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Mini Review

OVERVIEW OF *in vitro* PRESERVATION OF POTATO AND USE OF THE GENE BANK MATERIAL IN ESTONIA

Viive Rosenberg, Jaanika Edesi, Ketlin Liiv, and Katrin Kotkas

Department of Plant Biotechnology EVIKA, Estonian Research Institute of Agriculture, Teaduse 6 a, Saku 75501, ESTONIA
evika@evika.org

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At EVIKA, we have been preserving potato varieties, breeding lines and land-races in vitro *as meristem plants for more than 30 years. Various experiments have been conducted to determine the effects of medium components, growth conditions and other factors on regeneration and the sub-culturing interval of* in vitro *plants. Based on these experiments, the optimal preservation medium and long-term preservation conditions* in vitro *for many varieties have been developed. Every 3.0–3.5 months, the potato plants regenerated from meristems are transferred onto growth-regulator-free propagation medium. At present, there are 454 potato varieties, breeding materials, land-races and 1026 meristem clones in our gene bank. The interest in varieties as genetic resources and in those with coloured flesh tubers is increasing. In EVIKA's test field we have been testing meristem clones of variety 'Blue Congo'. We have demonstrated the use of that variety for making salads; baked, boiled, mashed potato and even for French fries. In addition, the use of the genetic resources was started in a farm, where. 2000 kg seed tubers were produced from 4580 meristem plants of variety 'Väike verev' in 2012. The main interests are: dark yellow flesh, content of antioxidants and use as a source for functional diet.*

Key words: *genetic resources, preserving* in vitro*, potato meristemplants, cryopreservation.*

INTRODUCTION

The process of breeding new varieties is continuing and the breeding methods are improving. More and more attention is being directed on the use and preservation of old varieties and land-races. It is widely understood that, in addition to the protection of the natural species under the threat of extinction, also the varieties created by breeders and the material of land-races need to be protected. At the international level the preservation of the genetic material is considered to be a strategic necessity of a country.

The Convention on Biological Diversity and Climate Change was established in 1992. Estonian Parliament ratified the Convention on 11 May 1994. The aim of the Convention is to protect and use sustainably the biological diversity, with fair and impartial distribution of the benefits gained from the use of genetic resources.

There are many different ways to preserve genetic resources. *In situ* preservation means that the genetic resources are protected in their natural habitat. The *ex situ* method supplements *in situ* preservation. In most cases *ex situ* preservation involves saving the seeds in gene banks. All e*x situ* methods are equally important, but in some cases maintaining seeds is complicated or even impossible. In field collections the genetic resources are preserved as growing plants, where they can be at risk from the natural forces, environmental hazards, diseases and pests. Changes in conditions of the environment have caused increasing damage, and especially to plant species with vegetative propagation via genetic erosion. Thus, the risks of diseased and ill quality material make the distribution and exchange of cultivated material complicated and unsafe.

In recent decades, *in vitro* methods have been developed and used to preserve the genetic material of plants. In this case, plants, micro plants or parts of plants are preserved in an artificial environment on medium or in liquid nitrogen at very low temperatures (cryopreservation).

In vitro methods are considered to be increasingly important for the preservation of genetic material of plants and particularly regarding plants with vegetative propagation, or for preserving species of which seeds cannot be preserved for different reasons. Today, we are not able to foresee the needs of people and changes in the environment. Therefore, it is very important to retain as many species, varieties and variations as we can. Greater diversity enables the users of genetic material to obtain the necessary characteristics and distinctive features for breeding.

The development of plant biotechnology has brought new possibilities to obtain disease-free plants. Preserving

healthy and disease-free plants in isolation ensures their better preservation and simplifies the procedure of plant quarantine when the material is exchanged by gene banks. The preservation of very small plant parts enables to lower labour and space costs. *In vitro* methods have been developed in recent times and are gaining importance, especially in preserving vegetatively-propagated plants and those plant species which are difficult to preserve as seeds. Maintaining the genetic resources is the only way to guarantee their availability for today's and future generations.

