hours. After incubation, exuded sugar (glucose) from a single seed is detectable with a piece of Tes-Tape paper (Fig.8 and Photo.2). The results of both tests are presented in Fig. 8. Seed viability and the amount of sugar exuded (Tes-Tape coloration) showed a significant and negative correlation.



T: Reaction of "Tes-Tape" test for individual seed. The coloration of "Tes-Tape" ranged from no change (0) = □, to light green (0.1–0.7) = ▨, green (0.8–1.2) = ▨ and dark green (1.3–4.0) = ■. The length of the column is represented with the number of individuals in each coloration group.

G: Daily germination and development of seedlings for ten days; the number of normal □, abnormal seedings ▨ and ungerminated seeds ■ were counted at the tenth day.

S: Percentage of germination in soil.

Fig 8. Relationship between "Tes-Tape" test for single rape seed and its germinability with an artificially aged seed. Seeds were treated under unfavorable conditions (30°C, 74% RH) for various durations (0 to 7 months) (TAKAYANAGI and MURAKAMI, 1969).

Direct correlation between a single seed viability and the amount of sugar exuded was also obtained.

This method was extended to apply to other cruciferous seeds such as *Brassica oleracea, B. pekinensis, B. rapa, B. juncea* and *Raphanus sativus* (TAKAYANAGI, 1977), white spruce seed (HOCKING and ETTER, 1969), peas (MATTHEWS and CARVER, 1971) and so on. However it was unsuccessfully applied to the seeds of Cucurbitaceae, Solanaceae and Gramineae so far tested.

(4) References

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5. Conclusion

For the conservation of natural resources and useful germplasm, a program for long-term seed storage is needed. Seed viability should be checked before and during the storage, and by this procedure we can preserve seeds safely for a long time. For this purpose, a simple and quick method is essential.

There have been several methods for the testing of seed viability without sprouting as already mentioned. Each of them is not always useful for every kind of seed. The seed exudate method explained in this chapter has several advantages. It is simple (with Tes-Tape), quick (within 6 to 7 hr.), applicable to small seeds such as cruciferous crop seeds and sensitive enough to test a single seed. If a tested seed is viable, it can be stored again after drying. This method is hopefully useful, not only for the Seed Storage Laboratory man, but also for seedmen in various fields. Another method will be also essential for specific kind of seeds.

We hope more effective and practical methods will be developed in the future.

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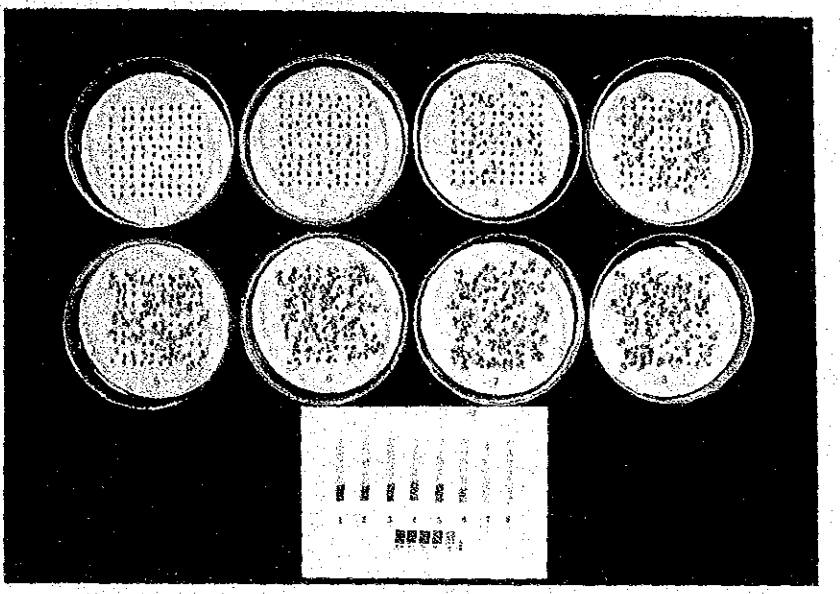

Photo 1. Mass test of rapeseed by Tes-Tape.

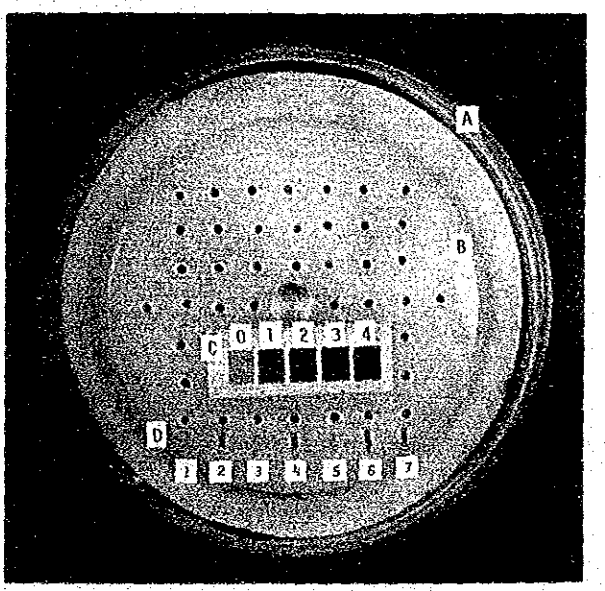

Photo 2. Single seed test of rapeseed by Tes-Tape.

IV Germplasm Preservation of Fruit Trees

by

Itaru KOZAKI, Mitsuo OMURA, Nagao MATSUTA and Takuya MORIGUCHI

Fruit Tree Research Station, Ministry of Agriculture,
Forestry and Fisheries

CONTENTS

1. Introduction

Usually, cultivars and wild relatives of fruit trees are maintained in orchards as genetic stocks. The adult trees, that are so preserved, provide flowers, pollen, fruits, seeds, and scions whenever they are required for cross-breeding, propagation, and the observation of many other traits. The most important advantage in the preservation of adult trees is that they are ready for immediate use for many purposes. Other germplasms, even nursery plants, take several years to grow into adulthood. However, there is a serious problem in preservation of whole plants; they require a large land area and a large amount of labour for their management. The second problem is that there is a tendency to lose some trees or cultivars through accident; such as frost, disease, pests, etc. Therefore, a more effective method for the preservation of germplasm has been long desired by fruit breeders.

