THE RESULTS IN THE FIELD OF THE *IN VITRO* CONSERVATION OF THE CULTIVARS WHEN USING CLASSIC AND MODERN CONSERVATION METHODS

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Summary: By means of the ***in situ*** conservation the vegetal material is exposed to genetic modifications as a consequence of the natural or artificial selecting. The *ex situ* conservation, by the use of traditional techniques, allows the keeping of seeds, plants or different plant organs elsewhere than in their originating places for a long period of time and in good conditions. The *ex situ* conservation may be in gene banks, in botanical gardens or in sanctuaries. The costs for such conservation are very high and it also runs the risk of losing the material because of pests, diseases or the unfavorable climatic conditions. The *in vitro* conservation eliminates all these disadvantages allowing the conservation of the vegetal material for a very long period of time. On the other hand by using this method the rapid multiplication of the stored material is possible. The *in vitro* conservation techniques can facilitate the application of some genetic handling procedures such as the putting at the disposal of a simple way to stock the experimental material in the form of in vitro cultures. The new conservation techniques give a new impetus to all in vitro culture conservation methods. The inoculums cultivated on aseptic media can be preserved in a certain morpho – physiological stage and their regenerative activity can start anew by using variety of types of techniques such as frost conservation (The frosting or the keeping of the inoculums at positive or low temperatures), cryopreservation; the conservation in conditions of inoculums slow growth; the inoculums dehydration (up to the surviving limit); the growths of the inoculums at low air pressure or in hypoxia; The goal of the present study is to find out appropriate conservation methods of the performing genotypes at different culture species.

Keywords: methods, conservation, *in vitro*, *in situ*

Coking's achievement from 1960 made possible the obtaining of large populations of protoplasts and thereafter the producing of somatic hybrids. 1966 is considered to be the beginning of modern researches of the *in vitro* vegetal explants. By means of the protoplasm at the same time with the discovery of some techniques there have been obtained resistant plants to agents such as pesticides, hydro and thermal stress.

The *in vitro* cultivation of the anthers (androgenesis) and of the ovules (ginogenesis) led to the obtaining of haploid plants. The modern *in vitro* multiplication practices assure both the obtaining in a short period of time of an infinite number of plants and the storage and conservation of the vegetal material in the so called gene banks.

The tissue, cells and protoplasm culture researches revealed amazing things about the vegetal cells genes and opened new developing perspectives.

In 1983 began in our country the *in vitro* propagations of potatoes. ***Cachiţă*** and his team devirused and multiplied some potato lines produced by I.C.P.C. Brasov.

The ex situ conservation

The seeds conservation

A large number of agricultural plants produce seeds that can be dehydrated up to a level which permits the storage at low temperatures (Ellis, 1990). Besides this procedure another is used, i.e. one that ultra dries the seeds (Ellis et al., 1990; Roberts, 1991). For the cultivars that produce seeds which can be dried and stored at low temperatures, this is the best way of conservation, as it is easily adapted and has a high degree of security.

There are three categories of cultivated plants that have seed storage poblems.

The first category includes those species that do not produce seeds and are propagated by the vegetative way (bananas).

The second category includes plants such as: potato, root crop and tubers Dioscorea spp., Manihot esculenta, Ipomoea batata and sugar beet Sacchatum sp. that has both sterile genotypes and also produces seeds that can be conserved. These species are generally propagated by the vegetative way (Simmonds, 1982).

The third category is formed by the species known as being "recalcitrant" such as the coconut, avocado, mango, cacao, and the species of the Dipterocarpaceae family (Chin and Pritchard, 1988; Roberts and King, 1982).

Traditionally the field gene bank, represents an ex situ storage method for this type of material. Although in some cases there are some advantages in storage, there are disadvantages that make the method unsure and limit its efficiency. The genetic material is exposed to diseases and pests and other calamities

The cultivated potato (Solanum tuberosum L.) can be multiplied both by the vegetal way and the generative one. Nowadays the potato production all over the world relies on the clonal multiplication. But this type of multiplication has also some disadvantages, such as:

- transmission of a large number of pathogens;
- a large quantity of seeding material;
- high costs for obtaining, transport and storage of he biological material;
- perishable material.