THE CULTIVATION OF MERISTEM PLANTS AND THEIR PRESERVATION *in vitro*

During studies conducted at the Department of Plant Biotechnology EVIKA of ERIA for more than 40 years, potato plants have been freed from viruses using the meristem method. Also, the method of preserving meristem plants *in vitro* and the method of propagation of plants using plastic rolls with peat have been developed. The method in short is as follows: the representative or owner of a variety brings 3–4 excellent quality tubers to EVIKA. 10–15 cm long plants are grown from the tubers. The plants are subjected to thermotherapy.

Meristem tissue of 0.2–0.3 mm in size is cut from apical or lateral buds of green plants that have passed through 6–8 weeks of thermotherapy treatment 37–39 °C 16 h photoperiod and 33–35 °C 8 h dark. Usually 20–30 explants are cut per variety. The explants are cultivated on Murashige-Skoog medium modified by EVIKA. The plants regenerate within 4–12 weeks. The first selection is made at the stage of the first meristem plants. Rapidly developed plants with stem length 8–12 cm and with developed root primordia are selected for propagation. The selected meristem plants are propagated *in vitro* by means of microcuttings. The progeny of each meristem plant is the basis for the meristem clone. One part of the meristem clone plants remains *in vitro*, the others, usually three of each clone, are used for virus diagnostics. The ELISA-test is used for potato virus X (PVX), potato virus S (PVS), potato virus M (PVM), potato virus A (PVA), and potato leafroll virus (PLRV) identification.

Our research has shown that some viruses are more easily eradicated using the meristem method than others. For example, 0.1-mm meristems of variety 'Jõgeva Kollane' were 100% PVY-free when regenerated into meristemplants. By increasing the size of meristems up to 0.7 mm, the number of PVY-free plants decreased to 11.7%. The same tendency was observed with other viruses as well — larger meristem cuttings tended to more frequently have virus infection. With PVX the results were as follows: meristem size 0.1 mm gave 86% successful regeneration, 0.5 mm — 12.8% and 0.7 mm provided no virus-free plants. With PVM the results were the following: meristems of size 0.1 mm regenerated 100% into virus-free plants, meristems of 0.7 mm — only 52%. The most difficult to eliminate was PVS: 0.1-mm meristems led to 6.6% virus-free plants, 0.3-mm meristems — 3.5% and all cuttings bigger than that were infected by viruses. Most of the varieties in our trials gave results similar to 'Jõgeva Kollane'. However, there were some exceptions.

The heat treatment of green plants prior to meristem operation significantly increased the effectiveness of virus eradication. Various heat treatment periods were tested: 4, 5, 6, 7, 8, 9, 10 weeks at temperature 37–39^{o}C with photoperiod 16 h and 33–35 ^{o}C during a 8-h dark period. It appeared that the eradication of all four tested potato viruses was most effective when the heat treatment was applied for 6–8 weeks and meristems 0.2–0.3 mm in size were subsequently used. Under these conditions, 100% of Y, M, S and 91.6% of X-virus free plants were established. Further studies with many varieties proved that success of virus-eradication depends strongly on the variety-virus combination. For some varieties it was sufficient to apply six weeks of heat treatment and for some varieties, viruses were more easily eradicated. PLRV was easily eliminated when green plant thermotherapy was applied and meristems of 0.2–0.3 mm in size were cultivated. As a result of these trials the system of virus eradication from seed potato was created, which consists of several cycles (Kotkas and Rosenberg, 1999).

The basic material for seed production in different areas of former Soviet Union was freed from viruses in EVIKA during the 1980s and 1990s. For that reason, many varieties were brought to the research centre and after treatment the plants were preserved *in vitro*. Similarly, varieties breed in Estonia and varieties of land-races found in gardens, were freed from viruses and preserved. In 1986, specialised plant biotechnology research centre was established and equipped, also with low-temperature (+4 – +6 °C) cold room for the long-term preservation of genetic recourses of plants.

At present, all potato varieties, breeding materials and land-races are preserved as virus-free *in vitro* meristem plants in EVIKA. The varieties are preserved as numbered meristem clones. There are 454 potato cultivars, breeding lines, land-races and 1026 meristem clones from varieties preserved *in vitro* in the gene bank as scientific material. Commonly, there are 5–10 plants per accession, which are duplicated in different storage rooms. The oldest meristem plants were introduced in 1977.