As storable germplasm, the utility of pollen, seed, scions and cultured tissue can be investigated. Most fruit trees, however, are heterozygous in almost all genes, so pollen and seeds are a means of preserving genetic variation rather than varietal characteristics, when compared to the whole plant or scion. Characteristics of these forms of germplasm are shown in Table 1.

Table 1 Characteristics of storable forms of germplasm of fruit trees.

Germplasm	Pollen	Seed	Scion	Tissue	Whole plant
Preservation space needed	very compact	compact	med.	med.	spacious
Transportation	easy	easy	med.		difficult
Propagation	pollination	complete	grafting or cutting		complete
Genetic variation	variable	variable	constant	mutation	constant
Generation (ploidy)	haploid	next dipolid	diploid	diploid	diploid
Evaluation	progeny				rapid
Longevity	long			short	long
Limitation	male-sterile, cross-incompat-ibility	sterile, recal-citrant	graft-incompat-ibility		laborious

Among these characteristics, longevity of the germplasm is the most important criterion for preservation. Therefore, we will describe the methods of preserving the viability of pollen, seeds and scions of fruit trees in the following sections.

2. Pollen Preservation

Pollen is a micro-capsule of plant genes. It is extremely small in size, but contains the entire genetic information of the plant. Therefore, to preserve pollen viability, is to preserve the plant's genetic resources.

The systematic storage of fruit tree pollen makes hybridization possible between cultivars that differ in their flowering season or between plants growing far apart from each other.

Research on pollen preservation has been developed by examining the optimal conditions for storage, as follows:

(1) the relative humidity and pollen moisture content,

(2) the storage temperature,

(3) the gaseous environment or vacuum conditions,

(4) the effect of diluent,

(5) the packaging for storage.

The proper combination of these conditions can prolong the viability of fruit tree pollen. The longevities that have been reported to date, are summarized in Fig. 1.

In general; low humidity, low temperature, low oxygen content or vacuum, and darkness, are favourable conditions for the preservation of pollen. High humidity and high temperatures cause the loss of pollen germinability. Under room conditions, the pollen of most kinds of deciduous fruit trees lose their germinability within a week's time. Under dry conditions, they could remain viable for several weeks or even several months. And, under dry and cold conditions, such as found under refrigeration, they could survive for over a year. In a deep-freezer (−20°C), the pollen of some deciduous fruit trees could be kept viable for a long period of more than ten years. The effect of storage conditions on the longevity of pollen is summarized in Table 2.

