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SUMMARY OF PHD THESIS

THE OPTIMIZATION OF MICROPROPAGATION TECHNIQUES FOR SOME FRUIT AND ORNAMENTAL SHRUB CULTIVARS

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CONTENTS

Keywords: *Lonicera*, *Amelanchier*, *Lycium*, *Rubus*, *Vaccinium*, hydroculture, CPPU

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ABBREVIATIONS

2-iP - N6-(2-isopentenyl)adenine
2, 4-D- 2, 4 dichlorofenoxyacetic acid
4-CPPU - N-(2-Chloro-4-pyridyl)-N-phenylurea
IAA –indolyl acetic acid
IBA - indolyl butyric acid
NAA –naftyl-acetic acid
BAP- 6-benzylaminopurine
BAR – benzyladenin-riboside
TDZ - thidiazuron
MSs - modified MS medium, gelled with wheat starch
mT - meta-topoline
NaDCC – natrium dichloroisocyanurate
NPK – fertilizer based on nitrogen, phosphorus and potassium
MSa/2 - modified MS medium, with macroelements concentration reduced to half, gelled with agar
PR – proliferation rate
MR – multiplication rate

FeNaEDTA – iron ethylenediamine tetraacetate
FeNaEDDHA – iron ethylenediamine hydroxyphenylacetate
Fig.- figure
H – height (of the plantlets)
MS - Murashige and Skoog Media (Murashige and Skoog, 1962)
MSa - modified MS medium, gelled with Plant Agar
SD- standard deviation
SE- standard error
WPM - Woody Plant Medium (Lloyd and McCown, 1980)
WPMm – modified WPM medium
ZEA – zeatin

INTRODUCTION

Plant biotechnology is one of the most important achievements of science and technology in the XXth century and it has an outstanding role in the development of modern agriculture and horticulture.

In vitro micropropagation is the branch of plant biotechnology which represents an ensemble of methods of plant propagation by the use of *in vitro* cultures of plant cells, tissues and organs. The use of this technique allows for the considerable increase of the propagation effectiveness in several species, and it also offers the possibility of eliminating the pathogens from the plant material.

Micropropagation is a means that has an immense potential for the conservation of biodiversity; by using *in vitro* cultures many rare, protected species can be propagated, for example endemic *Dianthus* species (Cristea, 2010) as well as rare species and varieties of cultivated plants which are less utilized and are on the brink of extinction. Micropropagation also has great importance for plant breeding and *in vitro* cultures are also indispensable for plant genetic engineering.

The development of effective micropropagation protocols for fruit species, especially for the ones that are difficult to propagate by classical means, can help the development of the Romanian nursery sector to produce planting material at European standards, in the context of the necessity of reconversion in the fruit plantations.

Culturing fruit shrub species on a larger scale, especially the less cultured ones addresed in the framework of this thesis, namely: *Amelanchier canadensis* – Fam. *Rosaceae, Lonicera kamtschatica* – Fam. *Caprifoliaceae, Lycium barbarum* – Fam. *Solanaceae, Rubus fruticosus* – Fam. *Rosaceae, Vaccinium macrocarpon* – Fam. *Ericaceae* is motivated especially by their importance as food crops, due to their high content in vitamins, minerals, antioxidants but also their frost tolerance (especially *Lonicera, Amelanchier* and *Lycium*) and their capacity to use low-fertility soils.

In this context, the PhD thesis entitled **The Optimization of Micropropagation Techniques for some Fruit and Ornamental Shrub Cultivars'** (**"Optimizarea tehnicilor de micropropagare « *in vitro* » a unor soiuri de arbuşti fructiferi şi ornamentali"**) addresses aspects regarding the micropropagation of the five species of fruit and ornamental shrubs, in order to elaborate effective micropropagation protocols useful for producing plant material.

The elaboration of optimized protocols for these species, by stimulating multiplication rates and the improvement of *in vitro* and/or *ex vitro* rhizogenesis and the improvement of *ex vitro* acclimatization, at the same time preserving the genetic uniformity of the micropropagated plants is justified scientifically and economically.

From a scientific point of view, this thesis brings new data for scientific literature, by the studies regarding the micropropagation of these species. The *ex vitro* acclimatization methods

which were applied, respectively acclimatization in float hydroculture and acclimatization in floating perlite are, also, elements of novelty and originality. Regarding the economic aspects, this work provides five effective protocols which can be successfully applied in fruit nurseries that have tissue culture laboratories, in order to produce container-grown planting material.

The structure of the thesis. The thesis contains 275 pages and it is structured into 10 chapters. It is divided into two parts:

The first part comprises 55 pages and synthesizes aspects regarding the current state of research in micropropagation, especially regarding the species that represent the object of the research in this study.

The second part of the thesis, having 185 pages, presents the author's research, respectively: the objectives, the materials and methods, the results obtained and the discussions, as well as the conclusions and recommendations resulted from the research.

I. THE SCOPE, OBJECTIVES AND MOTIVATION OF THE RESEARCH

The scope of our research was to elaborate optimized protocols in order to attain high effectiveness in the micropropagation of new fruit and ornamental shrubs in genera *Amelanchier*, *Lonicera*, *Lycium*, *Rubus* and *Vaccinium*, by stimulating multiplication rates, improving *in vitro* and/or *ex vitro* rhizogenesis and the *ex vitro* acclimatization techniques, at the same time preserving the genetic uniformity of the micropropagated plants.

In this sense, we pursued the elaboration of micropropagation technologies based on simple, accesible, effective methods used now on a large scale in micropropagation. These methods rely on the use of microcuttings from stem segments containing several nodes.

The objectives

In order to achieve our goal, by our research we addressed the folowing objectives:

- establishing the types of explants for the *in vitro* culture of the species that we studied, for the initiation and *in vitro* multiplication stages as well as for the *in vitro* rooting stage;
- establishing the basal media and the optimal plant growth regulator combinations for the *in vitro* culture of these species for the initiation, multiplication and *in vitro* rooting stages;
- testing some new gelling agents for the various culture media;
- the study of direct *ex vitro* rhizogenesis and *ex vitro* acclimatization in solid substrates (peat, perlite etc.);
- testing the techniques of *ex vitro* acclimatization in hydroculture, the method of "float hydroponics" and the method of direct *ex vitro* rooting and acclimatization in floating perlite in the species to be studied;
- the elaboration of effective *in vitro* micropropagation protocols for the five species of shrubs, these protocols being suitable for and usable by producers of planting material.

The motivation for choosing the research theme

Choosing the research theme **OPTIMIZATION OF MICROPROPAGATION TECHNIQUES FOR SOME FRUIT AND ORNAMENTAL SHRUB CULTIVARS** is motivated by the fact that the data regarding micropropagation of the species studied is scarce and, also, these species have been recently introduced into cultivation in our country as well as in some other European countries.

These species are remarkable by their value as food crops due to high content in vitamins, minerals and antioxidants and also by their tolerance regarding cold and hot weather and their capacity to grow in less fertile soils.

Having in view that these species are less cultured now, the difficulty regarding the production of planting material can be an impediment for establishing commercial plantations.

In this context, elaborating some effective protocols for the production of planting

material by micropropagation will permit the rapid introduction into large scale culture, of the new cultivars or of the cultivars in high demand on the market. On the other hand, the scientific importance of our research theme consists of acquiring knowledge regarding the morphology, anatomy, physiology of the species studied, in the stages of *in vitro* culture and in the *ex vitro* rooting and acclimatization stage.

II. THEORETICAL CONSIDERATIONS

2.1. A short history of plant *in vitro* culture and micropropagation

This chapter presents, chronologically, the progress in this domain. It is a remarkable fact that early research in plant tissue and organ culture began in the XIX-th century, when some Botanists tried to culture embryos and plant tissues (Gautheret, 1937). Gautheret mentions Van Tieghen (1878), Brown and Morris (1890), Hanning (1904), Buckner and Kastle (1917), Andronescu (1917), Dietrich (1924), Esenbeck and Suessenguth (1925), as well as White (1932), who isolated and cultured immature embryos, sometimes achieving considerable growth (Gautheret, 1937).

It is remarkable that in the '30ies, in Romania, at the University of Cluj, Solacolu and Constantinescu (cited by Gautheret amd by Shi-Tao Yie and Jui-Sen Yang) did *in vitro* cultures of mature embryos from non-germinated seeds (Gautheret 1937, 1957). Also, the first work in the reference list in Gautheret's book from 1937 belongs to Crăciun, who was a researcher of Romanian origin. So, Romanian researchers worked in the domain of plant *in vitro* culture in the interwar period and even during World War I.

The use of *in vitro* cultures for propagating planting material started in 1922, when Lewis Knudson managed the asymbiotic germination of orchid seeds on a culture medium composed of water, mineral salts, sugar and solidified with agar. Knudson's discovery lead to the possibility of massive production of orchids at a relatively low cost, by the technique of *in vitro* seed germination, which is far cheaper than symbiotic germination. The Knudson c culture medium is still used and sold.

In 1962 Toshio Murashige and Folke Skoog published the recipe for a culture medium optimized for the *in vitro* culture of *Nicotiana tabacum* tissues (Murashige and Skoog, 1962), which proved to be suitable for the *in vitro* culture and multiplication of a wide variety of species. Now this medium is used in the majority of micropropagation laboratories and it is adequate for a huge number of herbaceous species and for some woody species. The Murashige & Skoog (MS) culture medium made possible the apparition and development of the industrial scale micropropagation of horticultural species.

Later, some researchers elaborated various culture media for the micropropagation of some woody species: *Prunus* (Quoirin and Lepoivre, 1977), azaleas (Anderson, 1978), *Kalmia latifolia* (Lloyd and McCown, 1981), walnut (Driver and Kunyuki, 1984).

2.2. The current state of research regarding the micropropagation of some fruit and ornamental shrubs

The research regarding the micropropagation of the five species, carried out by several researchers in Romania and abroad, is presented in five subchapters.

Micropropagation of *Amelanchier sp.* was addressed by few researchers. *Amelanchier laevis* was propagated *in vitro* on media supplemented with BAP and NAA. Apical buds were

used as explants and proliferation rates of several tens of shoots per explant were achieved. The shoots were rooted *in vitro* on media supplemented with IBA (Lineberger, 1981).

In *Amelanchier grandiflora*, cv. 'Princess Diana', several types of culture vessels and culture systems were tested. Temporary immersion bioreactors provided the best results regarding the number of shoots/plantlet, plantlet biomass and shoot length (Krueger et al., 1991).

In *Amelanchier alnifolia* cultivars 'Northline', 'Pembina', 'Smoky' and 'Thiessen', Pruski et al. (1990) tested several basal media for initiation and MS medium proved to be optimal. BAP was tested as a growth regulator and optimal results regarding *in vitro* proliferation and plantlet quality were obtained at BAP concentrations close to 2 mg/l.

Micropropagation of *Lonicera sp.* From the papers presented it results that *Lonicera* species and, especially, Lonicera *kamtschatica* are a challenge for micropropagation, due to low proliferation rates and relatively low shoots, as well as due to some unwanted effects (necrosis of the apices, hyperhydricity when using higher BAP concentrations for the stimulation of *in vitro* proliferation), which necessitates further optimization of micropropagation technologies for this species.

Goji (*Lycium barbarum*) micropropagation is a real challenge because no papers were found regarding the *in vitro* multiplication by microcuttings consisting of shoot fragments, in this species.

In vitro cultures were started from seeds. Shoot regeneration from leaf explants by direct organogenesis was carried out (Hu et al., 2001) as well as somatic embryogenesis via callus, starting from root explants (Hu et al., 2008).

Blackberry (*Rubus fruticosus.*) micropropagation was studied by several researchers in Romania and abroad. For multiplication, generally, MS media containing plant growth regulators were used, especially BAP at various concentrations, mainly 1-3 mg/l, auxins (IBA or NAA) at low concentations and GA_3. For *in vitro* rooting, media supplemented with auxins (especially IBA) and with no plant growth regulators were tested (Bobrowski et al.,1996; Erig et al., 2002; Najaf-Abadi and Hamidoghli, 2009; Ružić and Lazić, 2006; Villa et al., 2006, 2009). In species *Rubus laciniatus*, cultivar 'Thornless Evergreen' BAP concentrations lower than 1 mg/l were tested, without the use of auxins and very high multiplication rates were achieved (Fira et al., 2009, a, b; Fira et al., 2010).

The blackberry shoots regenerated *in vitro* can be rooted directly *ex vitro* (Botár and Székely, 1985; Mihalache, 1996; Lepse and Laugale, 2009) in peat and perlite. In blackberry cultivar Gazda, the cytokinin CPPU gave optimal results in the multiplication stage and the shoots were rooted directly *ex vitro* in Jiffy7 pellets (Vescan et al., 2012).

In blackberry cultivars 'Thornless Evergreen' and 'Loch Ness' direct *ex vitro* rooting in hydroculture was achieved, by using tap water as rooting substrate (Fira et al., 2010, 2011).

The micropropagation of some *Vaccinium* species necessitated the use of special culture media elaborated for the *in vitro* propagation of the *Ericaceae* (WPM, Economou & Read, Anderson, Zimmermann & Broome) and less usual cytokinins (zeatin, 2-Ip, TDZ).

Debnath (2011) elaborated an efficient micropropagation protocol for three lowbush blueberry (*Vaccinium angustifolium*) genotypes, on solid as well as liquid media, in RITA temporary immersion bioreactors.

Marcotrigiano and McGlew (1993) achieved *Vaccinium macrocarpon* micropropagation by the use of only two stages: 1) the initiation-multiplication stage in which abundant shoot proliferation was induced on media containing high concentrations of 2-iP and low concentrations of auxins and 2) the *ex vitro* rooting and acclimatization stage, where the shoots regenerated *in vitro* were rooted and acclimatized directly *ex vitro* in various substrates.

2.3. Special techniques used in micropropagation

In this subchapter, some special micropropagation techniques are described, such as the use of bioreactors, the use of static liquid media with or without mechanic support, the chemical sterilization of culture media without autoclavation and sterilization in the microwave oven, the use of alternative gelling agents, the use of synthetic fertilizers as substitutes for MS basal media, the use of alternative sources of carbon and energy; possibilities for *in vitro* conservation are also presented, as well as special techniques of *ex vitro* acclimatization and the role of robotics in micropropagation.

Specialists at CIRAD elaborated the RITA system (Recipient á Immersion Temporaire Automatique or "Container with Automatic Temporary Immersion"), which is a relatively complex system made up of a variable number of plastic bioreactors, which are small, rigid vessels connected to an automated system of compressed air which regulates the delivery of culture media to the explants.

The SETIS system produced in Belgium and the Plantform bioreactor are based on the use of larger plastic bottles and it also operates on the principle of automatic temporary immersion, similar to the RITA system.