RESEARCH AND EVALUATION OF POTATO GENETIC RESOURCES

The aim of our research was to develop methods to prolong the sub-culturing interval *in vitro* without changes on meristem plant quality and productivity. The influence of medium components (kinetin, potassium, nitrogen, sucrose) on regeneration of plants *in vitro* and quality was studied. In the first generation test-field the influence of factors on the productivity and morphological characteristics of varieties were studied.

Different concentrations of potassium, nitrogen, kinetin and sucrose were added into the propagation medium. The re-

generation and preservation of plants were carried out in three different light and temperature conditions. The following quality characteristics of plants were evaluated: height of plants; number of internodes; length and number of roots. Four cultivars with different *in vitro* behaviour were involved in the tests. The acclimatisation of plastic roll plants, planting and cultivation were conducted according to the technology of growing first generation seed tubers developed in EVIKA. During the vegetation and harvesting period, the true-to-typeness was evaluated.

The height of stems was significantly influenced by the content of the medium. Height was lower in all tested media compared with control. The height of stems was more influenced by kinetin, than by potassium or sucrose. The height of stems was also significantly influenced by the preservation conditions and increased with longer preservation duration.

The number of internodes characterises the plant propagation rate and is significantly influenced by the medium, preservation conditions and duration. The average number of internodes per plant differs between cultivars and is mostly affected by kinetin and potassium concentrations.

The number of roots characterises the quality of the root system and rooting of plants in a plastic roll and in field conditions. KNO_3 and kinetin addition, separately and together, results in lower number of roots per plant. The best rooting was obtained when sucrose together with KNO_3 and kinetin were added into medium. The number of roots per plant of all varieties was more influenced by the medium and preservation treatments than by the mutual treatment of medium and preservation conditions. The number of roots is a more valuable characteristic than the length of the roots for *in vitro* preservation. The length of roots was found to depend significantly on the medium content. Kinetin and kinetin + KNO_3 decreased the length of roots. The number and length of roots were increased by an extended preservation period.

In the current study the plant sub-culturing intervals varied from 20...22, 53...55 to 100...102 days. Kinetin had a clear positive effect on plant tolerance to low temperatures and long sub-culturing intervals. During a long preservation period, plants grown without kinetin became elongated, tip leaves turned yellowish and dropped when planted out from test tubes. The potato plants grown in medium with higher concentration of kinetin had shorter internodes and smaller leaves. The roots were located close to the medium surface and some roots regenerated outside the medium. The cultivar Berber had callus on leaves and stem. It is known that the appearance of callus is associated with physiological changes caused by changes in the environment (Benson *et al*., 1989). Thus, the variety 'Berber' is more sensitive to lower temperatures than are the other tested varieties.

The plants from different media and preservation treatments rooted and developed similarly in the field. The number of tubers per plant was more influenced by the variety (genotype) than by the content of medium. The preservation duration had a clear effect on the number of tubers per plant. A long preservation period improved the formation of the root system *in vitro*, which resulted in a high number of tubers in the field (Table 1).

Table 1

EXAMPLE OF THE NUMBER OF TUBERS PER PLANTS OF SOME LONG PRESERVED PLANTS

Variety	Year of foundation	Tubers per plant of *in vitro* culture				
		1994	1995	1997	1998	1999
Ora	1977	9.5	6.8	7.2	7.5	7.7
Rector	1978	6.8	7.3	8.4	6.9	7.3
Dalia	1979	15 7	11.9	8.7	9.0	10.4
Xenia	1981	8.3	11.9	10.4	9.4	9.0

No differences in development, flowering time and growing intensity of plants regenerated on different media and multiplied with different preservation period were found (Kotkas *et al.,* 2002, 2008).

The research occurs in co-operation with the Jõgeva Plant Breeding Institute, Nordic Genetic Resources Centre and Baltic gene banks. EVIKA has initiated the search for material with Estonian origin from other gene banks. In 1998, Estonian varieties 'Brigadir', 'Jõgeva Valge', 'Linda', 'Suvik', 'Sangar' and a breeding line of Jõgeva 1968-32, were reintroduced as test tube plants and the variety 'Kalev' as tubers from Groß Lüsewitz Gene Bank in Germany. A toral of 17 preserved items of old varieties and land-races were sent to Germany.