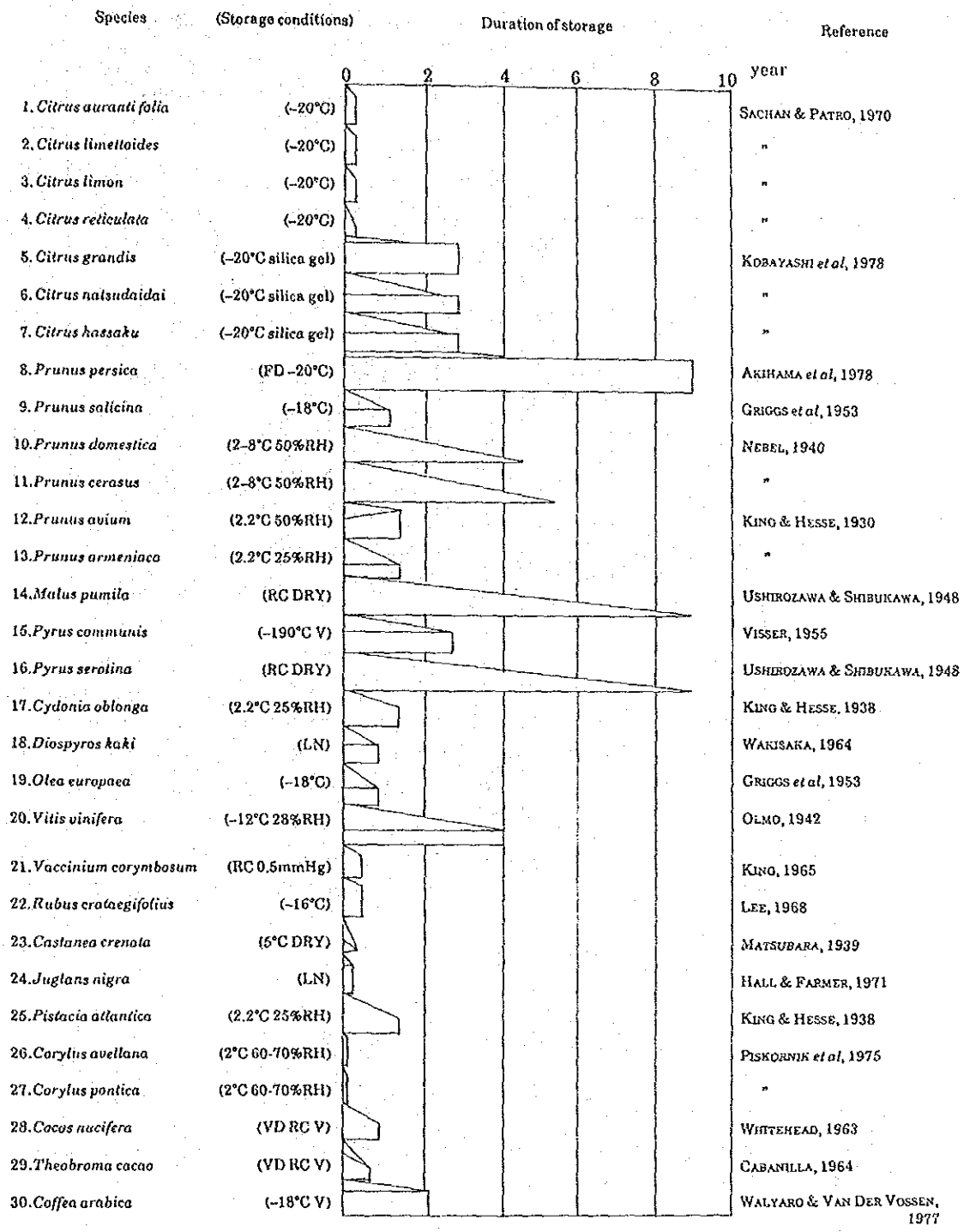

Fig. 1 Longevity of fruit tree pollen

Table 2 Storage methods and life span of fruit tree pollen

Treatment and storage conditions				Life span of long-lived pollen
Freeze-drying	Humidity	Temperature	Atomosphere	
-	high	room temp.	-	D
-	high	0-5°C	-	D
-	low	room temp.	-	M
-	low	0-5°C	-	Y
-	low	-20°C	-	10Y
-	-	super-low	-	10Y
FD	-	room temp.	V or N	M
FD	-	0-5°C	V or N	Y
FD	-	-20°C	V or N	10Y
FD	-	super-low	V or N	10Y

FD: freeze-drying, V: under vacuum, N: enclosed with nitrogen gas, D: several days, M: several months, Y: more than one year, 10Y: probably more than ten years, super-low: −80 to −196°C.

In addition to storage conditions, there are taxonomic differences in pollen logevity among families and species. For example, Gramineae plants, such as rice and wheat, have short-lived pollen. Fortunately, fruit trees of Rosaceae and Vitaceae have long-lived pollen. Among fruit tree families, Oleaceae and Rutaceae are those with intermediate-lived pollen. BREWBAKER (1967), reported that tricellular (trinucleate) pollen have a shorter life span than bicellular (binucleate) pollen. For such pollen, we have to conduct further studies on preservation techniques.

1) Freeze-drying of fruit tree pollen

After PFEIFFER (1955) reported a case of successful storage of freeze-dried *Lilium* pollen, freeze-drying has been applied to the pollen of many species. WHITEHEAD reported the successful room temperature storage of freeze-dried coconut pollen. In our results, however, storage at −20°C is even better for freeze-dried fruit tree pollen.

Several conditions affect pollen viability, such as:

(1) the prefreezing temperature and cooling rate,

(2) the moisture content of the pollen before freeze-drying,
(3) the duration of freeze-drying,
(4) storage conditions,
(5) rehydration.

The optimum treatment conditions vary among species and materials. We cite a few examples in Table 3.

Table 3 Freeze-drying procedures of peach and chestnut.

Material	Peach pollen pure	Peach pollen with anther	Chestnut pollen with catkin tissue
Collection time	flower just before anthesis	flower just before anthesis	just before full bloom of male catkin
Moisture condition	about 7% = pollen	about 7% = after 24 hr dehiscence	about 70% = fresh catkin
Pre-freezing	−10-−20°C	−20°C, but not necessary	inadequate
Duration of freeze-drying	8-10 min.	1-2hr	24hr
Storage	−20°C in vacuum vial	−20°C in vacuum vial	−20°C in vacuum vial
Rehydration	3hr	30min-4hr	30min-4hr

2) Viability test of stored pollen

(1) Germination test

A pollen germination test on agar or liquid medium is a common method of checking pollen viability in many plants.
The optimum medium differs somewhat for various materials;
e.g. peach pollen = agar 1%, sucrose 10%, for 6 hr at 20°C,
pear pollen = agar 1%, sucrose 10%, for 4-6 hr at 20°C,
chestnut pollen = agar 1%, sucrose 5%, boric acid 0.1%, for 48 hr at 30°C
citrus pollen = agar 2%, sucrose 15-20%, 20 hr at 25°C.

(2) Staining test

Vital staining is another effective method. MTT [3(4,5-dimethylthiazolyl-

2)2,5-diohnyl tetrazolium bromide] staining has been shown to have a good correlation with germination percentage in peach pollen (WERNER and CHANG, 1981). A fluorescent dye, FDA(fluorescein diacetate), is also used as a rapid check under a fluorescent microscope.

3) Pollination test

Pollination ability is the final and most precise estimator of pollen viability. For pollen that is hard to germinate on artificial media, a pollination test might provide better results.

Twenty to fifty flower buds before anthesis are emasculated, pollinated and bagged to prevent further pollination. Under field conditions, the opportunity for this test comes only once a year.

3. Seed Preservation

The seeds of fruit trees are also easy to collect and transport. The seeds of fruit trees are classified into recalcitrant and orthodox groups. The seed longevity and classification of some fruit trees are listed in Table 4.

Table 4 Seed longevity and classification of fruit trees

	Longevity	Storage condition	Classification	Reference
Apple	7years++	−3~−5°C	orthodox	SOLOVIEVA, 1966
Pear	7years++	−3~−5°C	orthodox	SOLOVIEVA, 1966
Peach	2-3 years	0~+5°C	orthodox	unpublished
Grape	4years++	−1°C	orthodox	unpublished
Persimmon	1.5years+	0°C	recalcitrant	KOTOBUKI, 1978
Chestnut	3.5years±	−1~+1°C	recalcitrant	JAYNES,1969
Citrus	3years	+4°C	orthodox?	HONJO and NAKAGAWA, 1978

1) Storage of Japanese pear seed

Japanese pear seeds can be desicated down to about 5% moisture content by drying (Fig.2). Fully dried Japanese pear seeds retain their germinability for more than 3 years even in liquid nitrogen(−196°C)(Table 5). After storage, dried seeds need to be rehydrated and chilled to break their dormancy.

In addition to dry storage, fresh or moist Japanese pear seeds can survive for 2 or 3 years at −3 to −5°C and require a shorter chilling period to break their dormancy.

