Management of the *in vitro* Genebank for old potato cultivars at the Institute of Crop Science in Braunschweig

GUNDA MIX-WAGNER

Institute of Crop Science

1 Tasks and Aims

By means of the Collection of Plant Genetic Resources (Braunschweig Genetic Resources Centre = BGRC) of the Institute of Crop Science of the Federal Research Centre for Agriculture (FAL) Braunschweig-Völkenrode (Germany), the Federal Minister of Nutrition, Agriculture and Forestry of Germany disposes in his scope of business of an independent Institution for the Conservation of Genetic Diversity of Crop Plants.

Maintenance of plant genetic resources is an important precautionary measure which serves the securing of plant genetical raw material for plant breeding and agriculture in Germany and worldwide. Related tasks and activities are of a long-term nature.

2 Historical Background and the Stock of the Collection

In 1970 the actual Institute of Crop Science of the Federal Research Centre for Agriculture Braunschweig-Völkenrode was charged with establishing a collection of plant genetic resources in a so-called "genebank". The number of BGRC-Accessions in different Groups of crop plants are demonstrated in Table 1.

3 In Vitro Collection of old potato varieties

Potato, a vegetatively propagated crop, are traditionally preserved in form of tubers or cuttings, which have only a very limited life. This is a great disadvantage, because a lot of work has to be done in maintaining genetic resources by seasonal regrowing. This method of propagation also means that during annual field cultivation the material is constantly in danger of being contaminated with diseases, and this definitely shortens the life of the samples. In spite of these disadvantages, however, we cannot abandon the preservation of vegetatively propagated species and the question therefore arose as to possible alternatives which would ensure economical storage and would also fully maintain the health of the material and the characteristics typical of the particular cultivar. In the BGRC-Collection 778 cultivars are maintained under slow-growth conditions and 140 cultivars are freeze-cryostored. These cultivars derive from 25 different countries. "Erstling" is the eldest cultivar which has been bred since 1891. It is followed by "Prof. Wohltmann", "Paul Krüger" and "Ella" (since 1898).

Forage Crop	7
Vegetables	2181
Drug Plants	43
Cereals (Gramineae)	28624
Cereals (other)	956
Grasses	3502
Medicinal Plants and Spices	1085
Small-Seeded Legumes	1336
Large-Seeded Legumes	7501
Root and Tuber Crop Plants	3223
In-vitro Potato Collection	778
	49236

Table 1: **Number of BGRC-Accessions in Different Groups of Crop Plants**

4 Preparation of the cultures and their storage

One aim of germplasm conservation is to insure the availability of useful germplasm at any time, therefore the collection is maintained under slow-growth conditions. The growing processes are here reduced to a minimum by limitation of a combination of growth factors. This storage procedure permits, if needed, a rapid multiplication for international distribution. Miller and Lipschutz (1984) summarized in the" Handbook of Plant Cell Cultures" several reports of different scientists who demonstrated the applicability of minimal-growth storage (slow-growth storage) to a wide range of Solanum species and cultivars.

Cryopreservation in liquid nitrogen for long-term storage has been at little more than an experimental stage for some twelve years. However, the technique has great potential and according to Chandel and Pandey (1991) more than 50 species have been successfully freeze-cryostored in form of cell or organ cultures. All available evidence suggests that the cryopreserved cell cultures are genetically stable (Withers 1989).

Slow-growth method for medium-term conservation (working collection)

Axillary buds are cut out from shoots of the potato cultivars which, for many different reasons, are not longer protected by "Variety Protection Registration". These single-node cuttings are laid on filter paper bridges in test tubes, (only one cutting per tube), filled with liquid culture medium and closed with aluminium foil. The cuttings are surface sterilized in 3% calcium hypochlorite solution for 10 minutes. All subsequent handlings are in sterile conditions. The basic medium of Murashige and Skoog (1962) supplemented with 2% saccharose are used as multiplication and also as storage medium (Table 2). After the medium had been adjusted to a pH of 5,8 it was autoclaved for 10 minutes at 120° C.