The TPS technology (True Potato Seed) relies on the usage of the botanic potato seed and it proved to be one of the most promissing strategies for producing an alternative planting material that assures the necessary potato seed.

In comparison to the traditional system, the potato culture with botanical seed has the following advantages:

- reduced planting volume;
- no problems with transportation and keeping of the seeds;
- no disease transmission from one generation to the other;
- seeds are all over the year accessible;
- low costs for the tubercles;
- rapid adaptation of a hybrid population compared with that of the clonal one;
- more tubercles for consumption.

Not long ago, the TPS technology was not well received as there were doubts about the generative multiplication.

Nowadays, due to the technical progress, the hybrid potato seed is at the disposal in large amounts, at a low price and can race the certified potato seed. So the hybrids must be considered first of all as a product for "getting better" the potato.

The potato botanic seed has become very promising in different parts of the earth. Today many countries use this way of potato multiplication. Therefore the efforts to improve the quality and the security of the conservation offered by the field gene banks are evident.

But there arises also a need of some alternative methods for the storage of the material that has problems. This happened at the beginning of the 1970 - ties, when the attention was drawn by the possibilities of the biotechnologies, especially by the in vitro cultures.

Germoplasm conservation

The potato germplasm is preserved traditionally through the tubercles. Numerous potato clones are to be found at the International Potato Centre from Lima Peru. The potato conservation requires a lot of time, intensive work, and large depositing rooms. The conservation of the genetic resources coming from the vegetative propagated plants was solved, when non-conventional *in vitro* preservation methods started being used at large scale. At first, meristem culture was mainly used for short time *in vitro* potato preservation (several months up to one year).

The cryopreservation of the biological material cultivated *in vitro*, at low temperatures was considered to be a progress for a large number of species (potato, strawberries, wheat, cabbage, and olives). So many economically interesting species have been cryopreserved successfully.

Modern preservation methods

Biotechnology through the eye of *in vitro* techniques, both biochemical and molecular, can offer a series of ways for the eradication and inventory of diseases. Typical examples for this can be for instance *Solanum tuberosum* L, *Manihot esculenta* and *Ipomea batata* (**Karta,** 1986; **Thottapilly** and **Rossel,** 1992). As a consequence of the predominant use of the vegetative propagation techniques there can be observed a tendency towards the accumulation of pathogens in successive generations. The meristem culture used alone or in combination with thermotherapy can eliminate the viral pathogens (**IPGRI,** 1994).

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In vitro preservation

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All *in vitro* preservation methods aim at diminishing or temporal stopping of the vital processes.

Cryopreservation means blocking the metabolism, the cells' functioning by means of freezing or frosting. In some circumstances, the gradual dehydration of inoculums is chosen and this requires the cell water removal by adding active osmotic oxygen substances or inoculums tissue drying using air.

Another method is based on creating a hypoxic medium or low pressure environment

All these preservation methods aim to preserve unaltered the hereditary background of the stored inoculums.

The inoculums cultivated on sterile media can be preserved in a certain morpho-physiological state which allows the reactivation of the regenerative function after the transfer on normal conditions using different techniques:

- low temperature preservation (freezing or inoculums storage at positive or low negative temperatures);
- preservation in conditions that insure inoculums slow growth;
- inoculums dehydration to the survival limit;
- growth of inoculums using low pressure conditions or hypoxia.

The *in vitro* cryopreservation

The preservation of the live material with keeping unaltered the vital biological functions after the integral de-freezing is the main concern modern vegetal and animal biology researchers.