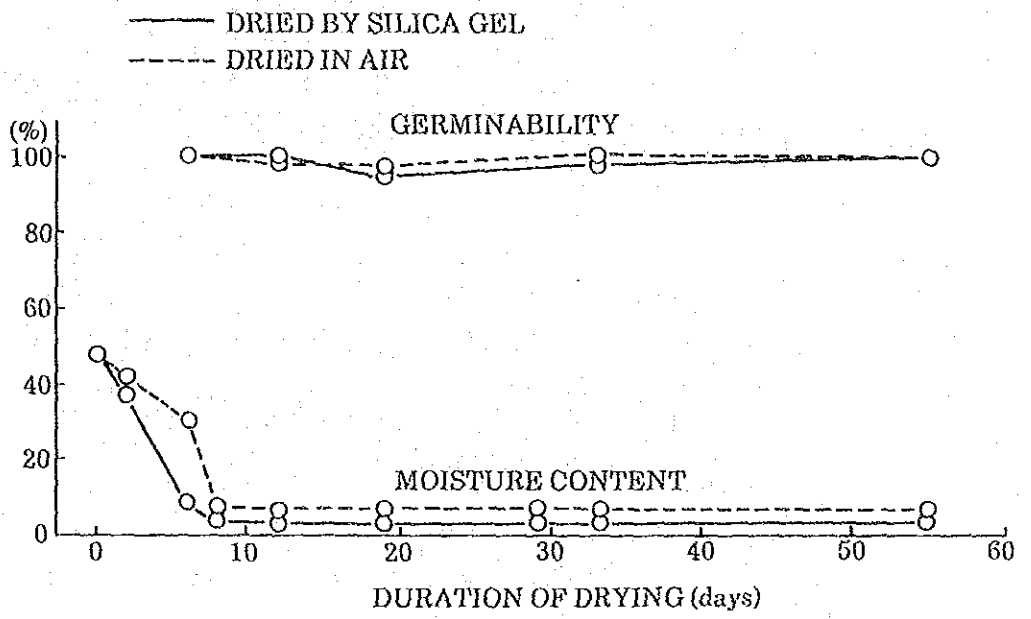

Fig.2 Changes of moisture content and germinability when Japanese pear seeds are dried in air or in desiccator containing silica gel.

Table 5 Viability of stored dry seeds for three years at different temperature

Moisture content(%)	Storage temperature	Germinability (%)			
		Duration of storage (years)			
		0.5	1	2	3
11.7	Control	42.5	0.0	0.0	-
	0°C	60.3	88.0	89.7	88.6
	−20°C	69.4	91.7	95.1	85.7
	−196°C	83.7	80.4	91.5	95.5
4.3	Control	85.3	90.3	90.4	84.9
	0°C	82.6	79.2	93.5	90.2
	−20°C	82.1	81.6	91.2	76.4
	−196°C	84.8	73.4	93.4	88.6

Control seeds were stored at room temperature.
Excised seeds were used for germination test.

2) Storage of citrus seeds

Citrus seeds are classified into orthodox, however, entire seeds rapidly lose their viability when dried like recalcitrant seeds.
But they can be preserved for 3 years at 1 to 4°C (HONJO and NAKAGAWA, 1978), but they can not survive in ultra-low temperatures.

In contrast to the whole seed with its testa, excised embryos of lemon tolerate drying down to 1.2% moisture content and freezing in liquid nitrogen(MUMFORD and GROUT, 1979).

4. Scion Preservation

Scions or cuttings are the most common form of germplasm that are used for the transportation and propagation of fruit tree cultivars and clones. The longevity of the scions usually last several months by the usual method. Cold storage, however, prolongs the longevity of fruit tree scions as shown in Table 6.

Table 6 Longevity of fruit tree scions in cold storage.

	Longevity	Storage temperature	Reference
Apple	2years+ +	0°C	HANIUDA et al,1978
Pear	5years±	−3~−1°C	OMURA et al, 1978
Peach	1-2 years	−1°C	
Grape	5years	−5-−3°C	OMURA et al, 1978
Persimmon	2years	−1-+5°C	
Chestnut	1years+	−1~+1°C	JAYNES, 1969
Citrus	2.5years+	+5°C	NAKATANI and IKEDA, 1980

Fresh and slightly desiccated scions of deciduous fruit trees are commonly wrapped in polyethylene bags and stored at 0 to −3°C. Lower temperatures prevent the budding and exhaust of scions and fungal rot, but also cause freeze injury. The pre-treatment of scions with fungicides does not affect their longevity so much. The storage temperature has an important effect on them, and the optimum storage temperature varies among species (Fig.3). Pear scions, that had been stored for two years, were able to be grafted and displayed good growth characteristics similar to that of fresh scions (KOZAKI, 1975).

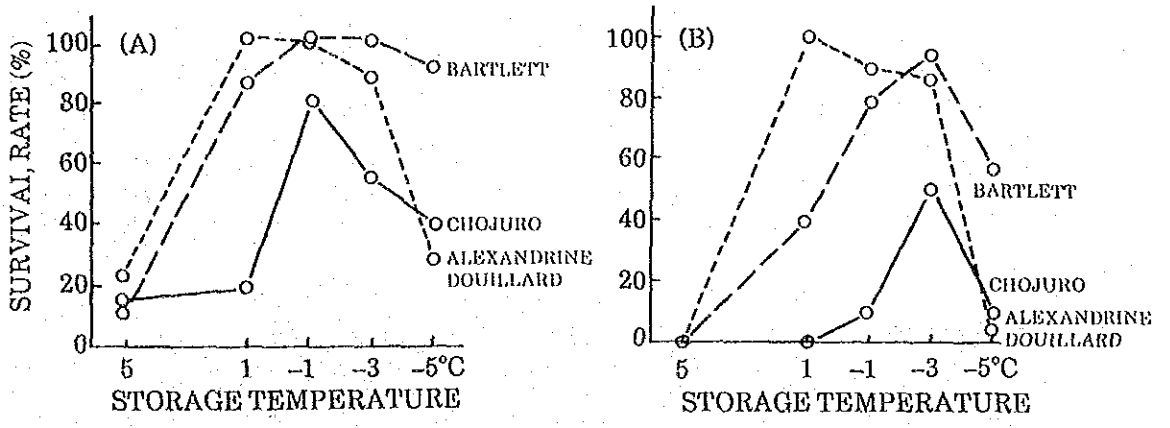

Fig.3 Effect of storage temperature on survival rate of pear scions in cultivars. Storage duration: (A) 2.5 years and (B) 4 years.

