"Alexandru Ioan Cuza" UNIVERSITY, IAŞI
FACULTY OF BIOLOGY

IUSTINA BRÎNDUŞA CIOBANU

# Cytogenetical and morpho-anatomical research to the local potato varieties *in vitro* cultivated through slow growth method

## PhD THESIS ABSTRACT

Scientific adviser:
Prof. Dr. GOGU GHIORGHIŢĂ

IAŞI – 2012

## CONTENTS

## Introduction

Because potato is an example of a species that form of seed germplasm preservation it is inadequate due to strong heterozygous and various degrees of sterility to store this plant genetic resources were discussed two methods of conservation in situ and ex situ. The two categories are forms of preservation of plant genetic resources valuable to ensure preservation of protected reserves on terms that provide certainty in terms of maintaining their genome [3]. In situ conservation, for a short period, it involves the maintenance of potato cultivars and varieties in the natural habitats by practicing a traditional agriculture. Form of ex situ conservation of potato germplasm conservation requires outside native habitat, over a long period of time by keeping its gene bank collections both in vitro and in the experimental field [6].

Two of the easy storage instruments in the germplasm collections are: conservation by slow growth and cryopreservation. They consist in maintaining cultures in physical stress or chemical conditions allowing expansion possible subculture interval without affecting the vitality of cultures [9]. The two approaches are based either on reducing the growth rate of inoculated or by stopping their growth limit survival [1].

The growth rate of potato plantlets *in vitro* may be limited by various methods including handling of nutrients in the culture medium, the use of growth inhibitors and osmotic agents [2], incubation temperature and/or low light intensity and photoperiod varied [4,5,10], reduction of $O_2$ concentration and type and size of vessels culture [8]. These methods can be used individually or can be combined.

## Chapter 1. The scientific objectives of the thesis

The study objectives included the following aspects: assess the potential of regeneration and *in vitro* multiplication of potato varieties under

slow growth; determining the effects of daminozide, mannitol and sorbitol on some morpho-anatomical and physiological indexes in the potato plantlets grown *in vitro*; highlight the differences between the evolution of the plantlets on culture media tested at different periods of cultivation *in vitro;* the identification of changes due to culture conditions and subculture period of experiment on the dynamics of cell division; establishing the frequency of chromosomal aberrations in the mitotic cells of root meristems; determination of *in vitro* plants acclimatization to *ex vitro* living environment; the evaluation regenerants of experimental field; identification of formulas the environmental and cultural conditions necessary to ensure increased interval between subcultivation.

## Chapter 2. Material and methods

### 2.1. The biological material

The biological material chosen for the study is represented by five local potato varieties (five genotypes) from collecting expeditions made by Gene Bank Suceava and maintained *in vitro* in its collection.

The explants used for culture initiation consisted microcuttings with 1-2 apical nodes collected from plantlets of five genotypes in active growth phase micromultiplication medium. The culture media used for conservation were based on Murashige-Skoog formulation [7] in ½ dilutions, with low concentrations of growth regulators, sucrose 2-3%, with the addition of daminozide, mannitol and sorbitol, resulting three variants of culture media. Control samples were maintained in the same ambient conditions, like the conservation samples, on Murashige-Skoog (1962) medium supplemented with 40 g/l sucrose and 6 mg/l daminozide (the medium $M_{14}$). After placing the inoculums, culture vessels were transferred in the growing room under the light of 2000-2500 lx, with a photoperiod of 16 hours per day, at a temperature of 20-22$^0$C. After 4 weeks, the samples

were transferred to the storage room and kept at 6-12$^0$C, under the alternation of light (photoperiod 10/24 hours), in white fluorescent illumination, having an intensity of about 1000 lx, for a period of 2, 7, 12 and 17 months.

**2.2. Research methods**

**Index of the plantlets growing**

The growth rate of the plantlets was assessed by measurements made in the number, length and branching of shoots, rooting, number of nodes viable number and size of microtubers, the number and size of ramifications thickened and the percentage of viable shoots from culture medium studied. Measurements were made after different periods of cultivation *in vitro*, respectively after 2, 7, 12 and 17 months.

**Techniques used to the studies of histo-anatomy**

To highlight the anatomy of the organs the plantlets were made the cross sections through plant material preserved in alcohol 70$^0$. The finest sections were analysed immediately on a slide dried in a drop of distilled water. The preparation thus prepared was analysed on Olympus microscope with a still camera incorporated.