According to the international regulations and standards, the preserved items in gene banks are to be described, evaluated and analysed on certain indicators. Pursuant to the international standard TG/23/5, the following data has to be accumulated: identification in plant passports (11 indicators), botanical characteristics (56), agronomical characteristics (13), conventional usage and quality (17). In addition, the resistance of the preserved plants to pests and diseases (58) also need to be tested and evaluated. Also information on cultivating history and generative characteristics need to be included. In total, 170 characteristics have to be described. The passport information of the preserved plants in EVIKA's potato gene bank is collected and the evaluation of botanical characteristics has been completed.

RESEARCH ON CRYOPRESERVATION

Cryopreservation is storage of germplasm at ultra-low temperatures, usually at temperature of liquid nitrogen (–196 ^{o}C), such that the viability is maintained and regeneration achieved after rewarming. Cryopreservation is suitable for different kinds of plant material: plantlets, seeds, shoot tips, meristems, callus, cell suspensions, pollen, roots etc. (Reed, 2002).

Cryopreservation is a viable alternative for the long-term storage of old potato varieties. Major advantages of cryo-

preservation are maintenance in small volume, protection from secondary contamination and avoidance of cumulative somaclonal variation through *in vitro* storage cycles. Additionally, labour costs in maintaining the cultures is reduced, as only the supply of liquid nitrogen is needed for maintenance of cryopreserved samples. Cryopreservation is mainly suitable for long-term preservation, because the plants are not immediately available when asked for, i.e. the samples need to be thawed and cultured before they can be passed to growers. For frequently requested varieties, it is appropriable to keep them as *in vitro* cultures to facilitate fast distribution and to use cryopreservation as a back-up storage method (Schäfer-Menuhr *et al*. 1996).

In EVIKA cryopreservation has been recognised as a promising method for long-term preservation of genetic resources. Cryopreservation is especially suitable for long-term conservation of plant genetic resources, because at cryogenic temperatures all growth and metabolic processes stop and therefore no alterations in the stored material will take place (Panis *et al.*, 2001).

In EVIKA we have started to work with cryopreservation of the potato varieties stored in the *in vitro* collection. Until now, ten varieties have been successfully cryopreserved by modified DMSO droplet method (Kaczmarczyk *et al*., 2008). For cryopreservation experiments, 3–4 week-old *in vitro* plantlets were used, form which shoot tips of 3–4 mm in size were excised for cryopreservation. From each variety 60–100 shoot tips were cryopreserved. All the varieties have survived cryopreservation and regenerated plants of each variety have been obtained. The survival of shoot tips varied between varieties with the highest survival 87% for variety 'King Edward' and the lowest (42%) for varieties 'Väike verev' and 'Blue Congo'. The regeneration into new plants also varied between varieties with the highest regeneration rate of 75% and 43% for varieties 'Kind Edward' and 'Anti', respectively. However, for some of the varieties the regeneration remained as low as 3%.

These experiments have been repeated several times and variation in survival and regeneration between different experiments have been observed. We will continue to study various aspects affecting survival and regeneration after cryopreservation. In addition, the potential of cryopreservation as a method for a back-up collection of potato genetic resources of Estonia will be evaluated.

CONCLUSIONS

The aim of our research is to develop ways to prolong the multiplication interval *in vitro* without changes on the meristem plant quality and productivity. Also, an aim is to study the influence of long-term *in vitro* storage on the genetic stability of cultivars. At this stage, we can conclude that preserving potato cultivars *in vitro* as meristem plants more than 30 years has not influenced yield productivity and characteristics of the cultivars.

The collected data provides opportunities for other institutions and breeders to use the material preserved in our *in vitro* gene bank. Also, the evaluated material can be utilized for diversification of food production. The materials preserved *in vitro* can be used as initial material for breeding, research, for propagation of disease-free material, for seed production and for establishment of field collections.