Winter buds of some species, such as the apple, pear, raspberry, currant and gooseberry, can be preserved in ultra-low temperatures (SAKAI and NISHIYAMA, 1978). The further technical development of these cryo-preservation methods are expected in the future.

Experiments on the long term preservation of soft or green scions, such as citrus, are very few. The season of the year in which these scions are collected greatly affects their longevity (NAKATANI and IKEDA, 1980), as shown in Table 7.

Table 7 Longevity and collection time of 'Risbon' lemon scions

Collection time	Jun.	Jul.~ Aug.	Sept.	Oct.~ Nov.	Dec.	Feb.
Longevity at 5°C	2	4-5	9	16	14-15	months 33

5. Preservation *in vitro*

Germplasm preservation *in vitro* would be the final method in future. It seems convenient if it is possible to keep germplasm *in vitro* under super-low temperature for a long term and to regenerate the whole plant from the stored tissue or cells.

The procedure in fruit trees of this method is not performed yet, but the possibility is proved in some species such as apple and pears.

In usual way, shoot-tips are aseptically isolated from dormant buds, sunk in distilled water with or without cryo-protectant (10% DMSO: dimethyl sulfoxide), prefrozen at −40°C, then immersed in liquid nitrogen. After the storage, the tissues in vials are rewarmed quickly in water at about 38°C, and placed on culture medium to regenerate and grow.

Publications on *in vitro* storage from IBPGR are useful to obtain the informations, however, this method is not perfected yet, but is being developed.

V Preservation of Genetic Resources of Vegetatively Propagated Plants by Tissue Culture

by

Seibi OKA

National Institute of Agrobiological Resources
Ministry of Agriculture, Forestry & Fisheries

CONTENTS

1. Introduction

The genetic diversity of plants is considered to be eroded rapidly on worldwide scale, while the plant genetic resources for crop breeding materials are recognized to become more important for human being in future. Recently, much active research has been done by means of biotechnology to develop the new methods for plant breeding. However, if there is not a wide range of genetic resources, it will not lead to satisfactory results, no matter how innovative is the technology being developed. The first step in the utilization of genetic resources for plant breeding is the collection of the resources. Important as well is the next step of efficiently and safely preserving the resources collected.

The International Board of Plant Genetic Resources (IBPGR) has developed international cooperative network for the promotion of the project of plant genetic resource preservation. For the initial period, IBPGR emphasized the collection and preservation of major grains and pulses (beans, etc.). Later IBPGR has also stressed its activities for vegetatively propagated crops, such as tuber crops, root crops, fruit trees, industrial crops, forest trees. Of them, the preservation of vegetatively propagated crops in the tropics and subtropics is especially emphasized by IBPGR. The reason for this emphasis is the possibility of loss of some species in these areas before any effective measures are taken to preserve these species. To solve this problem, preservation of plant genetic resources utilizing the tissue culture method has been discussed by specialists for genetic resources preservation, and related reports have already been published by IBPGR (1985) and others. With the assistance of IBPGR, some of the international organizations started to preserve the plant genetic resources by "*in vitro* tissue culture" at a large scale. Of course, the tissue culture method is applicable not only to tropical and subtropical crops, but also to every kind of vegetatively propagated crops. This paper, therefore, reviews current status of the researches on the preservation of vegetatively propagated crops by application of tissue culture method.

2. Preservation of Plant Genetic Resources in Fields and by Tissue Culture

Seed-propagated crops can be preserved in the form of seeds which are relatively compact and naturally storable for long period. In contrast, vegetatively propagated crops should be preserved in the form of whole plants in the "field genebank". There are two types of vegetatively propagated crops. One is the perennial type which can be maintained in the fields for long period

without replanting, and the other is the annual, or biennial type which must be dug out and replanted every year. For both types, the preservation of crops planted in the "field genebank" requires an adequate area of land and painstaking daily cares. As a matter of course, there is great danger of losing these precious genetic resources through damage caused by diseases, insects and/or adverse whether.

The "*in vitro* genebank" has, therefore, been conceived as a way to avoid such problems related with the field preservation. In this method, a part of the plant is removed and is preserved in a test tube, so that the plant can be reproduced whenever it is needed. This is based on the progress of the tissue culture technique of plants, in particular, the advancement of the technology which can reproduce perfect plantlets from a single cell, callus or tissues.

There are two types of preservation methods using tissue culture; (1) growth retardation method (slow growth and subculture preservation), and (2) freeze preservation (cryopreservation). The first method is being considered as potentially useful for the "active collection", i.e., the preservation of genetic resources for short or medium terms ranging from several months to several years for distribution of materials, and the latter method is for the "base collection". As a rule, the plant genetic resources preserved as the "base collection" have been evaluated their characteristics, and they are preserved for very long period for several decades or longer. Today, genetic resources are generally conceived to be preserved by a comprehensive combination of the "field genebank" and the "*in vitro* genebank" as shown in Fig. 1. As each of the preservation methods has its own advantages and disadvantages as shown in Table 1, it is necessary to make a proper choice of these methods to meet the requirements such as the kind of and the scale of genetic resources, facilities and the necessity of virus elimination, etc.