Static liquid media gave good results in the *in vitro* germination and plantlet growth in *Doritaenopsis* (Tsai and Chu, 2008), the micropropagation of *Picrorhiza kurroa* (Sood and Chauhan, 2009), *Stevia rebaudiana* (Kalpana et al., 2009).

Teixeira et al. (2006) tested natrium hypochlorite as a sterilant in culture media, in species *Ananas comosus*. The media with minimum 0.0003 % active chlorine gave zero contamination percentage and optimal proliferation rate, of 13.4, higher than in the control sterilized by autoclavation, where proliferation rate was only 6.6. Cardoso and da Silva (2012) tested the sterilization of culture media by using chlorine dioxide, in species *Gerbera jamesonii*. Contamination was zero in the multiplication stage and the treatment with 0.0050 % chlorine dioxide gave optimal results regarding multiplication rates.

Robots are used now at the Vitroplus company for the manipulation of fern plantlets. The Pic-o-Mat robot is used for transferring from the multiplication stage to the *in vitro* rooting stage in the framework of the Vitrotray and Vitroplug technologies and they are also used for *ex vitro* transfer for acclimatization.

The names of the Indian researchers Shashi Babbar and Ruchi Jain (Jain and Babbar 2002, 2011) are linked to an area of research of great practical importance, the use of alternative gelling agents, as they obtained very good results with the Isubgol product (Psyllium husk) and guar gum in the orchid species *Dendrobium chrysotoxum*.

Some researchers tested new, unusual *ex vitro* acclimatization techniques. In species *Curcuma longa*, *ex vitro* acclimatization in vessels containing Hoagland's hydroponic solution was carried out, while high air humidity was maintained (Zapata et al., 2003).

At the Fruit Research Station Cluj several new acclimatization techniques were elaborated, radically different from the usual ones by the fact that they do not necessitate high air humidity. One of the methods is based on culturing the plantlets in vessels containing water (Fira and Clapa, 2009), another one is based on culturing the plantlets in cell trays set to float on the surface of the water in small tubs and the third one consists of culturing either non-rooted shoots or plantlets previously rooted *in vitro*, in a layer of perlite that floats on the surface of the water in a tub (Clapa et al., 2013).

2.4. The practical importance, the advantages and disadvantages of micropropagation

The importance of micropropagation and its main advantages as compared to classical vegetative multiplication are (Cachiță-Cosma, 1987; Stănică et al., 2002):

- The possibility to obtain pathogen-free, especially virus-free plants by meristem culture;
- Immense efficacity and productivity, due to immense multiplication rates, in geometric progression, in several cycles per year;
- Potential role in plant breeding, due to the fact that a valuable selection can be propagated extremely rapidly and then introduced into large scale culture;
- It does not depend on season and weather, due to the fact that it is done in areas with controlled environment;
- Space is economized;
- It offers the possibility for the conservation of valuable genotypes in small spaces;
- It offers the possibility for the easy transportation of a large amount of germplasm independent of phytosanitary quarantine;
- It offers the means for the effective multiplication of some species where propagation by traditional methods is difficult and uneconomical;
- It offers real possibilities for saving and propagating rare plant species.

Micropropagation also has some disadvantages:

- It is more complicated and sophisticated than traditional methods and it requires special training;
- The initial costs for setting up a micropropagation unit are relatively high;
- Special laboratory equipment is needed, as well as expensive and very pure chemicals;
- There is the danger of flooding the market with species and cultivars that are fashionable at a given time or which are easy to propagate *in vitro*, in the detriment of other species and cultivars.

Apart from these, micropropagation has all the advantages and disadvantages of the production of container-grown planting material, because in the great majority of cases the final product, the plants produced by micropropagation consist of container-grown plants. The main advantages are (Stănică et al., 2002):

- The cultural techniques allow for the mechanization and standardization of operations;
- The plants are easier to manoeuvre as compared to the ones cultured in the soil, in the field, they do not need to be taken out of the soil in case of transplanting or sale;
- The container-grown plants can be transported, sold, delivered and planted all year round with the exception of very cold or very hot periods;
- Better use of the terrain due to the very high density of plants per area unit;
- The roots of the container-grown plants are better developed, they form a dense network in the earth ball in the container, which provides very high survival percentages, close to 100 % ;
- The necessity for stratification or storage in special conditions is eliminated.

2.5. Some biological features and the importance of the cultivation of the species of fruit shrubs studied

Amelanchier species (Fam. *Rosaceae*, subfam. *Maloideae*) are ornamental due to their many small white flowers that appear early in spring and their beautifully coloured leaves during autumn. Most species originate from North America. They also produce tasty, edible fruit, rich in vitamin C, minerals and anthocyanins (Jurikova et al., 2012).

Lonicera kamtschatica (Fam. *Caprifoliaceae*) originates from Northern Syberia and the Kamtschatka Peninsula. It is extremely frost-tolerant. *Lonicera* fruit contains significant amounts of vitamins and phenolic compounds which have antioxidant effect (Palikova et al., 2008; Malodobry et al., 2010) and the fruit extracts have great capacity of neutralizing free radicals (Rop et al., 2011).

Lycium barbarum (Fam. *Solanaceae*) has medicinal role. *Lycium barbarum* root bark contains kukoamine (Funayama et al., 1995), which is a hypotensive alkaloid.

The blackberry (Fam. *Rosaceae*, subfam. *Rosoideae* or *Ruboideae*) has great importance as a food crop due to the fruits rich in vitamins, anthocyanins, tannins (Lee et al., 2012). These contain higher amounts of vitamin C than blueberries and apples (The Vitamin C Foundation, 2013) but are superseded by some vegetables.

Blackberry cultivar 'Loch Ness' is one of the best in the world. The fruits have very good flavour and very good quality, this cultivar is very productive, as it was situated on the third place regarding production/plant after cultivars 'Chester Thornless' and 'Black Satin', it has large fruit, as it scored the third place after cultivars Black Butte and Karaka Black (Wójcik-Seliga and Wójcik-Gront, 2013), according to research where a large number of cultivars were tested.

The cranberry (*Vaccinium macrocarpon*, Fam. *Ericaceae*) is remarkable by high phenolics content as compared to other solid or liquid foods. Such, in cranberry juice the concentration of phenolics is about ¼ higher than in grape must or red wine (The Cranberry Institute, 2013). *Vaccinium macrocarpon* fruits also have bacteriostatic and bactericidal effect. The fruit extract had bactericidal effect upon *Escherichia coli* in minced meat (Wu et al., 2009).

III. MATERIAL AND RESEARCH METHODS

This chapter, by its seven sub-chapters describes the species and cultivars studied, the culture conditions, the general procedures in the stages of micropropagation common to the five specis as well as the specific procedures for each species in the *in vitro* culture initiation, stabilization, multiplication, rooting and acclimatization stages.

The experiments were carried out in the period 2010-2013 in the framework of the *In Vitro* Culture Laboratory of the Fruit Research Station Cluj.

The plant material consists of 6 cultivars from 5 species of fruit and ornamental shrubs as follows:

- *Amelanchier canadensis* – Fam. *Rosaceae* - cultivar ‚Rainbow Pillar'.
- *Lonicera kamtschatica* – Fam. *Caprifoliaceae* - cultivars ‚Duet' and ‚Atut'
- *Lycium barbarum* – Fam. *Solanaceae* - cultivar ‚Ning Xia N1'.
- *Rubus fruticosus* – Fam. *Rosaceae* - cultivar ‚Loch Ness'
- *Vaccinium macrocarpon* – Fam. *Ericaceae* - cultivar ‚Pilgrim'.

3.2.Common, general procedures used for all the species studied

The culture media were modified Murashige & Skoog 1962 (MS) (Table 1) for *Amelanchier canadensis*, *Lonicera kamtschatica*, *Lycium barbarum* and *Rubus fruticosus* and Woody Plant Medium, after Lloyd şi McCown (WPM) for *Vaccinium macrocarpon* (Table 2).

The type of vessel used for *in vitro* culture initiation: glass test-tubes, with about 5 ml of medium. For all the species, in the initiation and stabilization stages the culture media were gelled with agar which, due to its transparency, allows for detecting contamination.

The explant disinfection method: treatment with 20 % ACE bleach in sterile deionized water, followed by minimum 4 rinses with sterile deionized water.

For the multiplication stage, in all species glass jars with screw caps with antibacterial filters were used.

Table1. The composition of modified MS basal media

	MSa*	MSa/2**	MSs***	MSsq****
Component	Concentration	Concentration	Concentration	Concentration
MS macroelements	full	reduced to half	full	full
MS microelements	full	full	full	full
FeNaEDTA	36.7 mg/l	36.7 mg/l	36.7 mg/l	-
FeNaEDDHA - Sequestrene 138	-	-	-	100 mg/l
Myo-inositol	100 mg/l	100 mg/l	100 mg/l	100 mg/l
Vitamin B1	1 mg/l	1 mg/l	1 mg/l	1 mg/l
Vitamin B6	0.5 mg/l	0.5 mg/l	0.5 mg/l	0.5 mg/l
Nicotinic acid	0.5 mg/l	0.5 mg/l	0.5 mg/l	0.5 mg/l
Sugar	30 g/l	30 g/l	30 g/l	30 g/l
Plant Agar	6 g/l	6 g/l	-	-
Wheat starch	-	-	50 g/l	50 g/l
pH adjusted to 5.8				

*MSa –modified MS medium, gelled with agar
**Msa/2 - modified MS medium, gelled with agar, half-strength macroelements
**MSs – modified MS medium, gelled with wheat starch
****MSsq - modified MS medium, gelled with wheat starch, Sequestrene 138 as iron source

The most used acclimatization methods for all the species were the two methods elaborated at the Fruit Research Station Cluj: the method of float hydroculture and the method of acclimatization in floating perlite.

Statistical analysis. For statistical analysis, monofactorial ANOVA was used ($p \leq 0.05$). The software was Gnumeric, produced by The Gnome Foundation. In the charts, a, b, c, d represents the statistical significance. Some charts also contain error bars that represent standard errors

Table.2. The composition of modified basal WPM media

	Concentration	
Component	WPM	WPMm
WPM macroelements	Full concentration	Full concentration
WPM microelements	Full concentration	Full concentration
FeNaEDTA	36.7 mg/l	-
FeNaEDDHA	-	100 mg/l
Myo-inositol	100 mg/l	100 mg/l
Vitamin B1	2 mg/l	2 mg/l
Vitamin B6	1 mg/l	1 mg/l
Nicotinic acid	1 mg/l	1 mg/l
Sugar	30 g/l	30 g/l
Plant Agar	5 g/l	5 g/l

In order to elaborate effective protocols for each species, the research methods and experimental treatments were established for each stage of micropropagation (*in vitro* culture initiation and stabilization, *in vitro* multiplication, rooting and acclimatization).

RESULTS AND DISCUSSION

IV. *IN VITRO* PROPAGATION OF SPECIES *AMELANCHIER CANADENSIS*

4.1. *In vitro* culture initiation and stabilization

***In vitro* culture initiation** of *Amelanchier canadensis* was carried out in June, starting from mature plants. The optimal explants type consisted of shoot fragments containing the apical bud.

In the **stabilization stage**, MSa supplemented with 1 or 2 mg/l BAP caused the regeneration of short, underdeveloped axillary shoots; reducing BAP concentration to 0.5-0.7 provided, in the first stages, the intense proliferation of well-developed axillary shoots, 2-5 cm in length. It was noticed that the plantlets regenerated from microcuttings consisting of fragments from the apical part of the axillary shoots were better developed and more vigorous than the ones regenerated from the basal part of the axillary shoots.

4.2. Multiplication

The culture media MSs supplemented with 0.5 or 0.7 mg/l BAP (Fig. 1) provided very high proliferation rates, exceeding 100 shoots/explant.

The optimal explant type in the multiplication stage proved to be the microcuttings consisting of shoot fragments from the apical parts of the shoots, containing the apical bud.

When using whole axillary shoots 4-5 cm in length immersed in oblique position into the mass of MSs culture media, two explants/culture vessel proved to be the optimal number, while the optimal BAP concentration in this case was 0.3 mg/l, as this provided the regeneration of well-developed, normal shoots and the highest percentage of standard-sized shoots, minimum 2 cm in length.

Fig. 1. *Amelanchier canadensis* on MSs medium with 0.7 mg/l BAP, from explants from the apical part of the shoots: A – culture vessel, B – plantlets transferred *ex vitro*, C- shoots regenerated from one plantlet

Among other types of cytokinins tested, BAR and mT proved to be adequate for the *in vitro* multiplication of *Amelanchier canadensis*, as well as 2-iP at the concentrations of 10 and 20 mg/l, but 2-iP provided lower multiplication rates as compared to BAP. Kinetin proved not to be suitable, as it did not provide viable plantlets. The recommended BAR concentration is 0,48 mg/l.

All the alternative gelling agents (fibre agar, Psyllium husk, guar gum, mixture of wheat starch and Phytagel) provided good results. Phytagel at the concentration of 2.2 g/l was not adequate, as it caused the regeneration of poorly developed, hyperhydric plantlets. Spectacular results were obtained with guar gum at 20 g/l (Fig. 2), which provided intense proliferation of thin but very well developed, lignified axillary shoots, more than 90 % of which got rooted in floating perlite. The proliferation rate provided by guar gum was by far the highest, 108.1 shoots/explant, followed by the one provided by the media gelled with starch, 86.4 shoots/explant (Fig. 3).

Table 3. The results regarding the influence of BAP, BAR and mT upon *in vitro* proliferation in *Amelanchier canadensis* – average number of shoots/plantlet

Treatment*	Total no. of shoots < 2 cm	No. of shoots 2-3.9 cm in length	No. of shoots 4-5.9 cm in length	No. of shoots ≥6 cm	Proliferation rates
A1. 0.3 mg/l BAP	0	29.4	9.5	0.8	39.7
A2. 0.5 mg/l BAP	0	32	9.8	1.5	43.3
A3. 0.7 mg/l BAP	**119.3**	47.2	4.8	0.5	52.5
A4. 1 mg/l BAP	35.2	55.9	12.2	0.9	62
A1'. 0.48 mg/l BAR	10.9	25.6	7	0.6	33.2
A3'. 1.11 mg/l BAR	49.3	68.4	16.8	3.2	88.4
A4'. 1.59 mg/l BAR	80.3	84.4	14.8	0.6	**99.8**
A1". 0.32 mg/l MT	2.4	3.3	0.9	0	4.2
A2". 0.54 mg/l MT	1.7	4.8	22	1	8
A3". 0.75 mg/l MT	7.2	15.3	8	2.2	25.5
A4". 1.07 mg/l MT	164	**157**	**49**	**12**	21.8

*The differences between A1" and A2" regarding proliferation rates were statistically significant, as well as the ones between A2" and A4" ($p<0.05$).