After culturing for about four weeks at temperatures of 20-22° C, a light intensity of 4 klux and a 16-hours day, the plantlets are removed from the test tube, segmented and further cultured until

KNO	0,019 M
KH_2PO_4	1,25 mM
NH_4NO_3	0,2 M
$MgSO_4 \cdot 7H_2O$	1,79 mM
$CaCl_2 \cdot 2H_2O$	3,0 M
H_3BO_3	0,1 mM
$Mn\ SO_4 \cdot 4H_2O$	0,1 mM
$Fe\ SO_4 \cdot 7H_2O$	0,05mM
$Na_2EDTA \cdot 2H_2O$	0,1 mM
$COCl_2 \cdot 6H_2O$	0,1 µM
$CuSO_4 \cdot 5H_2O$	0,1 µM
$ZnSO_4 \cdot 7H_2O$	0,03 mM
$Na_2MoO_4 \cdot 2H_2O$	1,0 µM
KJ	5,0 µM
Sucrose	0,06 M
PH	5,8

Table 2: **Medium for Slow-Growth Culture of Potatoes**

ten plants of each cultivar are available for maintenance under slow-growth conditions. Before the ten plantlets of each cultivar are transferred to the slow-growth conditions, an intermediate culture duration of four weeks at 20-22° C° should give the opportunity getting strong plantlets with a good root system, which greatly benefits the storage at 10° C. After about four weeks, when the plantlets have reached a size of 3-4 cm, they are transferred to the slow-growth conditions. The cultures are stored at 10°C with a light intensity of 2 klux and a 16-hrs day.

After being stored for about two to three years, depending on the cultivars, the cultures are moved for recovery from the slow-growth conditions to the intermediate culture condition. After few days the axillary buds are cut out of the survived parts of the plants. Now the whole slow-growth culture procedure starts from the beginning (Mix 1984).

Seven hundred and seventy-eight cultivars have so far been transferred to slow-growth conditions. Now ten plantlets of each cultivar are stored, each plantlet in a separate test tube on a filter paper bridge. When we first started to transfer the whole *in vivo* collection under *in vitro* conditions, only five plantlets of each cultivar were stored. Several times we encountered difficulties due to contamination and high request and therefore later we increased the number of stored plantlets per cultivar to 10.

After two years *in vitro* maintenance, it was observed that several different growth pattern were identified, depending on the cultivar. Plants from about 60% of the cultivars, e.g. "Beta", "Lori" and "Möve", form one or more tubers after about six months and from these tubers new plantlets develop. After six months another 35% of the cultivars, e.g. "Cosima", "Pavo" and "Ackersegen" sprout from the old single-node cutting or from a new axillary bud and the original plants die off. The remaining 5% of the cultivars, e.g. "Erstling", "Mensa" and "Voran" suffer growth depressions of different intensity. This is the result of the low storage temperature (10° C), as in these cultivars there was a positive effect on growth when the storage temperature was increased to 15° C. In the case of these few cultivars an intermediate propagation is undertaken after a year, i.e. outside the otherwise normal two- to three-years cycle.

If this were not done, a large amount of space and energy would be necessary to store parts of the collection under different temperature ranges. There are still some cultivars which do not fit into this grouping.

The growth behaviour of the cultivars under the slow-growth *in vitro* conditions renders it necessary to check the collection at intervals of one to two months. Visual inspection of cultures under slow-growth will check for viability loss and microbial contamination, but meaningful, monitoring of stability by eye will be difficult as vigour is intentionally being depressed. Therefore all potato cultivars are transferred to *in vivo* growth at appropriate intervals to verify assumptions made *in vitro*.

The experiments showed that *in vitro* plantlets of potato can be stored at temperatures between 4-15°C. The optimal temperature depends on the cultivar, but it is impossible for a large collection to offer each accession the optimal storage temperature. Therefore the decision was made to store all cultivars at 10° C, and keep an eye on these cultivars which preferred 15° C. It is also possible for saving energy to thrust in the storage phase a total dark phase of up to half a year. Under the above mentioned conditions it is possible to store the cultivars up to three years. The storage duration is dependent on the behaviour of the cultivar under the storage conditions. After three years of storage, a subculture of about four weeks at a temperature of 20-22°C is necessary until the culture can be again transferred to slow-growth conditions (Table 3).

Cryopreservation for long-term storage (base collection)

Beside the slow-growth conservation the idea arose to establish an *in vitro* base storage through cryopreservation.

The project " Refinement of cryopreservation techniques for potato" started in September 1991 and is sponsored by the International Plant Genetic Resources Institute (IPGRI) formerly IBPGR. It is a co-operation between the Institute of Crop Science of the Federal Research Centre of Agriculture in Braunschweig-Völkenrode (FAL) and the German Collection of Micro-organisms and Cell Cultures in Braunschweig (DMS). The aim of the project is to compare different techniques and hopefully perfect a technique which could be applied to a broad spectrum of potato cultivars, because it is envisaged that cryopreservation, once available as a reliable, routine storage method, would be used in a complementary way alongside with different conservation methods (e.g. field genebank, *in vitro* storage and seed conservation) to provide secure, accessible and efficient conservation of the potato genepool.