The "*in vitro* genebank" means the "genebank in a test tube" and implies a preservation of cells, calluses or tissues in sealed test tubes. In most cases, there is actually no problem with this terminology. However, as I will describe later, it is possible to preserve intact tissues, and to use tissue culture technology only in the process of regeneration of plants after preservation; therefore the term "preservation by tissue culture" is to be used hereafter rather than the "*in vitro* genebank".

Table 1 Comparison of field genebank with *in vitro* genebank

	Advantages	Disadvantages
Field genebrank	The evaluation of the characteristics of the genetic resources, and their preservation, can be done simultaneously. The field genebank can immediately provide parents for mating.	A field genebank requires a large field area, and involves much labor and many expenses. There are also plant quarantine restrictions, when transferring plants to/from foreign countries. A field genebank is also susceptible to damage caused by adverse weather, by animals/insects, and by disease, etc.
In vitro genebank	With an *in vitro* genebank, many genetic resources can be preserved in a small space. These resources can be preserved as virus-free stocks. Genetic resources, to which the method of micro-propagation is applicable, can be multiplied rapidly. An *in vitro* genebank will be free of damages by adverse weather, disease, insects (or animals). The international exchange of genetic resources can be done easily.	The evaluation of characteristics must be done separately from preservation. Regeneration of plant and flower bud formation require a considerable time. There is some danger of the occurrence of genetic mutations (particularly in cases of callus preservation).

3. Preservation Method by Growth Retardation

In this method, the subjects for preservation are cultured cells, callus and shoot apex. For cultured cells and callus, differentiation of adventitious buds or somatic embryo genesis is needed to regenerate plants. The regenerative

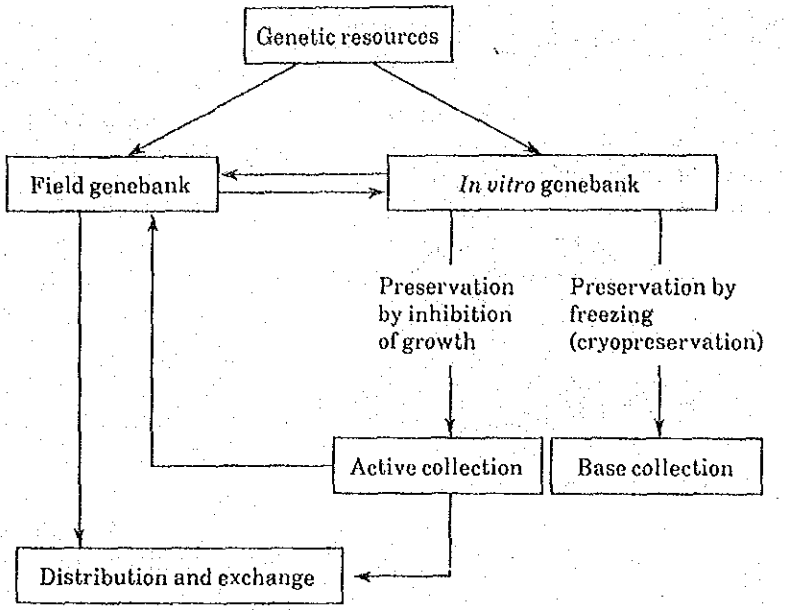

Fig. 1 Relationship between field genebank and *in vitro* genebank

pathway, however, has not yet been established for all kinds of plants. For shoot apex, however, the culturing operation is relatively easy, since the materials already have organized structures. Therefore, meristem culture (shoot apex culture, stem tip culture) methods have already been developed for many species of plants. In order to maintain these tissues and organs under ordinary culture conditions, it is essential to subculture every fixed term. The term of subculturing cycle is usually from 1 to 2 months for callus and between a few days and 2 to 3 weeks for cultured cells. Usually quite a large number of cultivars is handled for preservation of genetic resources. Thus, the preparation of culture media and the subculturing, etc., require a large amount of labor. In addition, chances of contamination increase, not only during *in vitro* culture, but also during transplanting.

Another big problem related to the tissue culture is the occurrence of genetic variation or mutation during the tissue culture period, especially in cultured cells and in callus. This is an extremely hazardous problem from the viewpoint of the preservation of genetic resources. Mutation occurrence during the tissue culture period is considered to increase with the frequency of cell division. Therefore, "growth retardation" is desirable for preservation of plant

genetic resources due to prolongation of intervals for subculturing. No matter how much the subculturing intervals are extended, however, as long as cultured cells or callus is used, the redifferentiation of adventitious buds or of somatic embryos remains indispensable. In actual use of cultured cells or callus for preservation, it is always necessary to consider the possibility of the occurrence of mutations. On the other hand, in subculturing of shoots, the mutation occurrence is considered to be much less than that in cultured cells or in callus, though the possibility of mutation occurrence can not be fully denied. Therefore, most of the researches on the growth retardation for preservation of plant genetic resources have so far used shoot apices as their materials.

1) Growth retardation with cultured cells and callus

In liquid cell suspension cultures which must be continually agitated in order to supply oxygen to the cells and is originally used to achieve rapid growth rates, it is difficult to set up conditions for prolongation of subculture intervals. So the growth retardation method is not used for liquid cell suspension cultures.

There was the classical experiment done by CAPLIN (1959) in which callus was cultured in agar medium with overlayer of liquid paraffin. This idea was derived from the preservation method for micro-oganisms. He tried to inhibit growth of callus by the restriction of oxygen supply, and to inhibit moisture evaporation from the surface of the culture medium. He successfully subcultured the callus of carrot and other plant species at interval of 3 to 5 months for over 3 years, without any decrease in growth capacity. This method has not received much attention, but recently the results of re-trials of this method have been reported. AUGEREAU *et al.* (1986) placed callus of coffee and other materials under liquid paraffin layer for 6 months, and then cultured these callus with ordinary conditions. As a result, 70% of them began to grow again. They also stated that this term of growth retardation could be further extended. MORIGUCHI and KOSAKI (1987) preserved grape callus under a layer of silicone for 270 days, and later obtained a 72% survival rate. Plant regeneration from the callus, however, was not accomplished in either of the above cases.