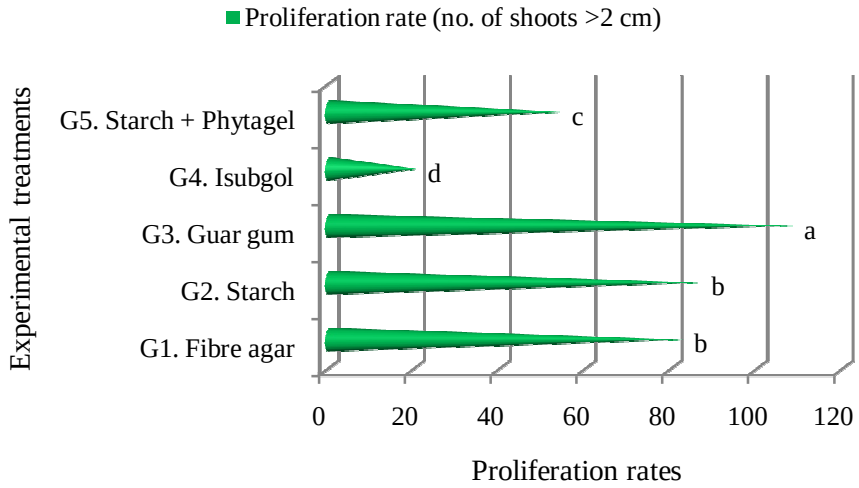


Fig. 2. The results regarding the *in vitro* proliferation of *Amelanchier canadensis* under the influence of various gelling agents

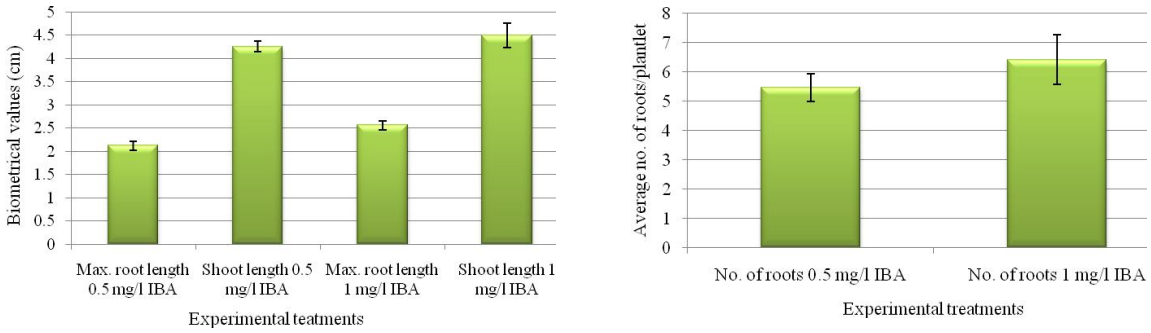
Fig. 3. *Amelanchier* cultured on media supplemented with: A, A1) fibre agar; B) Phytagel; C, C1, C2) wheat starch; D, D1, D2) wheat starch + Phytagel; E, E1, E2) Guar gum; F, F1, F2) Psyllium husk

4.3. Rooting and acclimatization

For ***in vitro* rooting** in *Amelanchier canadensis* the optimal culture medium was MSs supplemented with 0.5 mg/l IBA, as this provided rooting percentages of more than 90 % and well-developed plantlets and roots. Fig. 4 presents information regarding the biometrical characteristics of the regenerated plantlets.

Ex vitro acclimatization in float hydroculture gave good results, more than 80 % acclimatization percentages in the plantlets that were rooted on MSs supplemented with 0.5 mg/l IBA.

***Ex vitro* rooting in floating perlite** in *Amelanchier canadensis* proved to be extremely efficient, with rooting percentages of more than 90 % in most cases, such eliminating the necessity for *in vitro* rooting. For direct *ex vitro* rooting in floating perlite, it is recommended to use shoots excised from plantlets that were cultured for three months on the multiplication media.



A B

Fig. 4. The results regarding stem length, the length of root clumps (A) and the number of main roots/plantlet (B) in *Amelanchier canadensis* rooted in MSs media supplemented with IBA

V. *IN VITRO* PROPAGATION OF *LONICERA KAMTSCHATICA*

5.1. *In vitro* culture initiation and stabilization

In the initiation stage the optimal explants type were the apices excised from the apical buds. The culture media used for *Lonicera kamtschatica in vitro* culture initiation were MSa/2 or WPM supplemented with 0.5 mg/l BAP, gelled with agar.

For ***in vitro* culture stabilization** and inducing proliferation MSs media supplemented with 0.5 mg/l BAP were tested. Due to evidence of chlorosis, MSsq was tested (the iron source FeNaEDTA was substituted with 100 mg/l Sequestrene 138 - FeNaEDDHA). After transfer to this medium, the plantlets became healthier in appearance, bright green, chlorosis was reduced.

5.2. Multiplication

In the multiplication stage, among the cytokinins tested, BAP proved to be usable at 1 mg/l, but it provided mediocre multiplication rates (5.53 microcuttings/initial explant in cultivar ‘Atut’) whereas CPPU at 0.5 and 1 mg/l proved to be more effective. Meta-topolin and zeatin are

not recommended for the multiplication of this species, their addition to the media gave low multiplication rates (Table 5) and generated calli at the plantlets' base (Fig. 5).

It was found that the type of inocula influences multiplication rates in *Lonicera kamtschatica*. Such, in the multiplication stage, the microcuttings consisting of shoots or shoot fragments of minimum 2 cm with 3 nodes, 5-6 microcuttings/culture vessel gave good results (Table 4) and, also, the lateral shoots with 1-2 nodes and apical bud proved to be very viable explants for *in vitro* multiplication (Table 6).

For gelling the culture media for the multiplication stage it is recommended to use 50 g/l wheat starch, as the media gelled with Plant Agar (MSa) proved to be inadequate for the multiplication stage.

Table 4. The influence of explant type upon *in vitro* multiplication in *Lonicera kamtschatica*, cultivar 'Atut'

Treatment	No. of shoots/ vessel	Statist. signif.	No. of inocula/ vessel	Statist. signif.	Average height (cm)	Statist. signif.	Average PR	Statist. signif.	Average MR	Statist. signif.
V1-control	40		48.14		6.557		8		9.62	
V2	60.42	*	73.57	**	8.04	n. s.	12.09	**	14.71	***
V3	38.8	n. s.	50.4	n. s.	5.89	*	7.76	*	10.08	n. s.

n. s. – not significant statistically, * significant statistically ($p \leq 0.05$),** distinctly significant statistically ($p<0.01$), *** very significant statistically ($p<0.001$)

V1-3 node fragments (control); V2-short shoots 3-5 nodes; V3-whole shoots, 5 cm long

Table 5. The results regarding *in vitro* multiplication in *Lonicera kamtschatica*, cultivar 'Atut' under the influence of various cytokinins (average values per experimental treatment)

Treatment	No. of shoots/ vessel	Statist. signif.	No. of inocula/ vessel	Statist. signif.	Average PR	Statist. signif.	Average MR	Statist. signif.	Viable plantlets (%)	Statist. signif.
V1 (control)	17.5		27.66		3.5		5.53		100	
V2	9	00	16.66	00	1.8	000	3.33	000	100	n. s.
V3	6	00	8.33	0	1.2	000	1.66	000	90	0
V4	42.33	n. s.	50.83	n. s.	8.46	*	10.16	*	90	*
V5	58.83	n. s.	71.83	n. s.	11.76	**	14.36	*	66.66	n. s.

n. s. - not significant statistically, *significant statistically ($p \leq 0.05$),**distinctly significant statistically ($p<0.01$), – for values higher as compared to the control treatment; 0 - significant statistically ($p \leq 0.05$), 00 - distinctly significant statistically ($p<0.01$), 000 - very significant statistically ($p<0.001$) - for values lower as compared to the control treatment

(V1 -1 mg/l BAP (control); V2 -1 mg/l mT; V3-1 mg/l ZEA; V4-0.5 mg/l CPPU ; V5-1 mg/l CPPU)

Fig. 5. *In vitro* cultures of *Lonicera kamtschatica*, cultivar 'Atut': A, A1-1 mg/l BAP; B, B1 – 1 mg/l zeatin; C, C1, C2 – on media supplemented with 1 mg/l CPPU

The intense proliferation of axillary shoots is provided by the presence of cytokinin CPPU in the culture media gelled with wheat starch. The optimal concentration of CPPU proved to be 0.7 mg/l (Table 6). This concentration provided optimal proliferation and multiplication rates, exceeding 10 microcuttings resulted/plantlet, in both cultivars ('Atut' and 'Duet'), in both explant types we tested: in the standard microcuttings of minimum 2 cm with 3 nodes, as well as in the short lateral shoots with 1-2 nodes and apical bud.

Table 6. The results regarding the *in vitro* proliferation of *Lonicera kamtschatica* axillary shoots under the influence of the two adequate CPPU concentrations

Treatment	Plantlet height (cm)	Statist. signif.	No. of shoots/ vessel	Statist. signif.	No. of inocula/ vessel	Statist. signif.	PR	Statist. signif.	MR	Statist. signif.
V1	9.16		38		48		7.6		9.6	
V2	6.74	**	80.29	**	94.85	**	16.06	***	18.97	***
V3	5.28		30.71		35.85		6.14		7.17	
V4	6.12	n. s	62.85	**	68.57	*	12.57	***	13.71	***
V5	7.78		36.42		45.71		7.28		9.14	
V6	8.21	n. s	56.57	n. s	71	n. s	11.31	n. s	14.2	*
V7	7.46		29		37.85		5.8		7.57	
V8	7.26	n. s	53	**	63.85	**	10.6	*	12.77	*

n. s. - not significant statistically, *significant statistically ($p \leq 0.05$), **distinctly significant statistically ($p<0.01$), *** very significant statistically ($p<0.001$)
(V1: Atut, 0.5 mg/l CPPU, microcuttings (3 node shoot fragments); V2: Atut, 0.7 mg/l CPPU, microcuttings; V3: Duet, 0.5 mg/l CPPU, microcuttings; V4: Duet, 0.7 mg/l CPPU, microcuttings; V5: Atut, 0.5 mg/l CPPU, short lateral shoots (1-2 nodes + apical bud); V6: Atut, 0.7 mg/l CPPU, short lateral shoots; V7: Duet, 0.5 mg/l CPPU, short lateral shoots; V8: Duet, 0.7 mg/l CPPU, short lateral shoots).

5.3. Rooting and acclimatization

***Ex vitro* rooting in floating perlite** in *Lonicera kamtschatica* was not satisfactory at the beginning due to the small percentages of viable plants. In cultivar Atut, from the total number of 817 shoots planted into the floating perlite only 475 got rooted, that is 58.14 %, whereas in cultivar Duet the rooting percentage was 53.91 %.

For the *ex vitro* rooting and acclimatization stage of *Lonicera kamtschatica* shoots in floating perlite we recommend the use of 1 mg/l IBA in both cultivars, Atut and Duet. This auxin provided rooting and acclimatization percentages exceeding 60 %.

VI. *IN VITRO* PROPAGATION OF SPECIES *LYCIUM BARBARUM*

6.1. *In vitro* culture initiation and stabilization

In the *in vitro* culture initiation stage in Goji berry cultivar ‘Ning Xia N1’ by aseptic seed germination, the germination rate was 50 %. No contamination was noticed. The plantlets grew 5-8 cm in one month.

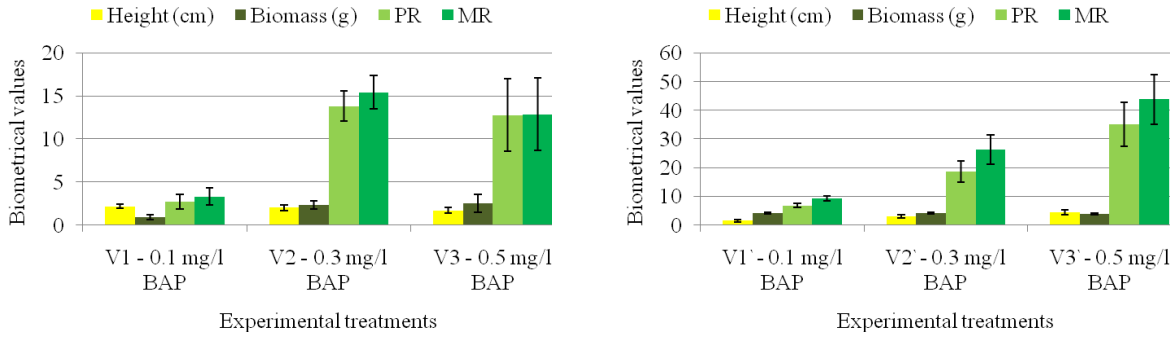
In the plant material obtained in the initiation stage and then subcultured on hormone-free MSa the explants containing the apical bud grew and generated well-rooted plantlets, up to 10 cm in height, whereas the microcuttings consisting of 2-3 node fragments generated leser developed plantlets, with short shoots (1-3 cm in lngth), reddish in colour. On the hormone-free MSs media, from each microcutting (with or without apical bud) a main shoot exceeding 10 cm grew, together with 1-5 shorter secondary shoots. The average proliferation rate was 3.95 and there was 100 % rooting. Due to their quality, the plantlets cultured on hormone-free MSs were subsequently used for starting *in vitro* multiplication experiments.

6.2. Multiplication

The results obtained in the experiments of *Lycium barbarum in vitro* multiplication show that wheat starch as a gelling agent had a crucial role for the success of the *in vitro* multiplication stage. BAP at the concentration of 0.3 mg/l proved to be optimal in this stage, as it provided high

proliferation and multiplication rates and well-developed shoots; the optimal explants were four-node shoot fragments (Fig. 6). V1, V2, V3 represent the two-node microcuttings, respectively MSs + 0.1 mg/l BAP; MSs +0.3 mg/l BAP; MSs +0.5 mg/l BAP. V1`, V2` and V3` represent the treatments with four-node microcuttings, respectively MSs + 0.1 mg/l BAP; MSs +0.3 mg/l BAP; MSs +0.5 mg/l BAP. The explants inserted vertically into the culture media provided more intense proliferation than the ones placed horizontally on the surface of the media (Table 7).

In case of cyclic multiplication on MSs+ 0.3 mg/l BAP, good results were obtained with either 2 cm long microcuttings (4 explants/culture vessel) or 4 cm long explants (2 inocula/culture vessel). The latter could be inoculated in several ways, ensuring good results in all the variants: placed horizontally onto the media, inserted in oblique position, inserted vertically. Figure 7 presents plantlets regenerated from long shoots.