The cryopreservation of the vegetal biological material, cultivated *in vitro* has numerous advantages:

- there is the storage possibility in a relatively small place of a great number of individuals without the contamination danger with phyto agents or zoo pathogens. During the inoculums preservation at the temperature of liquid nitrogen (-196ºC), no genetic modification takes place (**Kartha**, 1985);
- depending on the cryopreservation conditions, the keeping of the inoculums at very low temperatures can be theoretically carried out unlimitedly;
- the *in vitro* cultivated inoculums, being free of any type of germs, can be transported easily from one country to the other, thus avoiding restrictions imposed by the phytosanitary quarantines;
- the damages caused by freezing and defreezing have two major aspects:

- the formation of big ice crystals within the cells either during the freezing or the de freezing fact that can cause the rupture of the cell organits or the tearing of the whole cell.

- the rising of the solutions' concentration from within the cells in the course of freezing by the gradual losing of water from the cell's structure; in this way the cellular medium becomes noxious.

Achievement in inoculums conservation techniques by cryopreservation

Good results have been obtained at the conservation by freezing of the carrot callus, cells, embryos and plantlets. The results were also very good when the cells were in the „lag" growing stage or in an early exponential growing stage of the culture and were rapidly defrozen.

Tobacco anthers, callus and cellular suspensions were successfully frozen. Best results were obtained when glycerol10% or a mixture of glucose 0.2%, DMSO 3% and ethilenglycol 2.5% was used.

At the potato the combination of three cryopreservers (DMSO and glycerol, 5% each and saccharine 0.15M) gave better results than the exclusive use of DMSO 10%.

The plant regeneration takes place in a certain period of time. The surviving of the axial buds and of the stems apexes was 19-27%, and of the tubers 11-14%. The period of storage is from 4 weeks to 24 months and the de freezing took place rapidly (**Bajaj**, 1987).

The storage at cold is of great importance at the industrial nurseries where micro propagation and producing techniques are used in aseptic conditions of the seeding material. So that during longer or shorter periods of time, the plantlets stored in refrigerating rooms can be distributed to markets depending on the demand.

The inoculums conservation by imposing slow growing conditions

The slow growing represents another *in vitro* cultivated inoculums conservation form, but it is less practical. This technique can be compared to the traditional Japanese BONSAY method. The vegetal material cultivated *in vitro* must be kept at limit temperature conditions (at around 1-4˚C, or at temperatures over26˚C) using low oxygen pressure in the recipients and by administrating special phyto sanitary treatments (after **Nitzsche**, 1983) or substances that rise the osmotic pressure in the cells.

Results in this field

Mix (1981) mentions that for the potato callus he introduced into the culture medium N – dymethylanosuccinic acid and that he incubated the callus at a temperature of 10˚C at a luminosity of 4-5 Klucsi, he succeeded to extend the period of transferring the callus to over 2 years. The genetic stability seemed not to be altered. Mix grew the nodal segments of the potato stem on filter paper that was maintained in liquid medium. The inoculums were kept at 10˚C at a luminosity of 4-5 Klucsi, for 2 years. During this period of time some phenotypical modifications were observed. At solanaceae meristems under cultivated for a year (**Henshaw** et al., 1980), the surviving was of 14% at a temperature of 22˚C and of 61% at a temperature of 6˚C. Applying an alternative temperature regime, 12˚C during the day and 6˚C at night raised the surviving ratio to 83%. The storage at temperatures below 6˚C was unsuccessful. The rising of the saccharine concentration from 3% to 8 % and the augmenting of the medium volume from 3.5ml to 60ml, at the *Solanum* sp., meristem culture led to an improve of the meristems surviving ratio kept at 10˚C for one year. The regeneration percentage grew from 39% to 88%. The same culture type maintained at 22˚C (and adding an average of 6 mg of manitol or 5ml&l of abscisic acid) showed a growing of the surviving ratio after one year of culture conservation (**Henshaw** et al., 1975, 1980).