**Determinations of mitotic activity**

Cytogenetic studies were performed on biological material obtained on four culture media after 12 days, 2 and 12 month. To study mitosis la 12 days after initiation of culture root meristems were used by placing microcuttings obtained on the four solid medium variants in colourless glass vials with a capacity of 120 ml, maintained in growth room. The biological material used to study mitosis at 2 and 12 months was collected from plantlets maintained in storage room.

In order to achieve temporary microscopic preparations there have been the following stages comprised: fixing of the vegetal material in 2-3 ml of Carnoy solution in the refrigerator at 5$^0$C during 48 hours; hydrolysis

roots at warm, with 1N HCl, at $60^0$ C, for 25 minutes; staining of the biological material with 2 ml of dye Carr for 3-5 days in a refrigerator at $5^0$C; performing microscopic preparations by displaying of the material through the "squash" technique; examination of fresh preparations optical Olympus CX41 microscope with incorporated still camera.

**Acclimatization of *in vitro* regenerated plants**

Plantlets obtained after three months of *in vitro* culture medium $M_{14}$ were released medium with agars by washing easily with the water maintained at room temperature and then transferred into plastic cups with soil. After 30 days of culture in the growth room it was considered that the plantlets are sufficiently vigorous and they were transferred into the greenhouse.

**Statistical analysis**

The data obtained from observations and measurements have been statistically processed and interpreted using statistical analysis software SPSS for Windows.

## Chapter 3. The research results

### 3.1. Evolution of local varieties of potato plantlets after different periods of cultivation *in vitro* micromultiplication medium the restrictive conditions of the storage room

The observations made have revealed that with the advancement in subculture plantlets have shown a tendency of senescence expressed as by decreasing the percentage of viable shoots. The analysis on the whole of the rate of survival of shoots to genotypes studied shows that values have decreased compared to control, on average with 15.64% after 7 months, 40,58% after 12 months and with 59,06% after 17 months from inoculating the explants.

After each period of subculturing to microcuttings on the micromultiplication medium in the restrictive conditions of storage it noted, in general, a decrease in the length of shoots/plantlet, the average value of this parameter being all the more as the duration of subcultivation was higher. Reducing the height was accompanied by formation shorter internodes. The number of microtubers/plantlet increased progressively with the expand of the period for cultivation, highest values being obtained after 17 months of initiation of cultures.

**3.2. The dynamics of cell division after different periods of the inoculation *in vitro* the biological material on the micromultiplication medium**

The mitotic activity in the root meristems of the analyzed genotypes decreased compared to situation registered at 12 days of cultivation *in vitro*, but the most pronounced decrease in the mitotic index was found after two months of subculturing. Regardless of the genotype and cultivation period predominated as frequency prophases being followed distance to cells in other phases of mitotic.

The microscopic analyses of biological material have revealed the presence of various types of chromosomal aberrations, distributed randomly and depending on genotype and subculturing period. The percentage chromosomal aberration has varied between 0.29 to 0.55% at 12 days, between 0.39-0.74% at 2 months and between 0.39-1.04% at 12 months after initiation of culture.

**3.3. Preservation of slow growth under the medium with daminozide**

The main effects induced by daminozide were the size reduction shoots, the increase of the number of nodes/shoot, of the number of roots and microtubers/plantlet. Extension of maintaining the plantlets in conditions of conservation resulted in a precocious senescence of cultures as manifested by pronounced decrease in the survival rate to all potato

varieties analysed. Expressed in value percentage compared to the control medium, the decline ranged between 8,4–60% after 7 months, 28,6–73,7% after 12 months and 57,9–78,4% after 17 months storage.

**3.4. Preservation of slow growth under the medium with mannitol**

Mannitol has produced, in general, an effect of slowdown in growth to inoculums, reducing height of the plants through shortening of internodes and at the same time exercised an inhibitory effect on the rooting and tubering processes; at the same time, the leaves size became very short (1.5-2 mm), many of them growing on the surface of some branches having a hypertrophic and translucent aspect.

**3.5. Preservation of slow growth under the medium with sorbitol**

The sorbitol, added to the amount of 40 g/l, in the culture medium inhibited progressively the growth of plantlets producing compact shoots with short internodes and positively influenced the rooting process; at the same time had a beneficial effect on tolerance of plantlets to low temperatures and longer storage period. Survival average rate was of 85.50% shoots after 7 months, 79.50% and 61.12% at 12 months after 17 months of conservation.