A B

Fig. 6. The influence of various concentrations of BAP upon the *in vitro* proliferation and multiplication in *Lycium barbarum*, in the experimental series with two types of microcuttings: A – two-node microcuttings; B – four-node microcuttings.

V1, V2, V3 represent the two-node microcuttings, respectively MSs + 0.1 mg/l BAP; MSs +0.3 mg/l BAP; MSs +0.5 mg/l BAP. V1`, V2` and V3` represent the treatments with four-node microcuttings, respectively MSs + 0.1 mg/l BAP; MSs +0.3 mg/l BAP; MSs +0.5 mg/l BAP

Table 7. The results regarding *in vitro* multiplication in the two experimental treatments: inocula placed horizontally and inocula inserted vertically (average values/treatment)

Treatment	Plantlet height (cm)	Biomass (g)	Proliferation rates	Multiplication rates
Hs-horizontal	2.99	2.74	16.94	21.22
V-vertical	5.07***	5.72***	32.47**	46.56***

*** Differences very significant statistically ($p<0.001$); ** differences distinctly significant statistically ($p<0.01$)

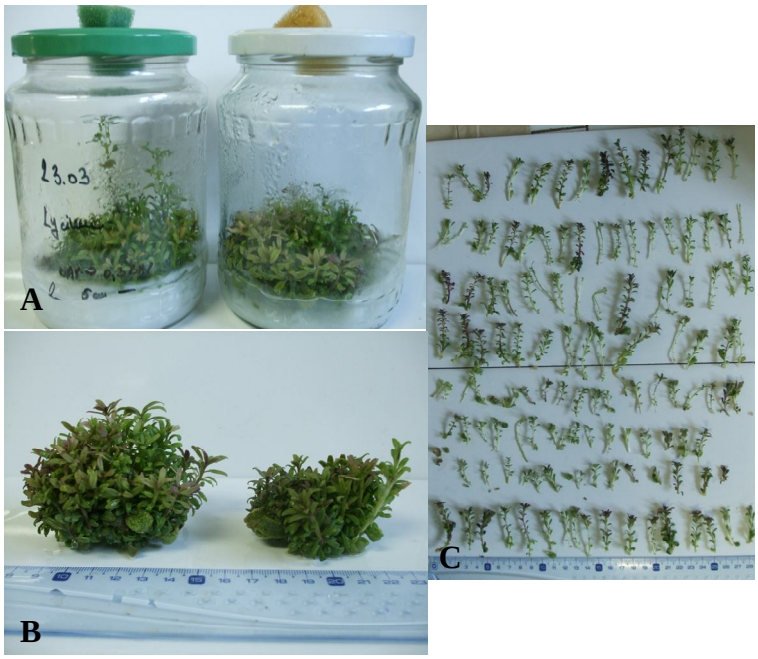


Fig. 7. Cultures of 5 cm long shoots: A – culture vessel, B- plantlets transferred *ex vitro* from the vessel, C – shoots that resulted from a single plantlet

6.3. Rooting and acclimatization

Lycium. barbarum is a species very suitable for *in vitro* rooting. The culture media for *in vitro* rooting was hormone-free MSs. The explants consisted of 1.5-2 cm long shoots or shoot fragments resulted from the multiplication stage on MSs + 0.3 mg/l BAP. It is recommended to use 40 microcuttings/culture vessel, as this treatment provided better developed plantlets as compared to the treatment with 60 microcuttings/vessel (Fig. 8). Fig. 9 represents cultures with 60 microcuttings/vessel. The duration of the *in vitro* rooting stage was 3 weeks, there was more than 90 % rooting and the plantlets were well developed, several cm in height, with a rich root mass.

The plantlets rooted *in vitro* were subsequently acclimatized *ex vitro* in float hydroculture in floating cell trays. *Ex vitro* acclimatization was 94.07 % and the plantlets had 90 % survival after potting.

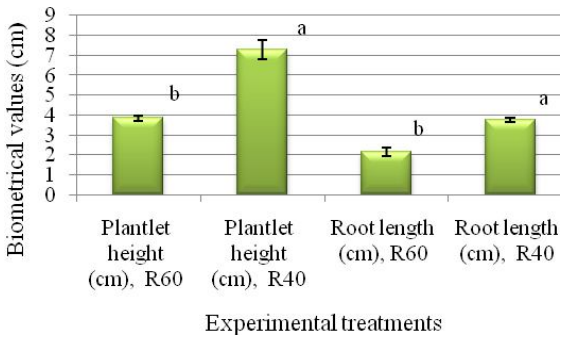


Fig. 8. The results regarding the size of the *Lycium barbarum* plantlets rooted *in vitro*: R60 – the treatment with 60 microcuttings/vessel; R40 – the treatment with 40 microcuttings/vessel

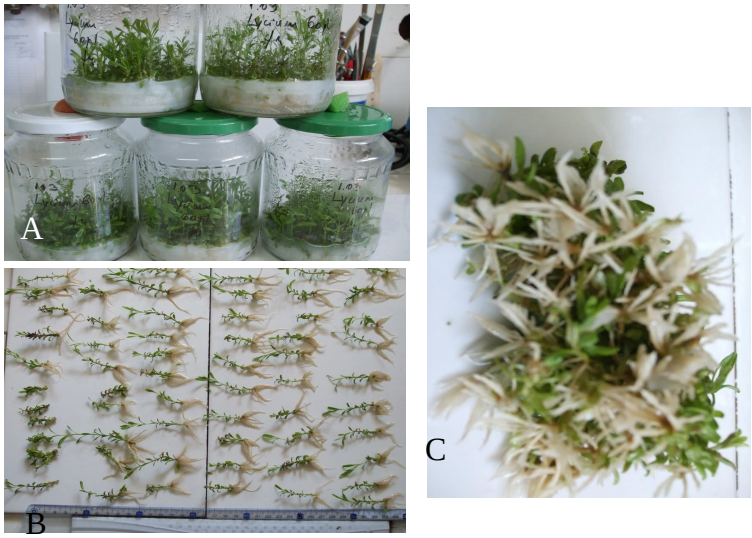


Fig. 9. *Lycium barbarum* rooted *in vitro*: A – culture vessels with 60 microcuttings; B and C – plantlets rooted *in vitro* and transferred *ex vitro*

As an alternative to *in vitro* rooting and subsequent *ex vitro* acclimatization, **direct *ex vitro* rooting in Jiffy7 pellets** was applied to the shoots resulted in the multiplication stage. The shoots were separated and 3-5 mm long whitish portions from the base of the shoots were excised. Then the small leaves were eliminated from a portion of 4-5 mm from the shoot bases. The microcuttings prepared in this way were cultured in Jiffy7 pellets in Multi Purpose Tubs and air humidity was kept by covering the cultures with the same type of vessels. *Ex vitro* acclimatization was carried out both in the growth room and greenhouse and in both places rooting percentages were more than 90 %.

VII. *IN VITRO* PROPAGATION IN SPECIES *RUBUS FRUTICOSUS*

7.1. *In vitro* culture initiation and stabilization

The use of axillary buds from greenwood primocanes (in April-September) provides the success of the initiation stage in blackberry cultivar ‘Loch Ness’. The axillary buds cultured on MSa + 0.7 mg/l BAP had 71.42 % regeneration percentage. The shoots transferred onto fresh MSa media regenerated well-developed and well-proliferated plantlets, with vigorous shoots.

In the *in vitro* culture **stabilization** stage the proliferation rates slightly exceeded 20 and the number of shoots/vessel was around 150 on MSa + 0.5 mg/l BAP.

7.2. *In vitro* multiplication

In the *in vitro* multiplication stage several experimental treatments were applied in order to established the optimal culture media, the culture vessels suitable for this stage, the adequate types of microcuttings, as well as the gelling agent that should provide economical proliferation rates and a high number of shoots suitable for acclimatization. The possibility of chemical sterilization by the use of NaDCC as an alternative to media autoclavation was also tested.

MSm media supplemented with 0.5 mg/l BAP and gelled either with wheat starch or agar proved to be very effective for the multiplication of blackberry cultivar ‘Loch Ness’.

The optimal explant type in the multiplication stage proved to be the microcuttings consisiting of shoots or shoot fragments 2 cm in length, with 4-5 nodes, whereas the fragments with 1 or 2 nodes were inadequate (Table 8 and Fig. 10). The optimal number of inocula/vessel

was 4. A higher number of inocula/vessel causes over-population and non-uniform growth and proliferation.

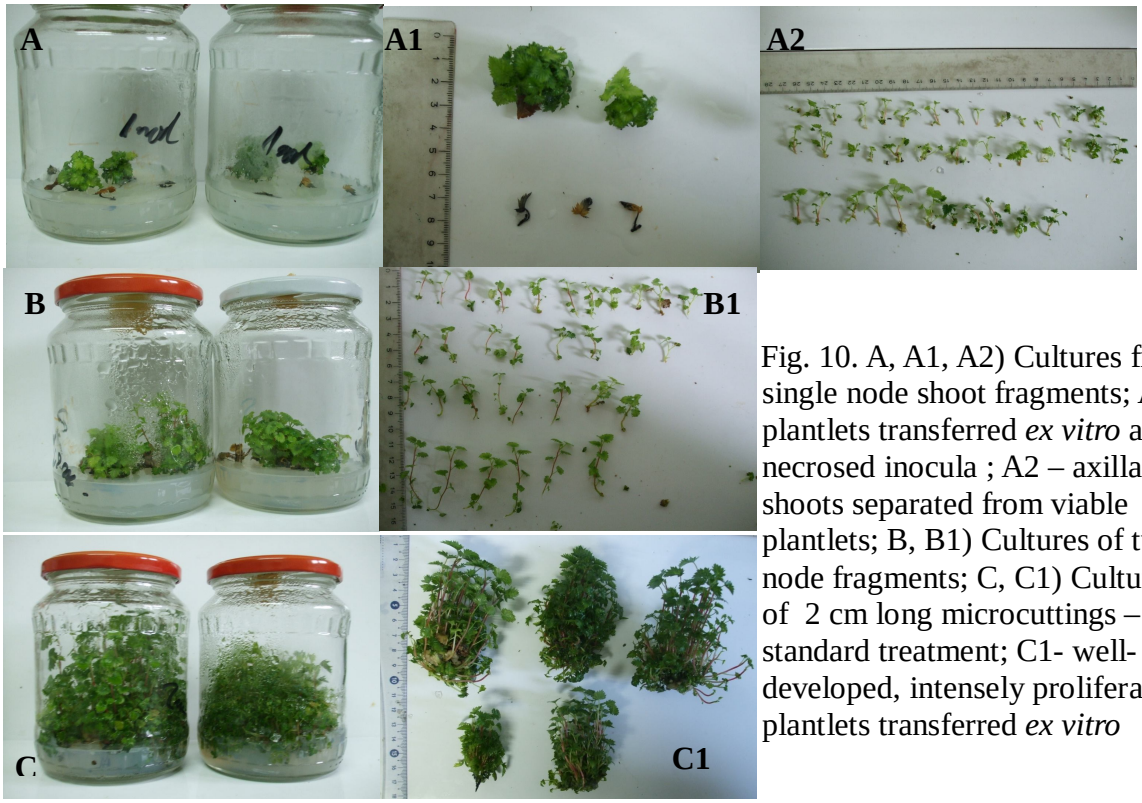


Fig. 10. A, A1, A2) Cultures from single node shoot fragments; A1- plantlets transferred *ex vitro* and necrosed inocula ; A2 – axillary shoots separated from viable plantlets; B, B1) Cultures of two node fragments; C, C1) Cultures of 2 cm long microcuttings – the standard treatment; C1- well-developed, intensely proliferated plantlets transferred *ex vitro*

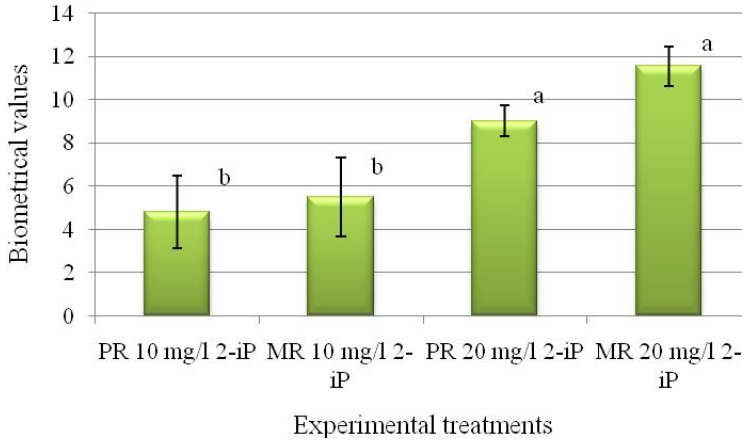
The position of the explant in the culture media had an important role in the multiplication stage. When the microcuttings were inserted vertically into the media gelled with 50 g/l wheat starch the proliferation and multiplication rates were superior as compared to the treatments where the microcuttings were placed horizontally onto the media.

Table 8. The results regarding the influence of explant type upon the *in vitro* proliferation rates in blackberry cultivar 'Loch Ness'

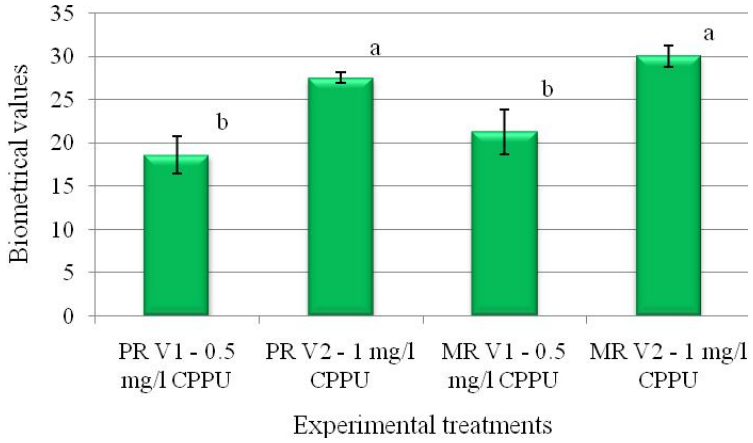
Treatment	Viable plantlets/ vessel (%)	Statist. Signif.	No. of shoots/ vessel	Statist. Signif.	No. of shoots/ plantlet	Viable shoots/ vessel *	Statist. Signif.	Viable shoots/ plantlet *
V1	45.71	000	28.42	000	6.6	4.42	000	0.89
V2	48.57	000	29.28	000	6.29	6.14	000	1.23
V3 (control)	100		234.42		46.89	234.42		46.89

*Shoots suitable for *ex vitro* rooting and acclimatization
V1- single-node explants ; V2- two-node explants; V3- 2 cm long microcuttings (control)
000 – differences very significant statistically in a negative sense as compared to the control ($p<0.001$)

The cytokinin that gave optimal results regarding *in vitro* growth and proliferation was BAP at 0.5 mg/l. Increasing BAP concentration to 0.7 mg/l increased multiplication rates but also caused deformations. The cytokinins CPPU and TDZ proved to be ineffective, as they yielded deformed plantlets. Meta-topolin provided lower multiplication rates as compared to BAP and the plantlets were deformed. Figure 11 presents information regarding multiplication on media with 2-iP and CPPU.