All tested medium components had an influence on the plant quality characteristics. Kinetin had a stronger effect than potassium, nitrogen or sucrose.

By modifying EVIKA's potato propagation medium by adding kinetin 0.2...0.3 mg/l, sucrose 20 g/l and KNO_3 4.0 g/l and maintaining plant material at temperatures 3...4 °C, 16h/8h, the sub-culturing interval can be extended up to 100 days.

In EVIKA cryopreservation has been recognised as a promising method for long-term preservation of genetic resources. Cryopreservation experiments with ten varieties have been repeated several times and variation in survival and regeneration between different experiments have been observed. We will continue to study various aspects affecting survival and regeneration after cryopreservation. In addition, the potential of cryopreservation as a method for back-up collection of potato genetic resources of Estonia will be evaluated.

Genetic resources can be used in plant breeding, research and educational purposes, as well as in production of disease-free seed and plants. If necessary, the varieties that are no longer (or yet) in use, can be utilised for agricultural or other purposes in the future.

The interest in varieties as genetic resources and in varieties with coloured flesh tubers is increasing. In our EVIKA test field, we have been testing meristem clones of variety 'Blue Congo' for more than 20 years. We have demonstrated the use of that variety for making different salads, baked, boiled, mashed potatoes and even making French fries. At first, buyers have spurned the coloured flesh and think that some chemicals have been used, but after tasting they are pleased with the taste and are willing to use this variety in the future. Also, dark-yellow flesh potatoes are appreciated, both because of the colour and the possible source of the antioxidants. The varieties have been accepted as a part of functional diet.

The two old varieties 'Väike verev' and 'Endla' in EVIKA's gene bank, which origins are unknown, have been listed as varieties for preservation in the National List of Varieties. Both of the varieties have been recognised as very tasty and suitable for fete dishes. In 2012, 4580 meristem plants were propagated and planted into a farmer's field. 2000 kg of seed potato was grown from these plants. Hence we have started to utilise the preserved genetic recourse material in production.

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PĀRSKATS PAR KARTUPEĻU SAGLABĀŠANU *in vitro* UN ĢENĒTISKO RESURSU IZMANTOŠANU IGAUNIJĀ

Jau vairāk nekā 30 gadus EVIKA veic kartupeļu šķirņu, selekcijas klonu un seno kartupeļu šķirņu saglabāšanu *in vitro.* Veikti vairāki pētījumi, lai noskaidrotu dažādu barotņu komponentu, augšanas apstākļu un citu faktoru ietekmi uz augu, kas iegūti no meristēmām, reģenerāciju un augšanas ilgumu *in vitro*. Pamatojoties uz šiem pētījumiem, daudzām šķirnēm ir izstrādāts optimāls barotnes sastāvs un izpētīti atbilstošākie audzēšanas apstākļi ilglaicīgai augu saglabāšanai *in vitro*. Vienu reizi 3 līdz 3.5 mēnešos no meristēmām iegūtie kartupeļu augi tiek pārstādīti barotnē, kas nesatur augšanas regulatorus. Šobrīd EVIKA gēnu bankā tiek saglabātas 454 kartupeļu šķirnes, selekcionāru materiāls, vietējās izcelsmes šķirnes un 1026 meristēmu kloni. Pieaug interese par dažādu ģenētisko resursu kolekcijā uzturētu šķirņu izmantošanu, īpaši par šķirnēm ar krāsainu mīkstumu. EVIKA izmēģinājumu laukā ir pārbaudīti šķirnes 'Blue Congo' meristēmu kloni. Mēs esam demonstrējuši šīs šķirnes izmantošanas iespējas salātu, vārītu, ceptu kartupeļu pagatavošanai, kartupeļu biezputras un pat kartupeļu frī pagatavošanā. 2012. gadā kartupeļu ģenētisko resursu paraugu audzēšana tika uzsākta saimniecībā, kurā no 4580 meristēmu augiem tika izaudzēti 2000 kg kartupeļu šķirnes 'Väike verev' sēklas bumbuļu. Galvenā interese pievērsta tumši dzeltenajam mīkstumam, antioksidantu daudzumam un kartupeļu pielietošanai funkcionālajā pārtikā.