**3.6. Comparative analysis of the results of growing the plantlets under slow growth after different periods of the conservation**

The culture medium with sorbitol was the most efficient in terms of numbers of viable shoots in the case preserving medium term *in vitro* the plantlets of potato, with the highest survival rate of shoots. By extending the storage period *in vitro* the number of ramifications thickened/plantlet increase, a very clear process on culture media with mannitol and sorbitol. The rooting process was favoured by the culture medium of daminozide, regardless of the storage period *in vitro* of slow growth to the plantlets. Generation of microtubers was slightly stimulated by maintaining the

plantlets grown *in vitro* on the culture medium with daminozide and certainly inhibited by the culture medium with mannitol and sorbitol.

### 3.7. Histo-anatomical aspects of local potato varieties plantlets maintained *in vitro* under slow

**The root structure.** Central cylinder may be triarch, with a large vessel of metaxilem that replaces pith, or diarch (two large vessels of metaxilem which replace by the pith).

**The stolon structure.** At the samples grown on culture medium supplemented with 40 g/l sorbitol, was noted a large number of very long uniseriate pluricellular tector hairs and rare secretory hairs, with tetracellular gland. In case of using the culture medium supplemented with 40 g/l mannitol the cortex is observed with large cell throughout it. At the level of epidermis there are observed secretory hairs with multicellular storeyed gland (2-3 storeys of cells) or very long secretory hairs, with multicellular pedicle (4-5 cells), uniseriate and pyriform gland. To cross sections performed in samples obtained by cultivating explants in the culture medium with 30 mg/l daminozide, there are noted greater conductive fascicles, primary xylem having more vessels; more cells in the cortex having oxaliferous sand.

**The shoot structure.** Compared to stolon, to experimental variants which were analysed, there are distinguished: a layer of hypodermic tangential collenchyma and elements more or less solitary of sclerenchyma in periphloemic position.

**The microtuber structure.** Fundamental parenchyma presents numerous large starch granules, in general simple, with centrically or eccentrically hilum, rare composed. The size of amiloplasts of cells increases as move forward towards the inside fundamental parenchyma.

**The ramifications thickened structure.** The internal cortex cells are very large, radial elongated, and at their level occur transversal walls of division; of the internal cortex level, there also, and the phenomena of suberisation of cores.

### 3.8. Cytogenetic investigations on the biological material grown *in vitro* slow growth method

The samples maintained on medium conservation with daminozide for 12 days after initiation of the experiment there has been a stimulating cell division reflected through the mitotic index which had higher values than of analysed control to all analysed varieties.

At 2 months it stand out increased values of mitotic index in samples grown on culture medium with 30 mg/l daminozide compared to variant control, which means that the stimulatory effect of daminozide on cell division was maintained during this time from inoculating explants. Extending the period of subculture up to 12 months it causes a decrease in the mitotic index, compared to control, to all genotypes of potato plants investigated, regardless of the composition culture medium conservation tested, while the frequency of the various phases of division does not support the significant changes towards it. An evident inhibitory effect on the pace of cell division shown in the case cultivation explants on the supplemented medium with mannitol.

The spectrum chromosomal aberrations identified in root meristems analysed after 12 days, 2 and 12 months for assessing the mitotic activity included: ana-telophase with chromosomal bridges, delayed and pyknotic chromosomes, cells with micronuclei and a small number of complex aberrations (ana-telophase with bridges and delayed chromosomes, ana-telophase with bridges and fragments).

### 3.9. Observations on neoplantlet acclimatization ability of local potato varieties *ex-vitro* living environment

After 30 days from transfer the plantlets in soil the rate of survival ranged between 80% and 100%, depending on genotype.

### 3.10. The evolution in the experimental field plants from minitubers

The obtained minitubers were planted in the experimental field in the year 2011. In the period emergence the variability was a accentuated between varieties as well as within the same variety, but during growth these differences were dimmed, plants have arrived at normal dimensions, having a more vigorous and vivid colors compared to plants from the basic of collection. Tubers harvested from plants obtained from minitubers specific characters kept varieties used in the experiment

### General conclusions

The inclusion in the Murashige-Skoog (1962) of the tested substances (daminozide, mannitol and sorbitol) and maintaining *in vitro* cultures up to 17 months of storage room conditions progressively inhibited the plantlets' increase by reducing the length of internodes and therefore the reduction of the height of the shoots have stimulated the thickening of ramifications and have determined the appearance of very small leaves. Extending conservation increased the ability of rooting and tubering to the plantlets, regardless of culture medium formula tested.