A



B

Fig. 11. *In vitro* multiplication in blackberry cultivar 'Loch Ness' under the influence of 2-iP (A) and CPPU (B)

For gelling the culture media, the following gelling agents were tested: Fibre agar, 6.8 g/l; Plant Agar, 6 g/l; wheat starch, 50 g/l; rice starch, 50 g/l; guar gum 20 g/l; Isubgol (Psyllium husk), 15 g/l; Phytagel, 2.2g/l; wheat flour 50 g/l and the combination wheat starch, 50 g/l + Phytagel, 500 mg/l.

Among the gelling agents tested, Plant Agar and wheat starch proved to be efficient, the most adequate being wheat starch at 50 g/l, having in view the optimal proliferation and multiplication rates, the lack of plantlet hyperhydricity and low cost. Adding 500 mg/l Phytagel to the starch did not have beneficial effect. Increasing starch concentration to 60 g/l was not beneficial, either. Guar gum at 20 g/l provided intense growth and proliferation, being a potential alternative to the use of starch as gelling agent. Figure 12 presents information regarding multiplication of blackberry cultivar 'Loch Ness' on media with alternative gelling agents.

All the explants cultured on liquid MSm media supplemented with 0.5 mg/l BAP underwent necrosis in two weeks.

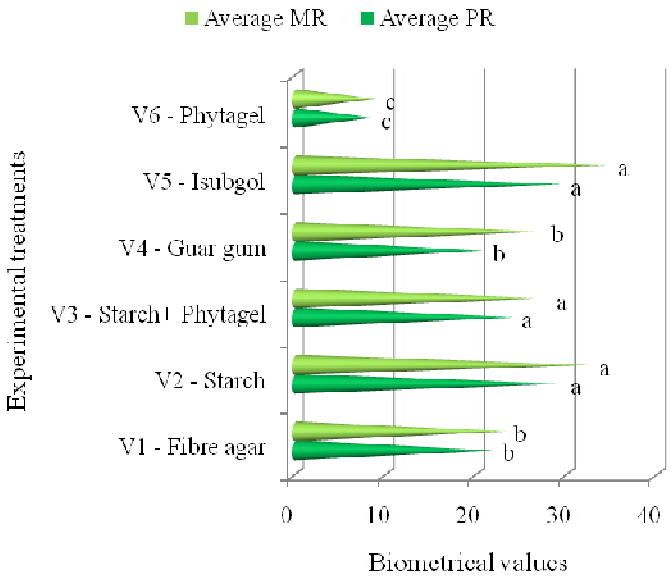


Fig. 12. Results regarding the proliferation and multiplication rates on media gelled with alternative gelling agents

In order to establish **the optimal type of culture vessel** for blackberry micropropagation, the following vessel types were tested: 720 and 320 ml glass jars, as well as three types of plastic vessels: Fresco, Jenny, round Multi Box vessels and lenticular vessels of various sizes. The best results were obtained by using 720 ml jars with vented screw caps (Table 9).

Table 9. The results regarding *in vitro* multiplication in various types of vessels (average values)

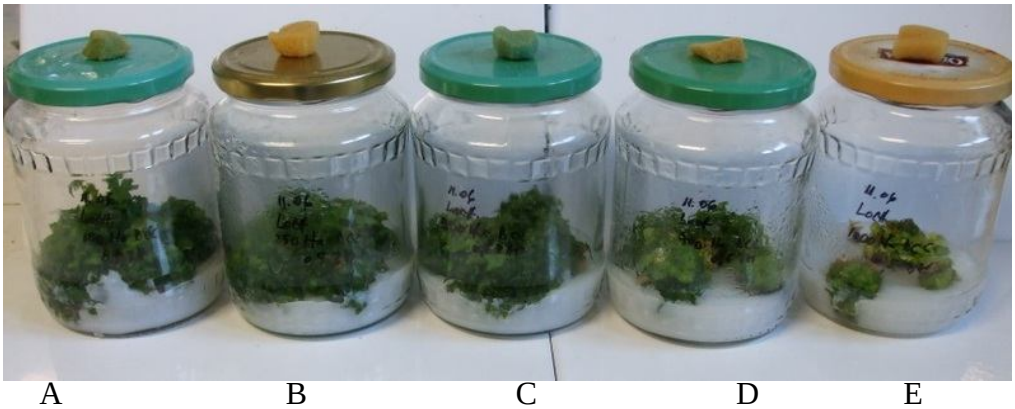
Treatment	Biomass / vessel (g)	No. of shoots/ vessel	No. of inocula resulted/vessel	Biomass/ plantlet	PR	MR
B 720 (control)	27.93	230.5	388	5.59	46.1	77.6
VF 500 ml	24.61	163.5	270.5	4.92	32.7	54.1
VF 800 ml	29.43	174.5	252	5.89	34.9	50.4
B 320	9.55	71	104	1.91	14.2	20.8

In order to evaluate the possibility for chemical sterilization as an alternative to the autoclavation of culture media, 12 experimental treatments using media with various concentrations of NaDCC were tested. The media were gelled with wheat starch. In the treatments with 5, 10 and 15 mg/l NaDCC there were high contamination percentages. The contaminations occurred progressively, during the whole culture period. In the treatment with 5 mg/l NaDCC there was 70 % contamination, in the one with 10 mg/l NaDCC there was 50 % contamination, whereas in the treatment with 15 mg/l NaDCC there was 60 % contamination.

In the treatments with 150-1000 mg/l NaDCC contamination was zero. Fig. 13 presents blackberry cultures on media with 150-1000 mg/l NaDCC.

NaDCC at high concentrations (150-1000 mg/l) prevented contamination but it had

inhibitory effect regarding plantlet height (Fig. 14). The treatments with 150, 250 and 500 mg/l NaDCC presented proliferation and multiplication rates superior to that of the control, whereas the treatments with 700 and 1000 mg/l NaDCC had inhibitory effect upon *in vitro* proliferation (Fig. 15). There was also a strong hyperhydricity and deformation and the number of viable plantlets regenerated/vessel decreased and such the plantlets could not be acclimatized *ex vitro*.



A B C D E

Fig. 13. 'Loch Ness' blackberry cultured on media sterilized with NaDCC: A- 150 mg/l; B- 250 mg/l; C – 500 mg/l; D – 700 mg/l; E – 1000 mg/l

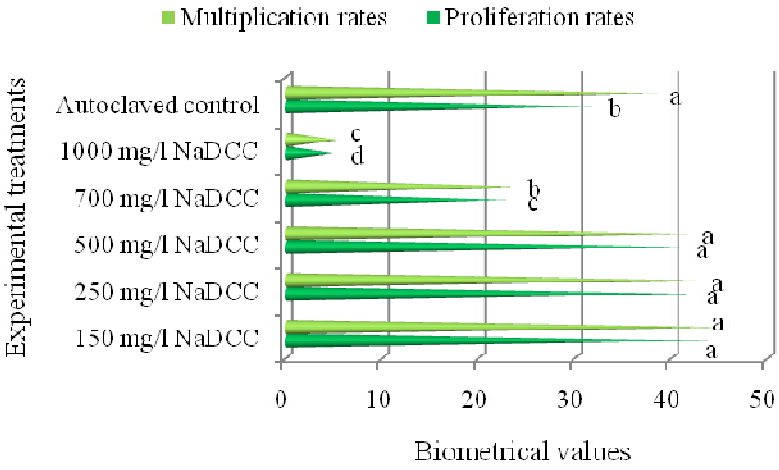


Fig. 14. Results regarding the proliferation and multiplication rates 'Loch Ness' blackberry on media sterilized with NaDCC

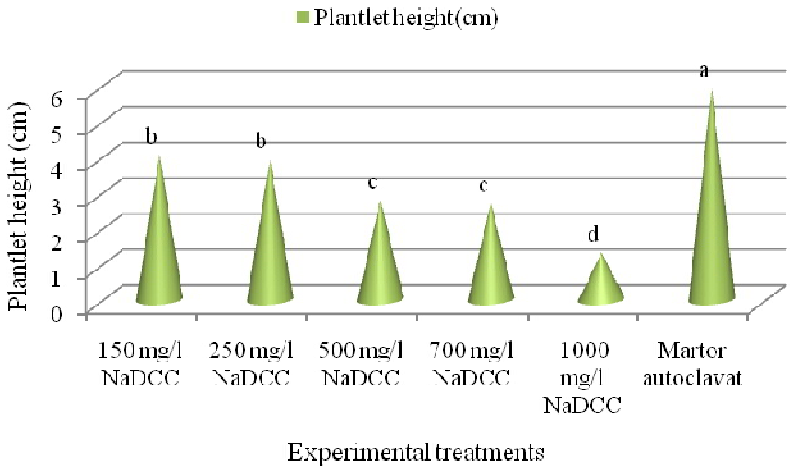


Fig. 15. Results regarding plantlet height in 'Loch Ness' blackberry on media sterilized with NaDCC

7. 3. Rooting and acclimatization in *Rubus fruticosus*, cultivar 'Loch Ness'

The axillary shoots regenerated *in vitro* were easily rooted *ex vitro*, by conventional methods (the use of solid substrates combined with ensuring high air humidity) as well as by the new, radically different *ex vitro* acclimatization methods elaborated at the Fruit Research Station Cluj.

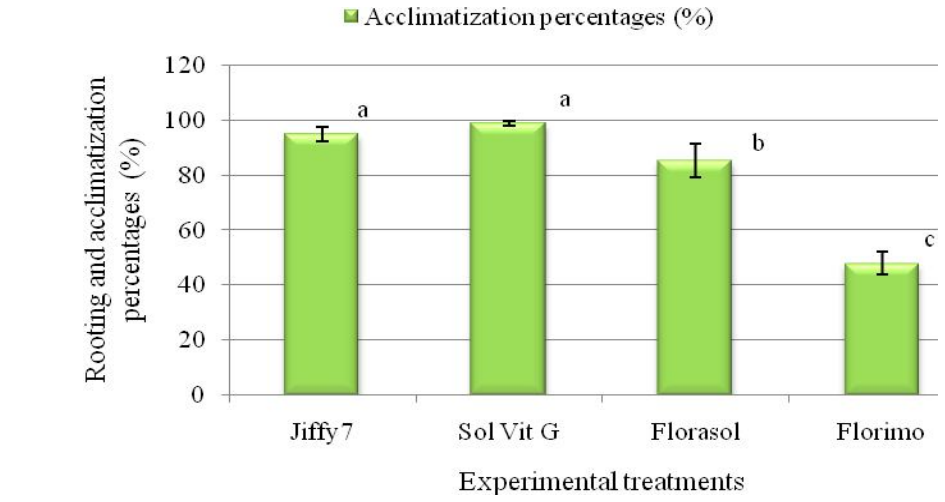
***Ex vitro* rooting and acclimatization in float hydroculture** (Fig. 16) of the shoots obtained in the multiplication stage provided rooting and acclimatization percentages of more than 70 % when applying it in the greenhouse, in the growth room or in open air in conditions of semi-shade, without ensuring high air humidity. It is recommended that the small shoots (1.5-2.5 cm) should be planted apart from the large shoots (3-5 cm) into cell trays with cells of 1 cm diameter.

Fig. 16. 'Loch Ness' blackberry rooted *ex vitro* in float hydroculture:
A – plants in hydroculture; B – bunches extracted from the cells; C- rooted plants separated from the bunches

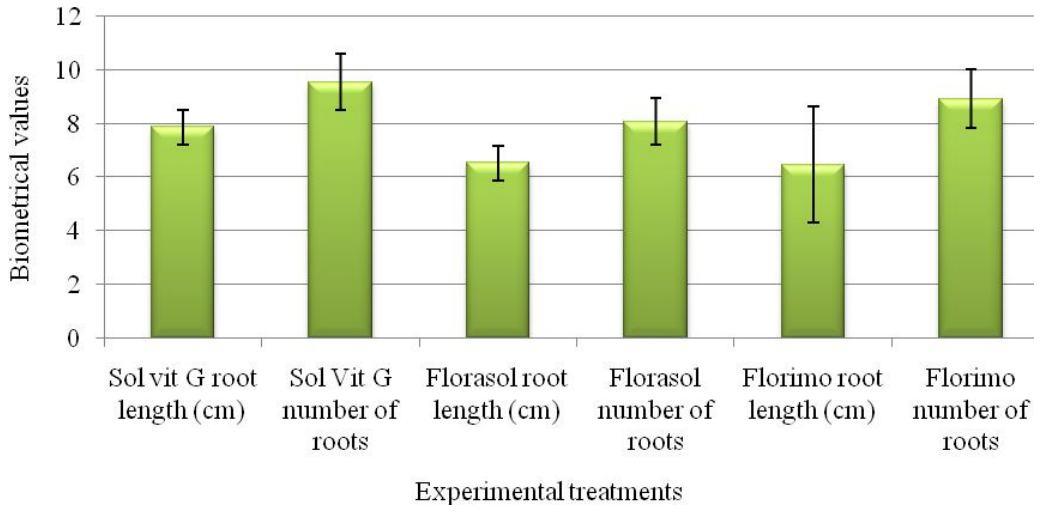
***Ex vitro* rooting and acclimatization in floating perlite** was very efficient in blackberry cultivar 'Loch Ness' and we recommend planting the shoots individually, one by one, as this provided rooting and acclimatization percentages of more than 90 %.

Among the **solid substrates**, Jiffy7 pellets, Sol Vit G (Fig. 17) and peat + perlite mixture

gave rooting and acclimatization percentages of more than 90 % and we recommend the use of peat + perlite mixture, having in view its availability, low cost and proven efficiency.