There are few reports concerning the preservation of callus under low temperature conditions. WITHERS (1978) reported the preservation of corn and redpepper callus at 4°C for 25 days, but this term cannot really be considered as long-term for preservation. But in this experiment, She treated the callus with

ethanolamine prior to exposing to low temperature in order to provide them low temperature resistance.

Cold acclimation for callus had previously been recognized as being effective for chrysanthemums and for apples. MORIGUCHI and KOSAKI (1987) succeeded in preserving the previously mentioned grape callus for one year at 10°C. If effective methods of cold acclimation are utilized, low temperature condition will by very promising measure for long term preservation of vegetatively propagated crops.

2) Growth retardation of shoot apex

The tissue culture method can be utilized most easily for preservation of vegetatively propagated crops. For this purpose, it is necessary to establish a meristem culture system stably maintainable for a given crop. This is not too difficult now. When a cytokinin such as benzyl adenine is used as a growth substance, meristem culture method may be established for most of the plant species. Even for woody plants such as persimmon and some of the stone fruits which were previously difficult to culture their tissue, 2ip and KT30 (urea-based cytokinin) are found to be highly effective for tissue culture; thus there is a steady increase year by year in the number of plant species for which meristem culture is possible.

The conditions for the highest rate of propagation should be found to establish a meristem culture system. To determine those conditions is necessary to propagate plants quickly after preservation. Further, rooting from cultured shoot apices, acclimation and planting into soil are also important procedure as the final steps of the tissue culture system.

For the purpose of preservation of plant genetic resources by tissue culture, the next stage after the establishment of the meristem culture system is rather the arrangement of conditions for slow-down of growth speed. In other words, this means minimizing the growth of the material per unit term. Sometimes, therefore, the growth retardation method is referred to as "minimum growth method". There are two ways to accomplish this; (1) utilization of a growth retarding medium, and (2) control of the physical environment.

(1) Utilization of growth retarding culture medium

In this method, growth is retarded by a shift in the composition of the medium to sub- or supra- optimum levels. To achieve this purpose, various methods can be used, such as decreasing the concentration of inorganic salts, varying the concentration of sugar, increasing the osmotic pressure of the medium by adding mannitol, removal of cytokinin and addition of growth retardants. The growth retardation by the use of chemicals can be done at normal temperature, but is sometimes combined with the low temperature condition.

Of the above methods, increasing the osmotic pressure of the culture medium using mannitol has given relatively good results. WESTCOTT (1981) achieved effectively slow growth of potato shoot. using 0.2 M mannitol. Generally, in the medium with additional mannitol, shoot apices become thicker and are stumpy in appearance. SILVA (1985) reported that pieces of potato tubers placed on an medium without any growth substances could be extended their dormant stage for 18 months, and developed regenerated plants, when transferred to a regenerating culture medium. There were some examples of successful preservation with the use of sugar-less medium and growth retardant. KARTHA *et al.* (1981) preserved coffee shoots for 2 years with the use of 1/2 MS medium without sugar at a normal temperature. We found that Japanese pear shoots were preserved for at least 6 months at a normal temperature using sugar-less medium. WESTCOTT (1981) stated that the growth retardants, such as B-9, hydrazide maleate and CCC were effective for potato shoot as well as the low temperature. But, the effective use of oligotrophic culture medium, or of growth retardant, is rather difficult.

(2) Control of physical environment condition

a. Temperature control

The most reliable and simplest method for growth retardation is a reduction in culturing temperatures. The optimum temperature for ordinary tissue culture is 25 to 28°C, but the growth rate gradually decreases as the temperature lowers. GALZY (1969) reported that grape shoots were preserved by meristem culture for 290 days at 9°C. Based on this result, MOREL (1975) estimated that 6 individual specimens of each of 800 cultivars of grape could be preserved in an area of only 2 m^2. In this

research area, tissue culture of potatoes at CIP (International Potato Center, Peru) is most advanced. ROCA (1977) preserved lateral buds of selected 17 potato varieties for a year at 6°C with an average survival rate of 60%. The researches done by CIP have later led to its practical germplasm preservation method in combination with the derivation of virus-free stocks. The media have also been standardized for short term preservation (8 months) and for long term preservation (2 years). In 1985, 4,500 materials were exported as "*in vitro* plant genetic resources".
MIX (1983) preserved nodal cuttings with bud of potatoes at 10°C for 2 years with a culture medium containing B-9 as a growth retardant. This method is actually used for the preservation of 350 potato cultivars in West Germany.

Many examples of growth retardation using low temperature conditions are given in Table 2. The longest period of low temperature preservation by meristem culture was attained in strawberries with a 100% survival rate after 6 years preservation by MULLIN and SCHLEGEL (1976). For the woody plants, shoot apices of radiata pine were preserved for 5.5 years at 4°C at the Forest Research Institute, New Zealand.

The preservation temperature varies according to the specific plant species, but the range of optimum temperatures are roughly estimated as follows: 0 to 6°C for temperate plant species, around 10°C for subtropical plant species, and 15 to 20°C for tropical plant species. For example, sweet potatoes originated from tropical area cannot be preserved at the temperature below 14°C. KOBAYASHI (1985) preserved 130 lines of sweet potato genetic resources at 15°C, with replanting those to fresh medium only once a year.