Cachita et al. tested the reaction of the vegetal inoculums cultivated *in vitro* at positive temperatures lower than 6-7˚C. Potato shoots, generated from cuttings, were stored in the dark and at continuous light for 11, 10, 9 and 8 months. When the inoculums passed to slow growth (6-7˚C), the stemlets newly formed from cuttings had a height of 3-5 cm and formed bushes. The culture medium on which the stemlet colony grew had BA 2 mg/l or with procaine 1mg/l or alar (B^9) 2.5 mg/l (**Cachiță** et al., 1985). In the course of the storage no interventions on the culture took place. The numerous stemlets formed mini tubercles at the apical zone. A month after their passing to light, at 25˚C there was observed that the stemlets necrotized and at the bottom of the culture new shoots appeared. About 60-70% of survivals and regenerations were observed only at the inoculums stored at cold (6-7˚C) for 6-7 months mostly on medium with BA 2 mg/l and alar 2.5 mg/l. From the dry stemlets small tubers were collected and afterwards these tubers were cultivated *in vitro* and generated a lot of shoots, thus proving that the shoots were viable (**Cachiță** et al. 1985).

Bioactive magneto – fluidic nanocomposites were integrated as components of the culture (nutritive) media for callus induction and plant regeneration of several species: *Chrysanthemum indicum* (Baciu et al., 2004), *Lillium regale, Mamillaria and Triticale* (**Butnaru**, 1994).

These studies and former researches have demonstrated the magneto – fluidic nanocomposite bioactivity and their positive effect when used in small concentrations as well as the repressive effect of high concentrations on callus induction and plant regeneration in *Triticale,* tomatoes, *Saintpaulia* and tobacco (**Butnaru** et al., 1995, **Butnaru** et al., 1995).

The method of storing the inoculums cultivated *in vitro* in slow growing conditions is insufficiently disseminated, but the researches have just begun. Comparative studies must be carried out and the factors analyzed that interfere in maintaining at the surviving level of the vital processes, needed for starting again the active life or for their diminishing when growth must be stopped so that inoculums can be stored at low activity.

Conservation by dehydration

The conservation by dehydration is taken from the normal processes in nature. So there are organs that lose water up to the surviving limit during ripening. These situations are typical for those instances when plants prepare themselves to overpass climatic dangers (drought or frost). Passing into hibernation or surpassing drought and frost are accompanied by water loss in the cells, by accumulation of glucids, lipids, and proteins through diminishing of the vacuoles volume, the reducing of the metabolic activity and by stopping the growth. All these processes are under phyto hormonal control (**Cachiță**, 1987).

At the pollen destined to conservation, water is substituted with organic solvents (acetone, ethanol).The maintaining of the pollen at 0 C, permits its storage for about 180 days. After the evaporation of the solvent the pollen can germinate (**Nitzsche**, 1983).

This method cannot be applied to the *in vitro* cultures but the dehydrated carrot callus could be kept *in vitro* at a temperature of 15 C and a low humidity for more than 2 years, and the cells did not lose their viability (**Nitzsche**, 1983). When the calls passes to a fresh medium in optimal ambient conditions his growth begins again and after about 4 weeks the plants differentiated themselves from the callus. **Nitzsche** noticed that even in this case the composition of the culture medium and mostly the saccharine concentration from the substrate play an important role.

The potato callus cultivated on MS medium with saccharine 0.15 M and abscisic acid 37.8 µM was dried in sterile air and maintained for 36 hours at - 80˚C. When transferred to normal conditions the callus began growing again and formed young shoots.

Conservation of the inoculums by their growth in low atmospheric pressure and oxygen lack (hypoxia)

The methods for the conservation at low pressures of the biological material suppose the lowering of the pressure of all atmospheric gases from the culture recipients around the tissue. Another technique reduces the pressure of oxygen partially. When the oxygen is reduced to around 50 mm Hg in the culture recipients the growth is reduced too. When the growth starts again in normal conditions no phenotypic modification was observed at the plantlets from the inoculums.

The storing of the biological material at low pressure is used for the conservation of foodstuffs, of flowers, and for other live biological products with active metabolism (after **Anonymus**, 1975).

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