A



B

Fig. 17. The acclimatization percentages in the treatments regarding various potting mixes (A) and the biometrical characteristics studied (B)

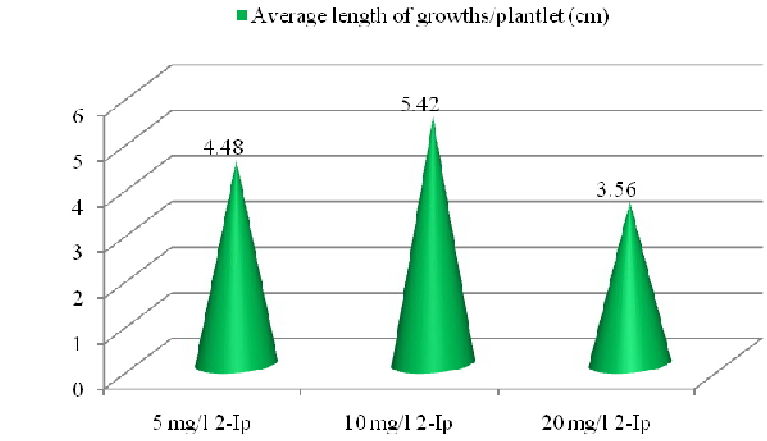
Float hydroponics has the potential to become an effective alternative to regular potting practices. In order to increase the size of plants, the complex Ferticare fertilizer at 2 g/l concentration was used and for this purpose it is recommended to use plants rooted and acclimatized in Jiffy7 pellets.

VIII. *IN VITRO* MULTIPLICATION IN SPECIES *VACCINIUM MACROCARPON*

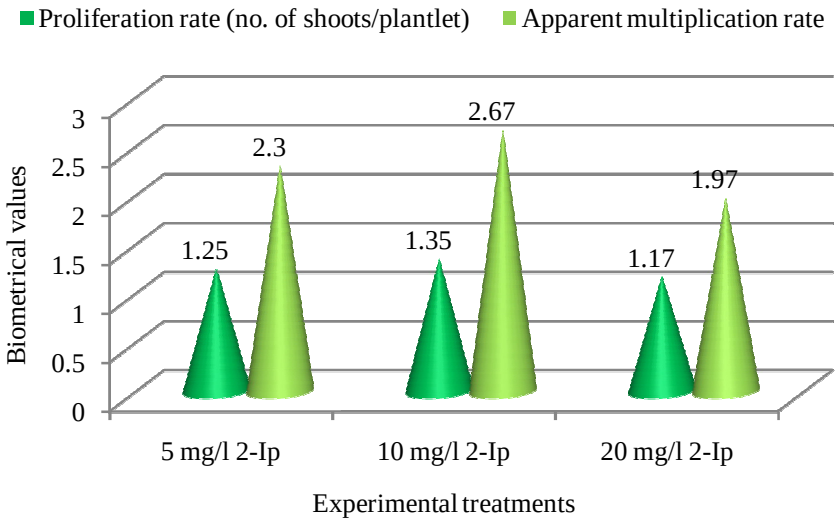
8.1. *In vitro* culture initiation and stabilization

***In vitro* culture initiation.** Our preliminary studies regarding *Vaccinium macrocarpon in vitro* culture initiation showed that the explants consisting of excised axillary buds yielded the greatest losses due to necrosis. Single-node fragments also provided poor results. For *in vitro*

culture initiation, multiple-node shoot fragments with intact leaves are recommended as explants, as the contamination percentages are low and the axillary shoots grow to large sizes in a short time and practical results are obtained in two months of culture. Increasing the concentration of 2-iP did not increase proliferation (Fig. 18).



A



B

Fig. 18. The results regarding *in vitro* growth and proliferation in the initiation stage in *Vaccinium macrocarpon*, under the influence of 2-iP: A - The average lengths of shoots/plantlet; B - The proliferation and multiplication rates on the three variants of initiation media

8.2. *In vitro* multiplication

For the multiplication stage, 720 ml glass jars with vented screw caps with antibacterial filter are recommended, with 100 ml medium/jar and 15 microcuttings/jar.

For *Vaccinium macrocarpon* cultivar Pilgrim the optimal culture medium proved to be Woody Plant Medium (after Lloyd and McCown) gelled with agar. Wheat starch and guar gum proved to be ineffective at the concentrations tested (Fig. 19 and 20, respectively), whereas the static liquid media without mechanical support gave results similar to the ones with 2-iP gelled

with agar, such the liquid media are potential alternatives to the media gelled with agar.

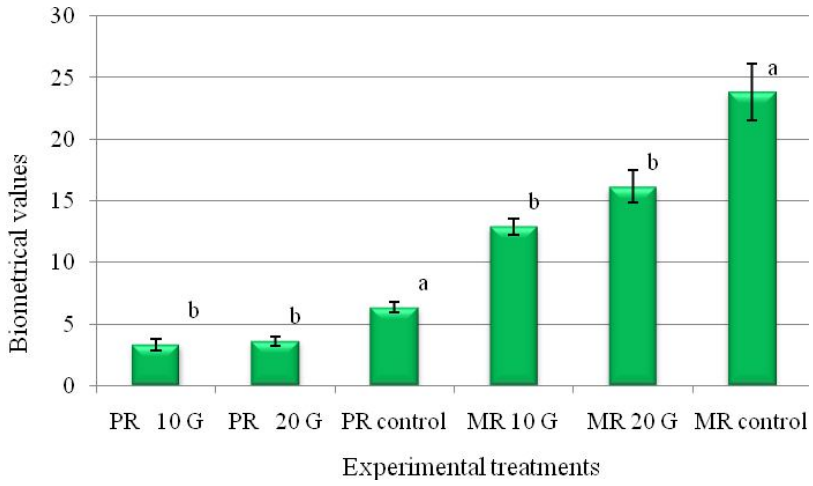


Fig. 19. *In vitro* multiplication in *Vaccinium macrocarpon* on media with guar gum: 10 G, 20 G are the treatments with 10, respectively 20 g/l

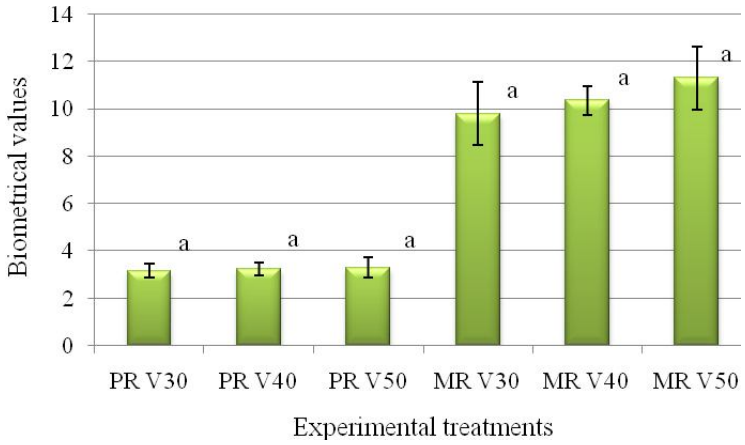


Fig. 20. The results regarding the *in vitro* multiplication of *Vaccinium macrocarpon* on media gelled with starch: V30, V40 and V50 – the treatments with 30, 40, respectively 50 g/l starch: PR – proliferation rates, MR – multiplication rates

The adequate growth regulators proved to be 2-Isopentenyladenine and zeatine. CPPU at the concentration of 0.02 mg/l gave results similar to the ones provided by 2-Ip (Fig. 21) and it has potential for being used, in the futre, for the micropropagation of this species, having in view the good results obtained and its low cost.

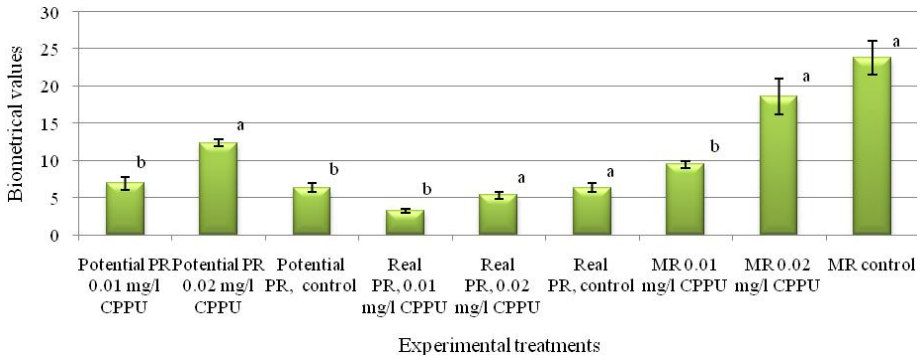


Fig. 21. The influence of CPPU upon *in vitro* multiplication in *Vaccinium macrocarpon*

Morphological features related to *Vaccinium macrocarpon in vitro* culture. In all the initiation and multiplication experiments, at the beginning just a small number of shoots grew from the microcuttings, in most cases just one/explant. The initial explants underwent hypertrophy and callusing and then buds and short shoots proliferated, which grew progressively in length during the 3-4 months of *in vitro* culture, most of them reaching to the caps of the culture vessels, some of them having far greater length than the height of the culture vessels. The plantlets' base gets callused and hypertrophied and adventitious roots appear at the base of the plantlets as well as from axillary buds on the shoots. In many cases the roots grow from axillary buds far above the media, even up to half the length of the shoot, sometimes from nodes situated even higher. The roots grow in length, then they touch the medium and in the contact zone they form small, green, button-shaped calli, which subsequently grow larger. These calli, after being transferred to WPM supplemented with 5 mg/l 2-iP generated neither roots nor shoots and they underwent necrosis in five weeks.

8.3. Acclimatization

Acclimatization and ex vitro rooting of ***Vaccinium macrocarpon*** shoots can be done in solid substrates (a mixture of acid peat and perlite 2:1 by volume, or Jiffy7 pellets) together with ensuring high air humidity.

For *ex vitro* acclimatization, float hydroculture proved to be effective only for the plantlets rooted *in vitro*, whereas for the individual shoots the most efficient option proved to be culture in floating perlite (Fig. 24), where rooting percentages of more than 90 % were obtained.

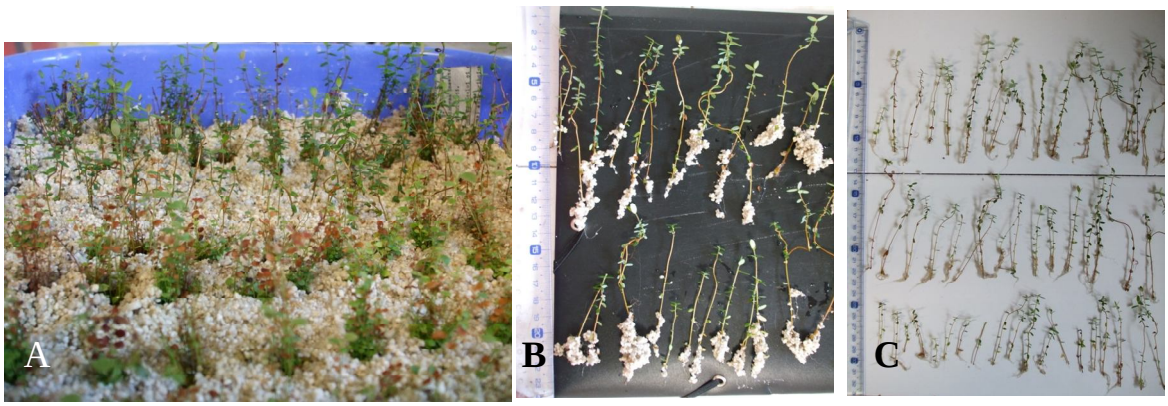


Fig. 22. Shoots rooted in floating perlite bed: A) as bunches; B, C) individually

IX. MICROPROPAGATION PROTOCOLS ELABORATED BASED ON THE EXPERIMENTS CARRIED OUT IN THE FRAMEWORK OF THE PRESENT THESIS

In this chapter we presented the micropropagation protocols for the five species (*Amelanchier canadensis* – Fam. *Rosaceae, Lonicera kamtschatica* – Fam. *Caprifoliaceae, Lycium barbarum* – Fam. *Solanaceae, Rubus fruticosus* – Fam. *Rosaceae, Vaccinium macrocarpon* – Fam. *Ericaceae*), which were elaborated on the basis of the results obtained during the experiments of initiation, multiplication, rooting and acclimatization caried out during the three years of research.

These protocols are very effective and they can be successfully applied in the fruit nurseries equipped with micropropagation laboratories, in order to produce container-grown planting material in these species.

The five protocols are presented schematically in Figures 23-27.

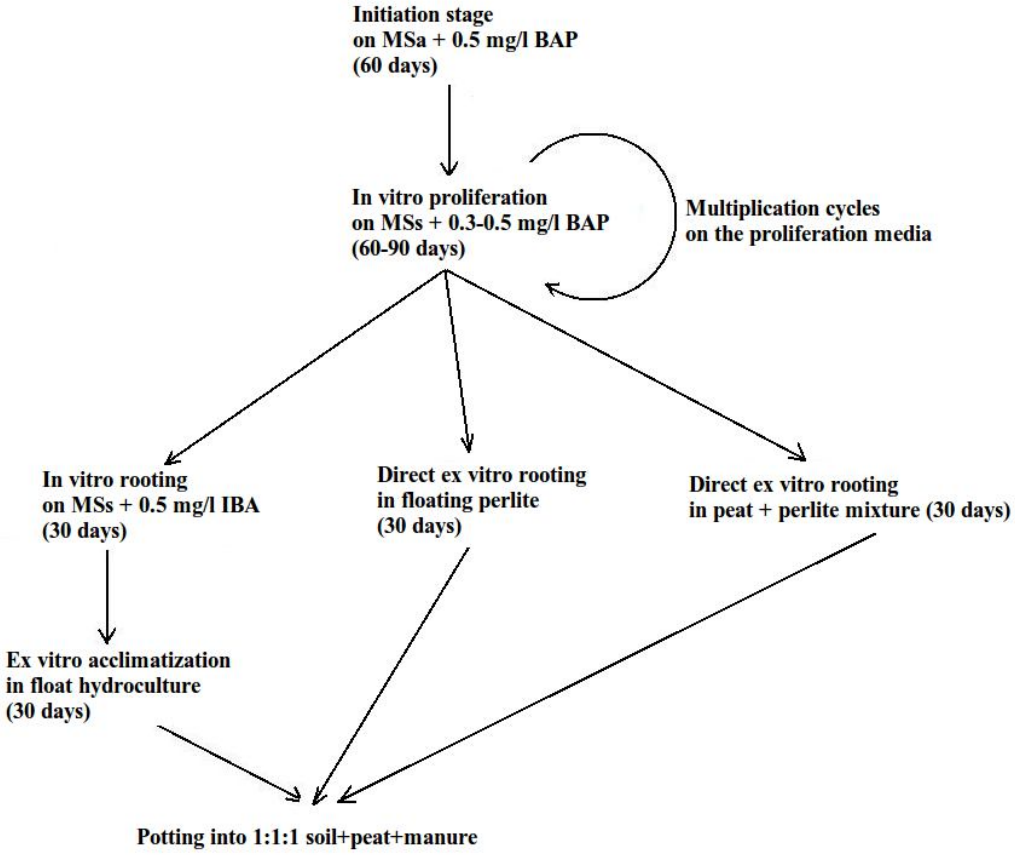


Fig. 23. Micropropagation protocol for *Amelanchier canadensis*, cultivar 'Rainbow Pillar'