The described method has been developed during the first period of the project. 140 potato cultivars were transferred during this time in liquid nitrogen. The number of meristems cryostored per cultivar varies from 36-456.

The method is designed for routine application and can be carried out in a laboratory for *in vitro* culture with low additional costs. Major investigations are liquid nitrogen containers for handling and storage and access to liquid nitrogen.

Solanum tuberosum L. (Slow-growth conditions)	
Pre-culture	nodal segments MS salts, 2% sucrose, solid medium 20-22°C, 16 hrs day, 4 klux
Storage	whole plantlets MS salts, 2% sucrose, liquid medium 10°C, 16 hrs day, 2 klux
Subculture interval	24-36 months

Table 3: **Protocol for a slow-growth maintenance**

For transferring the under slow-growth conditions stored cultivars to the cryopreservation condition the cultivars have to be potted in soil and grown under *in vivo* condition until they have reached the five to six leaf stage. Then the sterilized stems were taken into *in vitro* culture again and propagated further. The single-node cuttings are grown in culture vessels allowing very good air exchange. Murashige and Skoog basic medium supplemented with vitamins and 3% sucrose are used. The culture temperature should be 20-23° C. The light intensity should be high (4 klux) preferable from the top otherwise internodes become too long.

When all shoot tips of one culture vessel are cut off then the excising of the meristems has to follow. The material preserved are apical meristems, which can be regenerated into identical plantlets and the meristematic cells are small and compact and ideal for freezing. The excised meristems should have the size between 2-3 mm in length and 0,5-1 mm in thickness.

Place the excised meristems on a filter paper wetted with a Murashige and Skoog basic medium supplemented with vitamins, different hormones (IAA, GA3 and Zeatin) and 6% sucrose and incubate it over night at 23° C in a petri dish. The next day prepare a solution of 10% DMSO (dimethyl sulfoxide). Moisten a fresh filter paper with this solution and incubate for 2 hrs at room temperature. Prepare than the cryo vials, do not forget to mark the vials correctly. Prepare small aluminium foils for 6 drops of the above used medium and place in each drop one meristem. When all meristems are in the drops the aluminium foils are dropped directly in liquid nitrogen. The frozen meristems together with the aluminium foils are put into the cryo vials for storage.

During the course of experiments the following routine was established. 12-20 cultivars are propagated to a strength of 150-200 in vitro plants. All cultivar meristems are prepared in three sets of experiments which in total add up to about 30 cryovials (one vial = 12 meristems) of each cultivar. One vial, 12 meristems, of each experiment is cultured. This gives a rough estimate of the ability of the cultivar to survive freezing, what plant regeneration rate can be expected and whether the cells produce problematic substances like for example anthocanins. When the cultivars readily produce plants, the freezing experiment is repeated two (360 meristems) or four times (480 meristems) when plant production seems to be a problem.

With this technique the ice crystals are so small that they assumingly do not destroy the cell structure. A broad spectrum of potato cultivars survive freezing with a rate of 70-100 %.

For thawing, the foils with the meristems are dropped directly in room temperate MS medium. The meristems thaw instantly. For culturing, the meristems are placed in a drop of agarose surrounded with liquid medium. The same medium are used as for the pre-culture of the excised meristems (Schäfer-Menuhr 1994). The plant regeneration is lower then the survival rate of the meristems and depends more on the cultivars. Table 4 gives a few examples.

Since the potato cultivars are clonal propagates, a lower rate of plant regeneration is accepted as long as these plants are genetically identical. During the first part of the project the stability of the plants regenerated from cryopreserved apices was investigated by phenotypical observation of the adult plants and the tubers produced. The extension of the project allows the investigation of the genetic stability of the plants originating from frozen meristems on the molecular level. This will be DNA-fingerprinting techniques and cytometric analysis. So far there is one report of

Cultivar	BGRC	Number of meristems	Survival rate %	Number of plants	Tubers	Pheno.
Aguti	060056	252	72	29	2	o.k.
Altena	060061	216	78	58	1	o.k.
Amedo	060068	224	81	22	2	o.k.
Amiro	038063	36	71	38	1	o.k.
Anco	060210	120	80	27	2	o.k.
Andante	060070	84	89	36	1	o.k.
Bla Dalsland	060094	252	69	15	1	o.k.
Blanik	060228	264	64	22	1	o.k.
Cosima	031255	132	50	30	1	o.k.
Creta	060092	180	80	40	2	o.k.
Debora	038086	348	73	67	1	o.k.