Cassava shoot apex preservation is already in practical use. Cassava is a tropical tuber crop originated from Central America, and has become an important starch crop widely distributed in the tropical areas in the world. The meristem culture system has been established by KARTHA *et al.* (1975) with the aims of getting virus free stocks and of increasing propagation efficiency. More than 3,000 cassava genetic resources have been collected and preserved at CIAT, Republic of Colombia. Although the optimum temperature for cassava propagation is 25 to 30°C, shoot apex elongation rate becomes only 1/5 of the usual elongation rate when cultured at 20 to 22°C. When shoot apices are preserved at low temperatures below 18°C, viability will soon be lost unless light strength is decreased to around 500

Table 2 Preservation of shoots by the slow growth method

Material	Temperature °C	Storage period	Bibliography
Grape	9	290 days	GALZY (1969)
Strawberry	4	6 years	MULLIN, SCHLEGEL (1976)
Potato	6	1 year	ROCA (1977)
Japanese cedar	10	16 months	ISHIKAWA (1978)
Apple	1 – 4	1 year	LUNDERGAN, JANICK (1979)
White clover	2 – 6	15-18 months	CHEYNE, DALE (1980)
Potato	6 – 12	12 months	WESTCOTT (1981)
Coffee	26	2 years	KARTHA *et al.* (1981)
Sugar beet	5 – 10	1 year	MIEDEMA (1982)
Cherry	25	Several months	WILKINS *et al.* (1982)
Potato	10	2 years	MIX (1983)
Cassava	20 – 22	2 years	ROCA (1984)
Banana	15	12-15 months	BENERJEE *et al.* (1985)
Peach stock	– 3	300 days	MARINO *et al.* (1986)
Kiwifruit	8	52 weeks	MONETTE (1986)

lux. When the concentrations of 0.044μM of BA and 0.058μM of sucrose are changed to 0.22μM of BA and 0.12μM of sucrose, shoot apex growth rate is retarded. But the most of the shoot apices did not survive after 3 months preservation, when low temperature conditions were combined with the use of retardation medium. However, a high survival rate of the shoot apices was observed, when mannitol and sucrose were jointly added to medium in both high and low temperature conditions. So far, 1,500 of the genetic resources of cassava preserved at CIAT have been transferred to the tissue culture preservation system. The rooted, virus free stocks of cassava are distributed to many countries by air mail, as are the same of potatoes from CIP. From 1979 to 1982, CIAT sent more than 360 varieties (lines) of cassava to countries in the Americas and about 50 plants to countries in southeast Asia.

The shoot growth rate of temperate plants is retarded at lower temperature. But, there is an optimum range of the temperature, and excessively reduced temperatures are not necessarily good. For grape shoots, GALZY (1969) reported that the survival rate was 100% after 90 days of preservation at 9°C, but it decreased to 46% and to 13% when preserved at 7 and 2°C, respectively. MARINO *et al.* (1985) found that the shoots of peach and cherry were preserved for 120 days at 4°C, and when they were kept under below zero temperature condition (at -3°C), it was possible to preserve these shoots for 300 days. For *Dendrobium* (orchids), TANDON and SHARMA (1986) reported that the optimum temperature for preservation was -4°C. As stated above, the sensitivity to low temperatures varies with the plant species. The optimum temperature for preservation must, therefore, be determined with consideration of both the degree of growth retardation, and the survival rate after preservation. Shoot apex is usually preserved as whole shoots, but some crop species cannot survive under low temperature conditions unless they have already been rooted.

For some plant species, however, shoot apex can be preserved without lowering the temperature. The cases of coffee and Japanese pear were already described. WILKINS and DODDS (1983) reported that rooted shoots of cherry in liquid medium were preserved for several months at a normal temperature. Shoots which were preserved for long period at normal temperature, will be considered to be in a dormant-like condition. They stressed that preservation of rooted stock has the advantage of quick reproduction of whole plants. We observed that the apical buds of Japanese pear grew to a certain size at a normal temperature by meristem culture with sugar-less medium, then they stopped growing and formed buds which appeared dormant. When these dormant-like buds were subsequently cultured under the same condition, they were able to survive for over 6 months without shoot developing. In the cases of high survival rate for long period at a normal temperature without sugar, it can be assumed that the material itself has a certain capacity for photo-autotrophy. As a special case of temperature conditions for long term preservation, WESTCOTT (1981) reported that the temperature for the highest survival rates of potatoes varied between 6 and 10°C.

b. Control of light

The control of light conditions, as a part of the physical environment, sometimes has an effect on preservation. MIEDEMA (1982) found that sugar

beet shoots could not survive in complete darkness, therefore they must be subjected to weak illumination. ISHIKAWA (1978) reported that Japanese cedar shoots showed a stronger retarding effect on growth by illumination for 5 hours a day than that for 12 hours a day. CHEYNE and DALE (1980) showed another case, in which a difference in light duration has no effect on growth retardation of clover. Thus, the effects of light vary with the particular plant species examined.

c. Control of gas composition

This method was proposed by BRIDGEN and STABY (1981), who adapted the CA (controlled atmosphere) storage method being employed for the preservation of fruits, for the tissue culture environment. Drying of the medium is inevitable to a certain extent in the low temperature preservation method, but in the CA method, drying can be prevented by feeding the vessels with moist air (a moist atmosphere of mix gases). BRIDGEN and STABY (1981) cultured chrysanthemum shoot apex, tobacco shoot apex and tobacco callus under various conditions of atmospheric pressure and under varying partial pressures of oxygen. They found strong growth retardation for all materials examined under these various conditions as compared to the normal condition. However, as they them selves stated, the experimental term was short, only 6 weeks, so further examinations are required in order to determine the effect on long term preservation. With this method, there is some difficulty in assembling a complex culturing system for the supply of fixed composition gas.

(3) Preservation form and the prevention of drying of culture medium

One of the elements determining the duration of preservation at low temperature is the gradual drying of the culture medium. MULLIN and SCHLEGEL (1976) achieved the longest-yet preservation term of 6 years for strawberry shoots by liquid culture medium without subculturing. The culture containers were sealed with parafilm, and liquid medium was added,as needed, in amount of 1 or 2 drops, depending on the degree of drying. This operation seems to require a great deal of care, and the addition of large amounts of medium at any one time would have an adverse effect on the survival rate. MIX (1985) used both liquid and solid media at the first stage of his experiment for meristem culture of potato, then she used only liquid medium for preservation. Parafilm was used as a sealant for bananas by BANERJEE and LANGHE (1985), and for apples by