The preservation of potato plantlets *in vitro* of the medium term in the presence in the culture medium of the daminozide (30 mg/l) resulted in the most powerful inhibition of growth shoots and the number of ramifications, as well the most pronounced reduction in survival rate of shoots which reached 30.4% after 17 months of subculturing.

Mannitol and sorbitol have had a positive effect on increasing tolerance to the plantlets to the extension of subculturing interval, fact which gives certainty that use the two conservation culture media is suitable for medium term *in vitro* conservation under slow growth of the local potato varieties. The best variant of culture medium in this system of growth proved to be the supplemented by 40 g/l sorbitol which ensured the end of the experiment a rate of survival of 61.1% shoots allowing regeneration of a sufficient number of plantlets for the subsequent multiplication.

On measure extending period of cultivation *in vitro* the restrictive conditions of storage room, regardless of potato genotypes studied and culture medium conservation tested, the mitotic potential decreased and the frequency of cells with chromosomal aberrations in the root meristems increased, but their percentage was, in most cases, subunit. A higher frequency of chromosomal aberrations cells was recorded on variants of culture medium with 30 mg/l daminozide and 40 g/l mannitol.

Histo-anatomical observations have not revealed significant changes in the vegetative organs of the plantlets developed during cultivation explants on the culture medium formulas tested; differences occurring in some cases are rather quantitative.

Acclimatization neoplantlet *ex vitro* conditions was conducted with losses of up to 20%, depending on genotype, and the cultivation field of minitubers obtained in the greenhouse from *in vitro* regenerants of the led to a percentage of emergence of approximately 50% after the first month of culture.

Evaluation of experimental field by cultivating minitubers showed that propagation mode and *in vitro* conservation of plant in conditions of slow growth, has not produced phenotypic differences at the level of aerial parts or changes in harvested tubers, these maintaining specific characteristics varieties of a basic collection (maintained through the vegetative tubers).

## Selective references


1. **Cachiţă-Cosma Dorina, Halmágyi Adela, Cristea Victoria,** 1999 – *Conservarea germoplasmei vegetale in vitro*. În: Culturi *in vitro* la cormofite. Lucrările reunite al celui de-al VII-lea şi al VIII-lea Simpozion Naţional de Culturi de Ţesuturi şi Celule Vegetale (ed. Cachiţă-Cosma, Ardelean, Crăciun), Cluj-Napoca, pp.54-56.
2. **Cachiţă-Cosma Dorina, Constantinovici Dana,** 2008 – *Conservarea resurselor vegetale în Băncile de Gene sub formă de fitoinoculi*. În: Biotehnologii vegetale pentru Secolul XXI. Lucrările celui de al XVI-lea Simpozion Naţional de Culturi de Ţesuturi şi Celule Vegetale, Bucureşti 2007, Ed. Risoprint Cluj-Napoca, pp. 15-61.
3. **Cachiţă Dorina, Sand Camelia**, 2011 – *Biodiversitatea şi băncile de gene*. Vasile Goldiş University Press, Arad.
4. **Estrada, R., Schilde Rentschler, L., Espinoza, N**., 1983 – *In vitro storage of potato germplasm.* In: Hooker, W.J. (ed.): Research for the Potato for the Year 2000. (CIP) International Potato Centre, Lima, pp. 80–81.
5. **Gopal J., Chamail Anjali, Sarkar Debabrata**, 2005 – *Use of microtubers for slow growth in vitro conservation of potato germplasm*. Plant Genetic Resource Newsletter 141: 56-60.
6. **Khana, P.P., Singh Neeta**, 1991 – *Conservation of Plant Genetic Resources*. In: Paroda, R.S., Arora, R.K. (eds.): Plant Genetic Resources Conservation and Management Concepts and Approaches, New Delhi, cap. 9.
7. **Murashige, T., Skoog, F.,** 1962 – *A revised medium for rapid growth and bioassays with tabacco tissue cultures.* Physiologia Plantarum 15: 473-497.
8. **Ogbu, J.U., Essien, B.A., Essien, J.B., Anaele, M.U**., 2010 – *Conservation and management of genetic resources of horticultural crops in Nigeria: Issues and biotechnological strategies*. Journal of Horticulture and Forestry 2(9):214 -222.
9. **Toledo Judith, Espinoza N., Golmirzaie Ali**, 1998 – *Tissue Culture Management of in Vitro Plantlets in Potato Seed Production*. International Potato Center Training Manual, Lima, Peru, pp. 30-39.
10. **Westcott, R.J**., 1981a - *Tissue culture storage of potato germplasm. 1. Minimal growth storage.* Potato Research 24:331-342.