Table 4: **The influence of the exposure to liquid nitrogen on the regeneration and phenotypical appearance of potato cultivars**
Tubers: planted out and harvested 1 = 1 time, 2 = 2 times
Pheno: phenotype after planting out in comparison to unfrozen in vitro plants

Number of Samples				
Year	Institutes	Breeder	private person	E
1987	19	2	1	22
1988	17			17
1989	3			3
1990	100		31	131
1991	7		19	26
1992	144		11	155
1993			6	6
1994	34		3	37
1995	40		30	70
	364	2	101	467

Table 5: **Exchange of potato cultivars during the past nine years**

Harding (1991) who found no genetic changes on the molecular level in plants re-grown from 30 individual cryopreserved meristems of the potato cultivar "Golden Wonder".

Minimal technical facilities for slow growth and cryo preservation

Slow-growth: The *in vitro* facilities correspond to laboratory space and subculture area plus the storage and the growth room. There is an additional space in the greenhouse for tuber production. Three laminar flow benches are available for sterile transfers. The laboratories are equipped for culture medium preparation, microscopy. Two stereoscopic dissecting microscopes are available for meristem excision.

Cryopreservation: All facilities as for slow-growth are needed. Additionally liquid nitrogen containers for handling and storage must be available. The number of containers depends on the size of the collection. The major pre-requisite is permanent access to liquid nitrogen.

5 Evaluation and Documentation

A good documentation system is the key to the effective utilization of the material deposited in a germplasm bank. As a matter of fact, here, a collection of old potato cultivars is of concern. Therefore all evaluation data could be gathered from descriptive variety lists for storage in a computerized data base. In this data base, data are being handled by means of ORACLE which is a relational data base system. From BGRC all evaluation data are freely available without any restriction. In future, these data will be accessible for external users through a national German network of plant genetic resources.

Together with passport data, the evaluation data have been compiled in a brochure entitled "Evaluation Data on Old Potato Varieties of the BGRC In-Vitro Collection" Eds. G. Mix-Wagner and L. Seidewitz.

6 Exchange of potato varieties

The exchange of the existing material documented with available information and of the technology needed for its efficient utilization should be the ultimate goal of germplasm banks and related data banks.

The distribution of the requested potato cultivars is done in form of *in vitro* cultures on solid culture medium together with a manual for further cultivation of the *in vitro* plantlets. In general four separately cultured plantlets per cultivar are available for delivery. Sometimes it happens that the requesting person is not able to handle the *in vitro* plants, in this case 3-4 potato tubers of the requested cultivars will be delivered, but it takes extra time to produce the tubers. Since 1987 the Collection delivered 467 cultivars around the world (Table 5). One person in the gene bank is responsible for the shipping procedures either *in vitro* cultures or seeds.

Summary

In the BGRC-potato cultivar collection, 778 cultivars are maintained under slow-growth conditions (10°C, 16 hrs day, 1 klux). 140 cultivars are freeze-cryostored (-196°C) in liquid nitrogen. All passport and evaluation data of the old cultivars have been compiled in a brochure. Since 1987 the collection delivered 467 cultivars around the world.

In-vitro-Sammlung alter Kartoffelsorten des BGRC

Die In-vitro-Sammlung umfaßt 778 alte Kartoffelsorten aus 25 verschiedenen Ländern. 638 Sorten werden als kleine Pflanzen unter reduzierten Wachstumsbedingungen (10°C, 16-hrs.- Tag, 2 Klux) *in vitro* aufbewahrt.

140 Sorten lagern in Form von Meristemen in flüssigem Stickstoff (-196°C).

Alle Daten über die Sorten sind in einer Broschüre zusammengefaßt. In den letzten 9 Jahren wurden etwa 470 alte Sorten abgegeben.

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Author: Mix-Wagner, Gunda, Dr., Institut für Pflanzenbau der Bundesforschungsanstalt für Landwirtschaft Braunschweig - Völkenrode (FAL), komm. Leiter: Dir. und Prof. Professor Dr. Friedrich Weißbach.