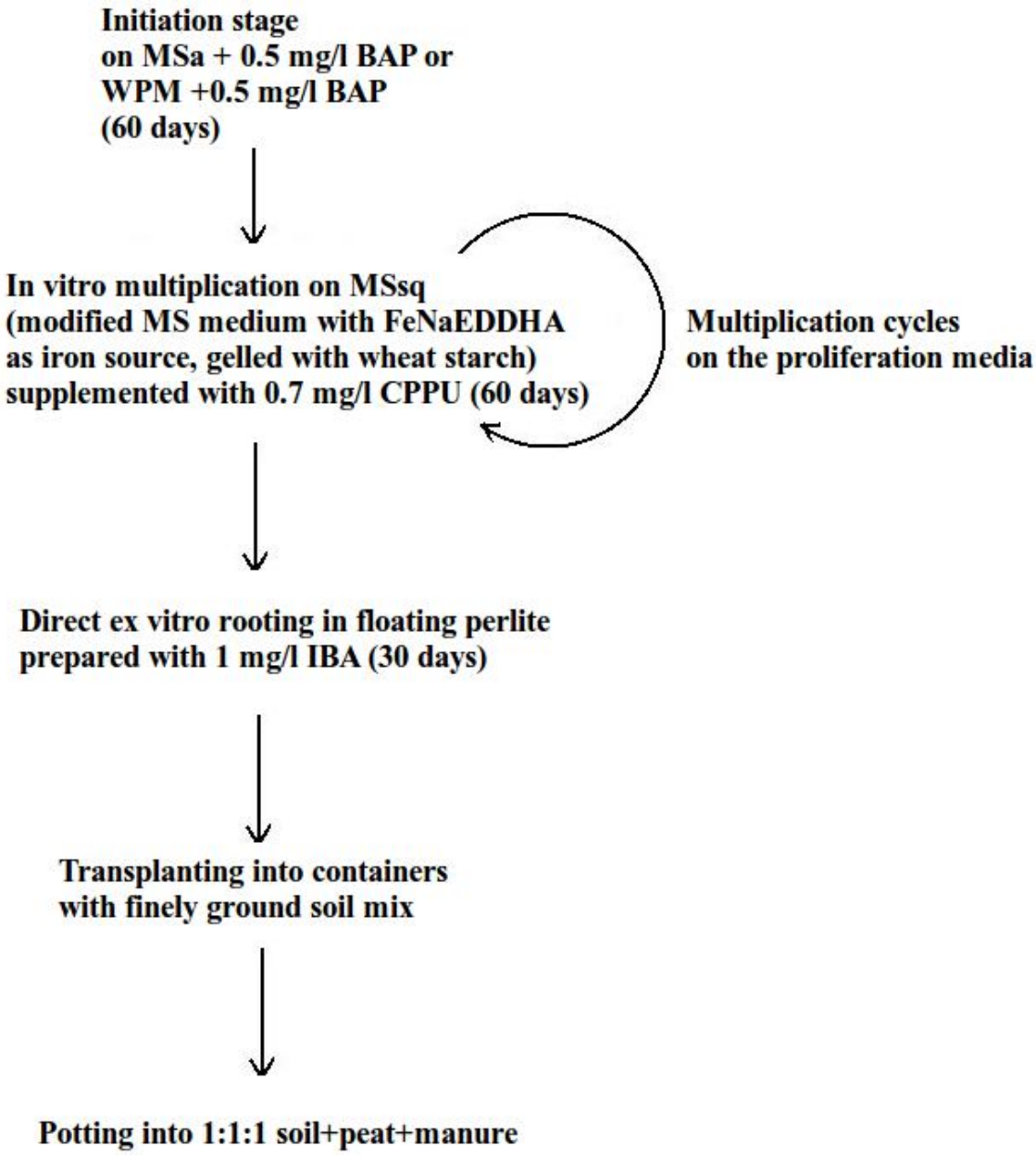


Fig. 24. Micropropagation protocol for *Lonicera kamtschatica*, cultivars 'Atut' and 'Duet'

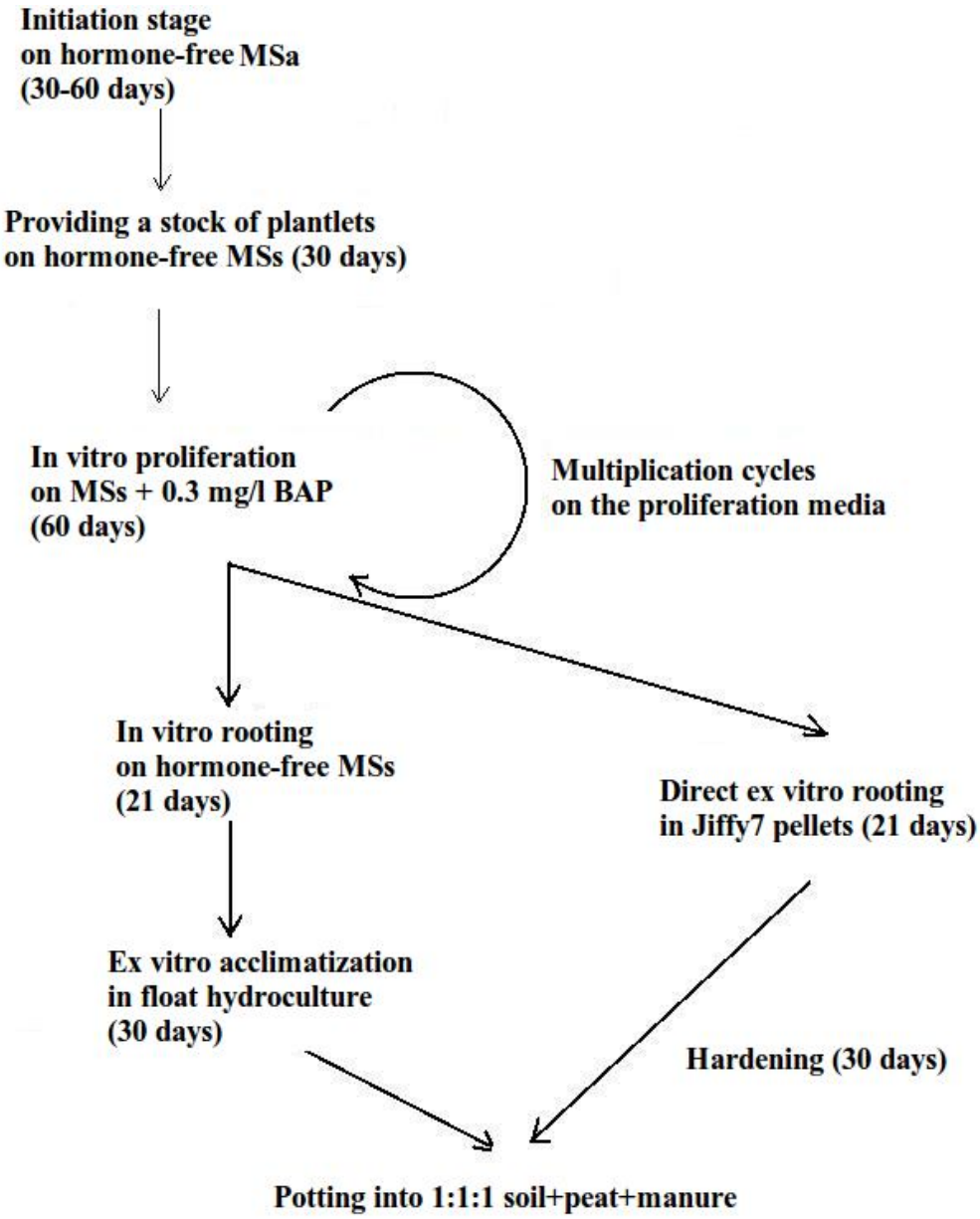


Fig. 25. Micropropagation protocol for *Lycium barbarum*, cultivar 'Ning Xia N1'

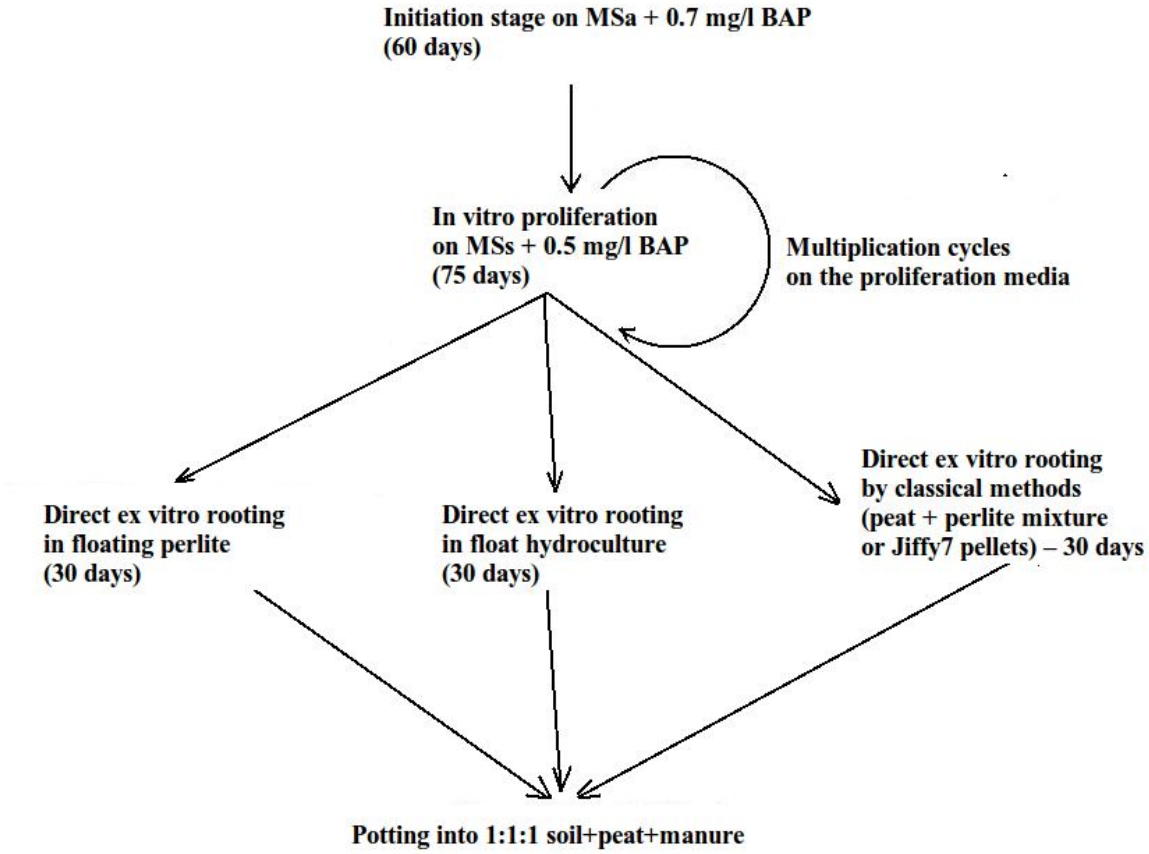


Fig. 26. Micropropagation protocol for blackberry (*Rubus fruticosus*), cultivar 'Loch Ness'

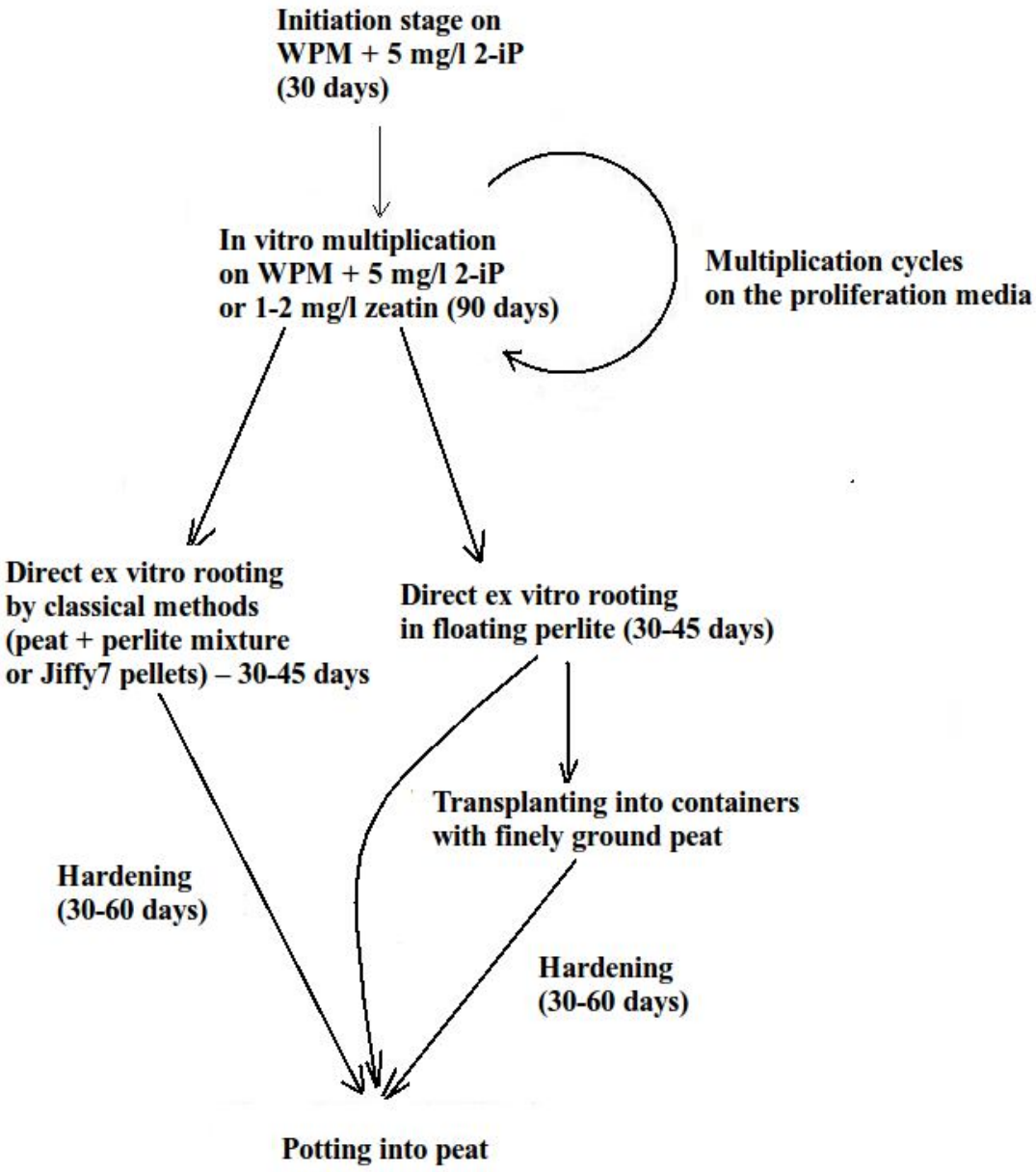


Fig. 27. Micropropagation protocol for *Vaccinium macrocarpon,* cultivar 'Pilgrim'

GENERAL CONCLUSIONS AND RECOMMENDATIONS

By the present thesis, studies regarding the micropropagation of species *Amelanchier canadensis* - Fam. *Rosaceae* - cultivar ‚Rainbow Pillar', *Lonicera kamtschatica* - Fam. *Caprifoliaceae* - cultivars ‚Atut' and ‚Duet', *Lycium barbarum* - Fam. *Solanaceae* - cultivar ‚Ning Xia N1', *Rubus fruticosus* - Fam. *Rosaceae* - cultivar ‚Loch Ness' and *Vaccinium macrocarpon* - Fam. *Ericaceae* - cultivar ‚Pilgrim' were carried out, for the first time in our country. The *ex vitro* acclimatization methods applied, respectively the method of *ex vitro* acclimatization in float hydroculture and *ex vitro* acclimatization in floating perlite are elements of novelty and originality in this domain.

The results of our research were a base for the elaboration of micropropagation protocols for the five species, protocols that can be applied successfully in the fruit nurseries equipped with *in vitro* culture laboratories, for the production of container-grown plant material for these species.

Based on the results of our studies we can state that:

- **The species** *Amelanchier canadensis* - Fam. *Rosaceae* - cultivar ‚Rainbow Pillar', *Lonicera kamtschatica* - Fam. *Caprifoliaceae* - cultivars ‚Atut' and ‚Duet', *Lycium barbarum* - Fam. *Solanaceae* - cultivar ‚Ning Xia N1', *Rubus fruticosus* - Fam. *Rosaceae* - cultivar ‚Loch Ness' and *Vaccinium macrocarpon* - Fam. *Ericaceae* - cultivar ‚Pilgrim' can be successfully micropropagated.

The *in vitro* propagation of species *Amelanchier canadensis*, cultivar 'Rainbow Pillar'

- For the initiation of *Amelanchier canadensis in vitro* cultures the optimal explant type is represented by shoot fragments containing the apical bud and the optimal medium is modified MS medium gelled with agar and supplemented with 0.5 mg/l BAP.
- In the multiplication stage, the modified MS medium gelled with wheat starch and supplemented with 0.5 or 0.7 mg/l BAP provided very high proliferation rates of more than 100 shoots/explants.
- The optimal explant type in the multiplication stage proved to be the microcuttings consisting of shoot fragments from the apical part of the axillary shoots, containing the apical bud. For the regeneration of well-developed plantlets, with vigorous shoots it is recommended to use minimum 2 cm long shoots or shoot fragments excised from the apical part of the axillary shoots regenerated *in vitro*. It is recommended to inoculate 4 microcuttings 2 cm in length into the culture vessels (720 ml jars). Approximately 2/3-3/4 of the basal part of the microcuttings should be immersed into the culture media.
- In the micropropagation procedures, larger shoots can be used, with the length of 4 cm. In this case it is recommended to inoculate 2 microcuttings/culture vessel. When using whole axillary shoots, 4-5 cm in length, inserted in oblique position into the mass of MSs culture media, the optimal BAP concentration was 0.3 mg/l, which provided the regeneration of normally developed shoots and a high percentage of normally developed, standard-sized shoots, more than 2 cm in length.
- The cytokinins BAR and mT proved to be adequate for the *in vitro* multiplication of *Amelanchier canadensis*, 2-iP at the concentrations of 10 and 20 mg/l also proved to be suitable, but 2-iP provided lower multiplication rates as compared to the ones obtained with BAP. Kinetin did not yield viable plantlets.
- The alternative gelling agents, namely fibre agar, Psyllium husk, guar gum and the mixture of starch and Phytagel gave good results. Phytagel at the concentration of 2.2 g/l caused the regeneration of poorly developed, hyperhydric plantlets. The highest proliferation rate (108.1 shoots/explant) were provided by the treatment gelled with guar gum, followed by the one provided by the treatment gelled with starch (86.4 shoots/explant).

- The shoots obtained in the multiplication stage could be rooted *in vitro* (the optimal medium was MSs supplemented with 0.5 mg/l IBA, as this provided rooting percentages of more than 90 % and well-developed plantlets) and then acclimatized in float hydroculture or they could be rooted directly *ex vitro* and acclimatized in floating perlite or in peat + perlite mixture, 1:1 by volume.
- *Ex vitro* acclimatization in float hydroculture had more than 80 % efficiency in the plantlets rooted *in vitro* on MSs supplemented with 0.5 mg/l IBA and direct *ex vitro* rooting in floating perlite provided rooting and acclimatization percentages of more than 90 % and such the necessity for *in vitro* rooting was eliminated.

The *in vitro* propagation of species *Lonicera kamtschatica*, cultivars 'Atut' and 'Duet'

- In the initiation stage the optimal explant type was the apex excised from the apical bud and the culture media recommended are MSm or WPM supplemented with 0.5 mg/l BAP and gelled with Plant Agar.
- The optimal medium for the multiplication stage is MSs supplemented with 0.7 mg/l CPPU and, as culture vessels we recommend 720 ml glass jars with screw caps, equipped with antibacterial filter which should enhance gas exchange.
- As a gelling agent for the culture media in the multiplication stage it is recommended to use wheat starch at the concentration of 50 g/l, as media gelled with Plant Agar proved to be inadequate for the multiplication stage.
- In the multiplication stage, among the cytokinins tested, CPPU at the concentrations of 0.5-1 mg/l proved to be suitable for this species. BAP proved to be usable at the concentration of 1 mg/l but it provided mediocre multiplication rates (5.53 microcuttings/ initial explant in cultivar 'Atut'), whereas meta-topolin and zeatin provided low multiplication rates and callus formation at the base of the plantlets.
- The optimal type of inocula in the multiplication stage proved to be the shoots or shoot fragments minimum 2 cm in length, with 3 nodes. It is recommended to insert 5-6 microcuttings/vessel. The lateral shoots containing 1-2 nodes and the apical bud also proved to be very viable explants, suitable for *in vitro* multiplication, but they caused uneven proliferation, with great differences among explants.
- The optimal culture period for one multiplication cycle is of two months (60 days). It is not recommended to exceed this period, because negative phenomena appear: necrosis of the apices, shoot and leaf necrosis).
- In the *ex vitro* rooting and acclimatization stage, in floating perlite, it is recommended to use IBA at 1 mg/l concentration for both cultivars, 'Atut' and 'Duet'. This provided rooting and acclimatization percentages of more than 60 %. It is recommended to use partial protection by partially covering the tubs with a transparent plastic lid.
- *Ex vitro* acclimatization is achieved in about 3-4 weeks in a gowth room with the temperature of 23 $\pm$ 3 ºC, 2400 Lux cold flourescent light, 16-hour photoperiod.

The *in vitro* propagation of species *Lycium barbarum*, cultivar 'Ning Xia N1'

- The initiation of *Lycium barbarum in vitro* cultures was successfully carried out using seeds harvested from ripe fruits. After disinfection, the seeds should be inoculated onto hormone-free MS media gelled with agar, in 320 or 720 ml jars. Inoculation is done by spreading the seeds on the surface of the culture media. The germination percentage was about 50 %.
- For the stabilization of *in vitro* cultures the plantlets derived from the initiation stage should bec ut into 2-3 node microcuttings, the leaves should be eliminated and these microcuttings should be inoculated into 720 ml jars containing hormone-free MSs media. Vigorous plantlets result, which can be subcultured at 1-1.5 month intervals.
- In the *in vitro* multiplication stage the results show that wheat starch had a decisive role for

the success of this stage. Modified MS medium, supplemented with 0.3 mg/l BAP proved to be optimal for the multiplication stage, as it provided high proliferation and multiplication rates and well developed shoots.

- The plantlets resulted in the multiplication stage on Mss with 0.3 mg/l BAP have shoots with short leaves, only a few mm in length, short internodes and , in most cases they do not have roots. These shoots proved to be very effective for further propagation on media of the same composition and they presented great versatility as they could be inoculated vertically, horizontally, in oblique position, one or two explants/vessel could be used (preferably two). In case of using short shoots, 1.5-2 cm in length, it is recommended to inoculate four microcuttings/vessel.
- Among the gelling agents tested, Isubgol gave good but inconsistent results, with high proliferation and multiplication rates in some plantlets. Vege-Gel and wheat starch proved to be inadequate as gelling agents for the *in vitro* multiplication of *Lycium barbarum*.
- In species *Lycium barbarum*, *in vitro* rooting and subsequent *ex vitro* rooting in float hydroculture can be successfully applied. Direct *ex vitro* rooting in Jiffy7 pellets of the shoots regenerated in the multiplication stage can also be applied and the rooting percentages are more than 90 %.
- Direct *ex vitro* rooting in Jiffy7 pellets of the shoots obtained in the multiplication stage is recommended only if there are adequate conditions for acclimatization, in high performance greenhouses or growth rooms, as the *Lycium barbarum* shoots are tender, sensitive and they quickly get de-hydrated.
- For *in vitro* rooting on hormone-free MSs media, 40 microcuttings/culture vessel are recommended, because this provides the regeneration of larger, better developed and better rooted plantlets and work with these is easier. In the *in vitro* rooting stage on hormone-free MSs the internodes get elongated, the leaves grow in length and more than 90 % of the shoots get rooted.
- For the *ex vitro* acclimatization of the plantlets rooted *in vitro*, float hydroculture is recommended, because this method proved to be very efficient as it provided very high acclimatization percentage, of more than 90 %.

The *in vitro* propagation of species *Rubus fruticosus*, cultivar 'Loch Ness'

- For the initiation stage the axillary buds should be excised under the binocular, any trace of bark and wood should be eliminated as well as 1-2 layers of leaf primordia and the buds should be inserted into MSa culture media supplemented with 0.5 mg/l BAP, in test tubes containing about 5 ml of medium each.
- In the multiplication stage very good results were obtained by using MSa or MSs culture media supplemented with 0.5 mg/l BAP and, as culture vessels, 720 ml jars with screw caps equipped with antibacterial filters are recommended. The polypropylene vessels proved to be less effective.
- The optimal explant type proved to be the 2 cm long microcutting, with 4-5 nodes and the 1-2 node fragments proved to be inadequate. The optimal number of microcuttings/vessel was four. A higher number of inocula/vessel causes over-population and non-uniform growth and proliferation.
- The results obtained in the multiplication stage, when using the cytokinins CPPU and TDZ show that these are ineffective for this blackberry cultivar, as the plantlets were deformed. Meta-topolin provided lower multiplication rates as compared to BAP and the plantlets were deformed.
- Gelling the culture media with a mixture of starch and Phytagel did not have a beneficial effect in the multiplication stage and increasing starch concentration to 60 g/l also did not have beneficial effect.
- Guar gum at the concentration of 20 g/l provided high proliferation and multiplication rates,

thus it proved to be a potential alternative to the use of starch as gelling agent in the culture media.

- Our research regarding the *in vitro* multiplication of blackberry cultivar 'Loch Ness' on media sterilized chemically with NaDCC, without autoclavation, show that blackberry cultivar 'Loch Ness' is sensitive to NaDCC and growth and proliferation are inhibited. NaDCC stunts the growth of this cultivar and causes hyperhydricity and deformation in the plantlets.
- The axillary shoots regenerated *in vitro* in the multiplication stage can be easily rooted and acclimatized *ex vitro* either by conventional methods (the use of sold substrates at the same time with ensuring high air humidity) as well as by the new, radically different acclimatization methods elaborated at the Fruit Research Station Cluj. Among the solid substrates, Jiffy7 pellets, Sol Vit G and peat + perlite mixture provided more than 90 % *ex vitro* acclimatization. Peat + perlite mixture is recommended, having in view its availability, low cost and proven efficiency.
- *Ex vitro* rooting of the blackberry axillary shoots, in float hydroculture in floating cell trays gave rooting percentages of more than 70 % while applied in the greenhouse, in the growth room or in open air in semi-shadow, without ensuring high air humidity. It is recommended to plant the small shoots (1.5-2.5 cm in length) apart from the long ones (3-5 cm), in trays with 1 cm diameter cells.
- *Ex vitro* rooting in floating perlite was very effective in blackberry cultivar 'Loch Ness', where it is recommended to plant the shoots one by one and in this way this method provides rooting and acclimatization percentages of more than 90 %.
- Float hydroponics has the potential to become an efficient alternative to conventional potting, in case of using plants rooted and acclimatized in Jiffy7 pellets. In order to increase the size of plants, they were successfully cultured in hydroponics by using 2 g/l of Ferticare complex fertilizer as nutrient solution.

The *in vitro* propagation of species *Vaccinium macrocarpon*, cultivar 'Pilgrim'

- For *Vaccinium macrocarpon in vitro* culture initiation multiple-node shoot fragments with intact leaves are recommended as explants because these provide high regeneration rates and contamination is low and, as culture medium, WPMm + 5 mg/l 2-Ip.
- Among the variants of culture media tested in the multiplication stage, WPMm containing either zeatin or 5 mg/l 2-iP provided reasonable proliferation rates and relatively well-developed shoots.
- Among the gelling agents tested, wheat starch and guar gum proved to be ineffective at the concentrations tested, whereas the static liquid media without mechanical support provided results similar to the ones on agar-gelled media with 2-iP, so these are potential alternatives to agar-gelled media.
- In the multiplication stage the regeneration of axillary shoots occurs slowly, progressively, the axillary shoots are regenerated one after another and then they elongate rapidly, growing up to the caps of the culture vessels and reasonable proliferation rates are obtained in 3-4 months of *in vitro* culture.
- In the multiplication stage the plantlets can be stored *in vitro* for five months on the multiplication media at laboratory temperature, without subculturing. At the same time, growth, proliferation and *in vitro* rooting continues, slowly.
- Rooting and acclimatization can be done by classical methods in solid substrates (acid peat + perlite mix, 2:1 by volume or in Jiffy 7 pellets) at the same time providing high air humidity, or in tubs containing floating perlite, in the latter case the rooting percentages are more than 90 %.
- *Ex vitro* acclimatization in hydroculture in floating cell trays proved to be efficient only when using plantlets already rooted *in vitro*.

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